ANTIGENIC MIMICRY AND AUTOANTIBODIES IN RHEUMATIC FEVER

Thesis presented by

QUENTIN GAVIN EICHBAUM

in fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

PAEDIATRICS AND CHILD HEALTH

and

MEDICAL BIOCHEMISTRY

in the

FACULTY OF MEDICINE

UNIVERSITY OF CAPE TOWN

May 1990

CERTIFICATE OF SUPERVISORS

In terms of paragraph eight of the "General regulations for the degree of Ph. D.", we as supervisors of the candidate Q. G. EICHBAUM, certify that we approve of the incorporation into this thesis of material that has already been published or submitted for publication.

Signed

Professor D W Beatty
Associate Professor and Senior Specialist
Department of Paediatrics and Child Health

Signed

Dr M I Parker
Senior Lecturer
Department of Medical Biochemistry

ACKNOWLEDGEMENTS

I wish to acknowledge and express my sincere appreciation to the following:

- My supervisor Professor David Beatty for his patience, enthusiasm and guidance; for his untiring efforts to obtain funding for the project; and for the keen and supportive interest he has taken in my career.

- My co-supervisor Dr Iqbal Parker for his encouragement and support and for skilfully and enthusiastically introducing me to molecular biology.

- Professor Wieland Gevers for providing the facilities to do aspects of this work, and for his keen support and the interest he has shown in my career.

- Margaret Cooper and Renée Allin who (at different stages of the project) persisted in casting gels and setting up ELISA plates.

- Miss Jane Hughes for assistance in screening and characterizing the monoclonal antibodies, and for her superb vigilance in seeing to the material needs of the Immunology Laboratory.

- Professor Pat Commerford (Head of the Department of Cardiology, Groote Schuur Hospital) for obtaining the sera from ischaemic heart disease and chronic rheumatic heart disease patients, and Professor Heinz Rode (Department of Paediatric Surgery) for obtaining child surgical control sera.

- The staff of the Rheumatic Fever Clinic at Red Cross Children's Hospital for their meticulous examination of patients and for always being so wonderfully obliging in "feeding" this project with serum.

- The technologists in the Department of Histopathology at Red Cross Children's Hospital, for always somehow managing to squeeze-in the cutting of a few more sections of heart tissue.

- The many doctors, medical technologists and secretarial staff members of Red Cross Children's Hospital who have been my friends, who have created a pleasant working environment and have done me innumerable favours.

- The hospital and laboratory workers of Red Cross Children's Hospital and the Department of Medical Biochemistry, for ensuring the smooth running of facilities.

- Ms Jane Hall of the Institute of Biostatistics (South African Medical Research Council) for performing the statistical analyses on the ELISA data.

- Ms Rose Cooper for measuring antibodies to cardiolipin in the rheumatic fever patients.

- Dr James Dale of the University of Tennessee, Memphis (USA) for measuring antibodies to the streptococcal M proteins, and for the gifts of the rabbit antisera against these M proteins.

- Dr John Zabriskie of the Rockefeller University (New York) for the gift of the purified streptoccocal cell membranes and cell walls.

- My colleagues, in the Department of Medical Biochemistry, Paul Goldberg, Rachel Judd, Johann te Water Naude, Arlene Smith, Juli Roussouw and Malcolm Collins for their assistance, many kindnesses and for the fun we shared.

- Brenda Cowley for her unstinting support and for putting up with being a "thesis widow" for so long!

- Ms Vicky Melly for her superb word-processing skills in typing the tables and references, and her patient assistance in printing the text.

- I am also deeply indebted to the generous support of the following funding bodies: the South African Arthritis Foundation; the South African Heart Foundation; the Medical Research Council; the Institute of Child Health, Red Cross Children's Hospital; and the University of Cape Town for various scholarships and bursaries.

CONTENTS

Title Page i
Certificate of Supervisor ii
Acknowledgements iii
Contents v
Abbreviations xiii
Dedication xv
Abstract xvi

1. INTRODUCTION

A. RHEUMATIC FEVER: OVERVIEW AND IMMUNOPATHOLOGY 2
1.A.1 Introduction 2
1.A.2 Incidence, Clinical Manifestations and Diagnosis 2
1.A.3 Pathogenic Concepts 4

B. ANTIGENIC MIMICRY, AUTOANTIBODIES AND AUTOIMMUNE DISEASE 6
1.B.1 Introduction 6
1.B.2 Antigenicity, Cross-reactivity & Antigenic Mimicry 6
1.B.3 Structural Properties of Cross-reactive Antigens 8
1.B.4 Autoantigens 10
1.B.5 Autoantibodies 11
1.B.5.1 Natural/Physiologic Autoantibodies 11
1.B.5.2 Origin of Autoantibodies 12
1.B.5.3 Pathogenic Autoantibodies 14
1.B.5.4 Polyclonal B cell Activation 15
1.B.6 Antigenic Mimicry and Autoimmune disease 16
1.B.7 Cross-reactivity: Serum and Monoclonal Antibodies 18

2. ANTIBODIES TO CARDIAC, NUCLEAR AND STREPTOCOCCAL ANTIGENS

2.1 INTRODUCTION 21
2.1.1 Myosin ELISA 22
2.1.2 Actin ELISA 23
2.1.3 Collagen Type II ELISA 24
2.1.4 Heart Extract ELISA 24
2.1.5 Group A Streptococcus ELISA 25
2.1.6 Streptococcal M Protein ELISA 25
2.1.7 ASO and anti-DNAse antibodies 25

2.2 RESULTS AND DISCUSSIONS 27
2.2.1 Study Design and Statistical Analyses. 27
2.2.1.1 Study Design 27

2.2.1.2 Patient Sera 27
2.2.1.3 Rabbit Hyperimmune and Nonimmune Control Sera 30
2.2.1.4 Patient Sera: Immunoglobulin Concentrations 30
2.2.2 Cardiac Autoantigens 32
2.2.2.1 Heart Extract ELISA 32
2.2.2.2 Myosin ELISA 34
2.2.2.3 Actin ELISA 36
2.2.2.4 Phosphorylase b ELISA 38
2.2.2.5 Anticardiolipin Antibodies (ACA) 40
2.2.2.6 Collagen ELISA 40
2.2.3 Group A streptococcal Antigens 42
2.2.3.1 ASO and anti-DNAse B Titres 42
2.2.3.2 Group A Streptococcus ELISA 42
2.2.3.3 M Protein ELISAs 45
2.2.4 Nuclear Autoantigens 48
2.2.4.1 Antinuclear Antibodies (ANA) 48
2.2.4.2 Extractable Nuclear Antigens (ENA) 52
2.2.4.3 Anti-DNA Antibodies 53
2.2.4.4 Immunocytochemistry: Biotin-Streptavidin-Peroxidase and Immunofluorescence 53

2.3 CONCLUSIONS 60

3. IMMUNOBLOTTING: CARDIAC AND STREPTOCOCCAL ANTIGENS AGAINST RHEUMATIC FEVER AND CONTROL SERA

3.1. INTRODUCTION 64
3.1.1 Immunoblotting of cardiac antigens: Crude Extracts, Sarcolemmal membrane and Purified Proteins 64
3.1.1.1 Extracts of Heart Tissue: Procedures 64
3.1.1.1.1 Crude Extracts 64
3.1.1.1.2 Sarcolemmal Membrane Extracts 65
3.1.1.2 Immunoblotting of Heart Antigen Against Human Sera and Monoclonal Antibodies 65
3.1.1.2.1 Crude Extracts of Heart 65
3.1.1.2.2 Sarcolemmal Membrane Preparations 67
3.1.1.2.3 Purified Cardiac Antigens 67
3.1.1.2.3.1 Myosin 67
3.1.1.2.3.1.1 Rabbit Antiserum 67
3.1.1.2.3.1.2 Monoclonal Antibodies 68
3.1.1.2.3.2 Tropomyosin 69
3.1.2 Immunoblotting of Streptococcal Antigens 70
3.1.2.1 Crude Extracts and Purified M Proteins 70
3.1.3 Special Applications of Immunoblotting 70
3.1.3.1 Affinity Purification of Antibodies 70

3.1.3.2 Immunization 71
3.1.3.3 Antigens Recognized by T Lymphocytes 71
3.1.4 Cross-reactivity and Immunoblotting: Some Pitfalls 71

3.2 RESULTS 82
3.2.1 Human Heart Detergent Extracts 82
3.2.1.1 Non-rheumatic Heart Extract 82
3.2.1.1.1 Preliminary Investigations 82
3.2.1.1.2 Main Investigation 83
3.2.1.1.2.1 Identification and Descriptions of Antigen Bands 83
3.2.1.1.2.2 IgG Binding 86
3.2.1.1.2.3 IgA Binding 86
3.2.1.1.2.4 IgM Binding 86
3.2.1.2 ARF Heart Extract 94
3.2.2 Cross-reactivity: Human Heart and *S.pyogenes* Antigens 94
3.2.2.1 Eluted Antibody: Reactivity with Immunoblotted *S.pyogenes* Antigens 94
3.2.2.2 Eluted Antibody: Immunofluorescence Staining of Heart 95
3.2.3 Sarcolemmal Membranes 95
3.2.4 Nuclear Antigens 99
3.2.5 Reactivity of Anti-Streptococcal Sera with Heart Extract 102
3.2.6 Additional Experiments 106
3.2.6.1 Reactivity to Human Collagen Types II, III and VI 106
3.2.6.2 Ca^{2+} ATPase Binding 106

3.3 DISCUSSION 108
3.3.1 Human Heart Detergent Extracts 108
3.3.1.1 Isotype Binding Patterns of Groups of Sera 108
3.3.1.2 Hypothesis: Raised IgM Anti-heart Antibodies 109
3.3.1.3 Individual Protein Bands Recognised 110
3.3.1.4 Specificity and Pathological Relevance of Antigens 112
3.3.2 Cross-reactivity: Heart and *S.pyogenes* M Proteins 113
3.3.2.1 Reactivity of HRA with Immunoblotted Streptococcal Antigens 113
3.3.2.2 Immunofluorescence Staining of Eluted Antibody Against Heart 114
3.3.3 Sarcolemmal Membranes 114
3.3.4 Nuclear Antigens 115
3.3.5 Rabbit Anti-*S.Pyogenes* Serum Reactivity with Heart Antigens 116
3.3.6 Additional Experiments 116
3.3.6.1 Ca^{2+} ATPase Binding 116

3.4 CONCLUSIONS 118

4. HUMAN MONOCLONAL ANTIBODIES DERIVED FROM LYMPHOCYTES OF ACUTE RHEUMATIC FEVER PATIENTS

4.1 INTRODUCTION 121
4.1.1 Rheumatic Fever: Use Of Murine And Human Monoclonal Antibodies 121
4.1.1.1 Mouse Monoclonal Antibodies 121
4.1.1.1.1 Heart and S.pyogenes 121
4.1.1.1.2 Myosin and Tropomyosin 121
4.1.1.1.3 Multispecificity of Anti-streptococcal mAbs 122
4.1.1.1.4 Reactivity with DNA 123
4.1.1.1.5 Human Sarcolemma 123
4.1.1.1.6 Other Streptococcal Serotypes (and Myosin) 123
4.1.1.1.7 Shortcomings of Murine mAbs 124
4.1.1.2 Human Monoclonal Antibodies in Rheumatic Fever 124
4.1.2 Problems in Development of Human Monoclonal Antibodies 125
4.1.2.1 Range of Immunogens and Poor Source of Lymphocytes 125
4.1.2.2 Instability of Human Hybridomas 126
4.1.2.3 Immortalization of Human B Cells 126
4.1.2.4 Expansion of Human Monoclonal Antibody Cell Lines 127
4.2 RESULTS 128
4.2.1 Screening and Characterization of Monoclonal Antibodies 128
4.2.1.1 ARF Patients 128
4.2.1.2 Hybrid Screening and Selection 128
4.2.1.3 Stability of Hybridomas in Culture 129
4.2.1.4 Karyotyping of Selected Hybrids 129
4.2.1.5 Characteristics of Immunoglobulin Produced 129
4.2.1.6 Propagation of Hybridomas in Nude Mice 132
4.2.1.7 Stability of Antibody Production 132
4.2.1.8 $CD5^+$ Cell Surface Marker 132
4.2.1.9 Anti-Streptococcal Antigen Reactivity 132
4.2.1.10 Selection of mAbs against Group A Streptococci 132
4.2.1.11 Cross-reactivity with other Streptococcal Groups (B,C,D,G) 133
4.2.1.12 Cross-Reactivity with Non-streptococcal Bacteria 134
4.2.2 Anti-Human Heart Antigen Reactivity 134
4.2.2.1 Binding to Heart Sections: Immunoperoxidase Sandwich Assay 134
4.2.2.2 Immunoblotting of Heart Extract and Sarcolemma 135
4.2.2.3 Organ Non-Specificity of mAb Binding 135
4.2.2.4 Nonspecific Binding to Mammalian Tissue Antigens 135
4.2.3 Cross-reactive Binding of Human mAbs to Heart and Streptococcal Ags 135
4.2.3.1 Immunoblotting 135
4.2.3.2 ELISA Inhibition Assay 139
4.2.4 Binding to Nuclear Antigens 139
4.3 DISCUSSION 141
4.4 CONCLUSIONS 146

5. CONSTRUCTION AND IMMUNOSCREENING OF λGT11 cDNA EXPRESSION LIBRARIES

5.1 INTRODUCTION 148

5.2 RESULTS 150
5.2.1 cDNA Synthesis and Construction of λgt11 Libraries 150
5.2.1.1 RNA Extraction 150
5.2.1.2 cDNA Synthesis 150
5.2.1.3 Cloning of cDNA into Bacteriophage λgt11 153
5.2.1.4 Analysis of cDNA Inserts 155
5.2.2 Screening of λgt11 cDNA Expression Libraries with Antibodies 155
5.2.2.1 Expression of Recombinant Fusion Proteins 158
5.2.2.2 Detection of Clones Reactive with ARF Patient Serum 158
5.2.2.3 Analysis of Positive Clones 161
5.2.2.3.1 Size Analysis cDNA Inserts 161
5.2.2.3.1 Size Analysis of Fusion Proteins 166
5.2.3 Reactivity of Fusion Proteins with Sera 166
5.2.3.1 Purified Plaque Assay: Specificity for ARF Sera 166
5.2.3.2 Fusion Proteins: Immunoblots against ARF and Normal Sera 170
5.2.3.2.1 Immunoblots of Bacterial Lysogen Lysates 170
5.2.3.2.1 Affinity Purification of Fusion Proteins 172
5.2.3.3 Plaque Selected Antibodies: Cross-reactivity with Streptococcal Antigens 172
5.2.4 DNA Sequence Analysis of Positive Clones 174

5.3 DISCUSSION 177
5.3.1 cDNA Synthesis and Construction of λgt11 Libraries 177
5.3.2 Screening of Libraries and Detection of Positive Clones 177
5.3.3 Characterization of Positive Clones 178
5.3.3.1 Specificity of Positive Fusion Proteins 178
5.3.3.1.1 Specificity of Purified Plaques for ARF Sera 178
5.3.3.1.2 Immunoblots of Bacterial Lysogen Lysates 179
5.3.3.1.3 Cross-reactivity of Plaque-Selected Antibodies with Streptococcal Antigens 179
5.3.3.2 cDNA Sequence of Positive Cloned Inserts 180

5.4 CONCLUSIONS 183

6. CONCLUSION

6.1 STUDY OBJECTIVES 185

6.2 NOVELTY OF THE APPROACH 185

6.3 SALIENT FINDINGS 185

6.4 DIFFICULTIES IN THE STUDY OF ANTIGENIC MIMICRY 189

6.5 RHEUMATIC FEVER AS A MODEL OF AUTOIMMUNITY AND IMMUNE FUNCTION 189

7. MATERIALS AND METHODS

7.A AUTOANTIBODIES AND IMMUNOBLOTTING (Chapters 2, 3) 192
- 7.A.1 Raising Rabbit Hyperimmune Sera 192
- 7.A.2 ASO and DNAse B Antibody Titres 192
  - 7.A.2.1 Anti-streptolysin O antibodies (ASO) 192
  - 7.A.2.2 Anti-DNAse B antibodies (DNAseB) 192
- 7.A.3 Group A Streptococcus ELISA 192
- 7.A.4 Actin ELISA 193
- 7.A.5 Collagen Type II ELISA 193
- 7.A.6 ELISAs to Cardiac Autoantigens 194
  - 7.A.6.1 Myosin 194
  - 7.A.6.2 Heart Extract 194
  - 7.A.6.3 Phosphorylase b 195
- 7.A.7 Antinuclear Antibodies 195
- 7.A.8 Bacterial Cell Cultures 195
- 7.A.9 Immunoblotting 195
- 7.A.10 SDS Polyacrylamide Gel Electrophoresis 196
- 7.A.11 Heart Antigen Extract 197
- 7.A.12 Sarcolemmal Membrane Purification 197
- 7.A.13 Affinity Purification of Antibody on Immunoblots 198
- 7.A.14 Nuclear Extracts from Cultured Heart Cells 198
- 7.A.15 Primary Culture of Human Heart Cells 199
- 7.A.16 Processing of Tissue for Immunocytochemistry 199
  - 7.A.16.1 Paraffin Wax Sections 199
  - 7.A.16.2 Frozen Sections 199
- 7.A.17 Immunofluorescence on Tissue Sections 200
- 7.A.18 Immunocytochemistry: Biotin-streptavidin-peroxidase 200

MONOCLONAL ANTIBODIES (Chapter 4) 201
- 7.A.19 Cell Culture 201
- 7.A.20 EAC Rosettes: B Cell Purification 201
- 7.A.21 Monoclonal Antibody Cell Fusions 202
- 7.A.22 Hybridoma Screening against Heart and *S.pyogenes* 202
- 7.A.23 Immunoperoxidase Sandwich Assay 202
- 7.A.24 Production of Ascites 203
- 7.A.25 Cryopreservation of Hybridoma Cells 203
- 7.A.26 Karyotyping of Hybridomas 203
- 7.A.27 Immunoprecipitation of Hybridoma Immunoglobulin 203

7.A.28 Indirect Immunofluorescence: Cell Surface Antigens 204
7.A.28.1 Cell surface immunoglobulin 204
7.A.28.2 CD5$^+$ Antigen 204
7.A.29 Tissue Absorptions 204
7.A.30 Bacterial Antigen Sonicates 205
7.A.31 Bacterial Detergent Extracts: *S.pyogenes* and *E.coli* 205

7.B λGT11 CDNA LIBRARIES: CONSTRUCTION AND IMMUNOSCREENING (Chap. 5) 206
7.B.1 Preparation of RNA and Synthesis of cDNA 206
7.B.1.1 RNA Extraction 206
7.B.1.2 Poly (A)$^+$ RNA Isolation 206
7.B.1.3 Synthesis of cDNA 207
7.B.1.3.1 S1 Nuclease Method 207
7.B.1.3.2 RNAse H Method 208
7.B.1.3.3 Random Hexanucleotide Priming Method 210
7.B.1.3.4 Calculation of cDNA Reaction Yields 210
7.B.2 Cloning of cDNA into λgt11 Expression Vector 210
7.B.2.1 T4 DNA Polymerase Blunt End Repair of cDNA 210
7.B.2.2 Methylation of Internal EcoR I Sites 210
7.B.2.3 Ligation of EcoR I Phosphorylated Linkers to cDNA 211
7.B.2.4 EcoR I Digestion of Linkered cDNA 211
7.B.2.5 Separation of Excess Linker Molecules 211
7.B.2.6 Ligation of EcoR I-Ended cDNA into λgt11 211
7.B.2.7 Packaging of λgt11 Recombinants 212
7.B.2.8 Plating and Titration of λgt11 Libraries 213
7.B.2.9 Evaluation of Libraries 213
7.B.2.10 Lambda DNA Extraction 214
7.B.2.10.1 DNA Extraction from Liquid Lysates 214
7.B.2.10.1.1 Liquid Lysate 214
7.B.2.10.1.2 DNA Extraction 214
7.B.2.10.2 Plate Lysates 215
7.B.3 Immunoscreening of λgt11 Libraries 215
7.B.3.1 Antibody Screening Procedure 215
7.B.3.2 Primary Antibody Dilution and Treatment 216
7.B.3.3 Removal of *E.coli* Cross-reactivity 216
7.B.3.4 Affinity Purification of Antibody on Western Blots 216
7.B.3.5 Purification of Positive Clones 216
7.B.4 Analysis of positive clones 217
7.B.4.1 Analysis of Fusion Proteins 217
7.B.4.1.1 Isolation of Recombinant Lysogens 217
7.B.4.1.2 Fusion Proteins 217
7.B.4.2 Analysis of DNA Positive Inserts 218
7.B.4.2.1 Subcloning of λgt11 Inserts into pUC19 218
7.B.4.2.1.1 In-gel Ligation of Insert into pUC19 218
7.B.4.2.1.2 Plasmid DNA Isolation 218
7.B.4.2.1.3 Small Scale (Rapid) Preparation 218
7.B.4.2.1.4 Large Scale Preparation 219

7.B.4.2.1.5 Preparation of Competent *E.coli* Cells 219
7.B.4.2.1.6 Plasmid DNA Transfection of *E.Coli* Cells 220
7.B.4.2.2 Sequencing of DNA 220
7.B.5 General Nucleic Acid Techniques 220
7.B.5.1 Formaldehyde Agarose Gel Electrophoresis of RNA 220
7.B.5.2 Agarose Gel Electrophoresis of DNA 221
7.C BUFFERS AND SOLUTIONS 222

APPENDIX A: STATISTICAL TESTS AND UNIVARIATE DESCRIPTIVE STATISTICS 230

APPENDIX B: CLINICAL AND DEMOGRAPHIC PARTICULARS OF ARF PATIENTS 242

APPENDIX C: ADMISSION CRITERIA FOR PATIENTS 243

REFERENCES 244

ABBREVIATIONS

A_{260}	absorbance at 260nm
A	adenine or adenosine
AAA	anti-actin antibodies
Ab	antibody
ABTS	2.2 azinodi-3-ethylbenzthiazoline sulphonic acid diammonium salt
ACA	anti-cardiolipin antibodies
AEC	aminoethylcarbizole
AET	2-(2 aminoethyl) isothiouronium bromide hydrobromide
Ag	antigen
AGN	acute glomerulonephritis
AMA	anti-myosin antibodies
APA	anti-phosphorylase b antibodies
ARF	acute rheumatic fever
ASO	anti-streptolysin O
ATP	adenosine 5'-triphosphate
BCG	Bacillus Calmette-Guérin
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BLOTTO	dry-milk powder immunoblot blocking reagent
bp	base pair
BSA	bovine serum albumin
C	cytosine or cytidine
°C	degree Celsius
cDNA	complementary deoxyribonucléic acid
CHAPS	3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane-sulfonate
Ci	curie
CONT-a	control adult
CONT-c	control child
cpm	counts per minute
CRF	chronic rheumatic fever (i.e. rheumatic heart disease) (-a = adult; -c = child)
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEAE	diethylaminoethyl
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DMSO	dimethylsulfoxide
DNAse	deoxyribonuclease
d/s	double strand
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
F(ab)	antigen-binding fragment
FCA	Freund's complete adjuvant
FCS	fetal calf serum
FIA	Freund's incomplete adjuvant
FITC	fluorescein isothiocyanate
g	unit of gravity
G	guanine or guanosine
GAS	group A Streptococcus
GKN	Dulbecco's PBS with added 2g/L glucose, 0.01g/L phenol red
H chain	heavy chain of immunoglobulin
H.A.T.	hypoxanthine, aminopterin, thymidine
HGPRT	hypoxanthine guanosine phosphoribosyl transferase
HRA	heart-reactive antibodies
HRPO	horseradish peroxidase

HSP	heat shock protein
HT	RPMI containing hypoxanthine $1x10^{-4}$M, thymidine $1.6x10^{-5}$M, glycine $3x10^{-6}$M, sodium pyruvate $1x10^{-3}$M
IHD	ischaemic heart disease
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
IPTG	isopropyl-1-thio-ß-d-galactoside
IQR	interquartile range
kD	kilodaltons
mAb	monoclonal antibody
MEM	minimum essential medium
m.o.i.	multiplicity of infection
mol.wt./M.W.	molecular weight
n	number in study or group
NBT	nitroblue tetrazolium
ND	not determined
OD_{260}	optical density at 260nm
oligo	oligonucleotide
PAGE	polyacrylamide gel electrophoresis
PBL	peripheral blood lymphocyte
PBS	phosphate-buffered saline
PEG	polyethylene glycol
Pep M5	pepsin extracts of M type 5 (6, 19, etc) virulence associated protein of streptococcus
pfu	plaque-forming units
p.i.	pre-immune
PMSF	phenylmethylsulfonyl fluoride
poly(A)$^+$	polyadenylated mRNA
POS 5	positive clone No. 5
POS 6	positive clone No. 6
POS 7/8	positive clone No. 7 or 8
PVC	polyvinyl chloride
PWM	pokeweed mitogen
RA	rheumatoid arthritis
RHD	rheumatic heart disease
RNAse	ribonuclease
RNasin	ribonuclease inhibitor
s.c.	subcutaneous
SDS	sodium dodecyl sulfate
SRBC	sheep red blood cells
Strep A	group A streptococci
Strep A-ELISA	streptococcal group A ELISA
T	thymine or thymidine
Tris	tris(hydroxymethyl)aminomethane
Tris-HCl	tris hydrochloride
UV	ultraviolet
Xgal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

FOR

MY FATHER AND MY MOTHER

FOR THEIR SUPPORT AND FRIENDSHIP

ABSTRACT

Antigenic mimicry or cross-reactivity between antigens of the group A streptococcus and human heart antigens may initiate an autoimmune response in certain individuals leading to cardiovascular damage and the production of heart reactive antibodies in rheumatic fever.

The objective of this study was to identify cross-reactive cardiac antigens, and to examine and compare the autoantibody profiles in seven groups of subjects. These groups, each of twenty individuals, were: children with acute rheumatic fever, rheumatic heart disease, acute glomerulonephritis and normal children; and adults with rheumatic heart disease, ischaemic heart disease and normal adults.

Enzyme-linked immunosorbent assays (ELISA) and immunocytochemistry were used to measure antibody levels to the following cardiac, nuclear and streptococcal antigens: crude extracts of human heart, myosin, actin, phosphorylase b, cardiolipin, type II collagen, nuclear antigens, DNA, heart muscle sections, whole group A streptococci and streptococcal M proteins.

It was shown that acute and chronic rheumatic fever patients have elevated titres of antibody to crude extracts of heart (as well as heart sections in immunocytochemistry), and to myosin, actin, phosphorylase b and nuclear antigens. Antibody levels to cardiolipin, collagen and DNA were not significantly raised. Antibodies to group A streptococcal antigens, particularly the M proteins, were also elevated in the groups of rheumatic fever patients. The finding that adult chronic rheumatic fever patients showed the highest levels of antibodies to several of these antigens suggested an underlying autoimmune polyclonal B cell activation response, or repeated exposure to streptococcal infection which may have contributed to the chronicity of the disease in these patients. It was noted that normal and related-disease control subjects also had antibodies to some of the tested antigens, although usually at much lower titres, indicating that these antibodies may form part of the natural/physiologic autoantibody repertoire. It is therefore uncertain whether these autoantibodies have a role in the pathogenesis of rheumatic fever.

Immunoblotting was used to determine whether rheumatic fever sera reacted with specific constituents in extracts of human heart, which were not recognised by control sera. Crude extracts of human heart as well as purified cardiac sarcolemmal membranes, nuclear extracts of cultured heart cells and cardiac myosin were examined.

The pattern of binding of IgM antibody (but not IgG and IgA) to constituents in crude extracts of human heart prepared using the detergent CHAPS, differed markedly in acute rheumatic fever sera (ARF) compared to chronic rheumatic

fever (CRF) and control groups of sera. CRF sera showed only IgG and IgA (but no IgM) binding to these extracts, which may be a consequence of a natural shift in antibody isotype production during chronic infection, or may represent a shift from natural IgM to pathological IgG autoantibody production. More specifically, IgM binding to a 33 kD doublet constituent in these extracts was virtually unique to ARF sera. Other cardiac antigens which appeared to be uniquely recognised by ARF sera were a 200 kD constituent in purified sarcolemmal membranes and a 140 kD nuclear antigen in extracts of cultured heart cells. Elution experiments showed some evidence of antibodies in ARF sera that were cross-reactive between a 38-40 kD antigen in crude heart extracts and streptococcal M5 protein.

The constituents recognised by these rheumatic fever patient sera, were also compared (in molecular weight) to constituents in similar heart extracts reported to be reactive with monoclonal antibodies and rabbit immune sera evoked by group A streptococcal antigens. The innovation of this immunoblotting study lay in the use of patient sera as opposed to such monoclonal antibodies and rabbit sera to identify cardiac antigens.

Human monoclonal antibodies were isolated from hybridomas developed by the fusion of lymphocytes from patients with acute rheumatic fever with mouse myeloma SP2 cells. These (IgM) monoclonal antibodies were selected for cross-reactivity between group A streptococcal antigens (by ELISA) and human heart tissue (by immunocytochemistry). However, they appeared to lack specificity, reacting with a wide range of cardiac, nuclear and bacterial antigens, and were possibly low affinity natural autoantibodies. One of the monoclonals showed strong binding to 43 kD constituents in both crude heart extracts and in purified sarcolemmal membranes. This constituent corresponds in molecular weight to a region in SDS and Triton X-100 extracts of heart reported to be reactive with murine monoclonal antibodies and rabbit immune sera evoked by group A streptococcal antigens, and may therefore be of relevance to rheumatic fever.

Two cDNA expression libraries in λgt11 were constructed and screened with rheumatic fever patient sera, using an alkaline phosphatase-conjugated antibody detection system. Three positive clones were detected using serum from an ARF (acute-on-chronic) patient with severe carditis and high titres of anti-streptococcal antibodies. The DNA from one of these clones was sequenced and showed 100 % homology with human cytokeratin 8, an α-helical coiled-coil protein, and 40-50% homology with human cardiac heavy chain myosin, tropomyosin and streptococcal M5 protein, which all have similar coiled-coil protein structures. Since keratin (antigenically related to cytokeratin) has been identified in other investigations as a member of one of two groups of autoantigens cross-reactive with streptococcal M5 and M6 proteins (as defined by human and murine monoclonal antibodies), this cardiac autoantigen may be of significance to rheumatic fever. Autoantibodies directed at such cytoskeletal

proteins are known to occur also in normal human sera, but this does not preclude their potential pathogenicity.

This study has therefore introduced a number of innovations into the investigation of antigenic mimicry in rheumatic fever by relying on patient sera and lymphocytes rather than on rabbit and murine products to identify cross-reactive cardiac antigens. It showed that rheumatic fever patients have autoantibodies to cardiac and nuclear antigens that differ from those of normal and related-disease control subjects. Specific cardiac antigens were identified both by immunoblotting and by immunoscreening of λgt11 cDNA expression libraries comprised of human heart cell genes. Antibody reactivity against these autoantigens may be unique to rheumatic fever sera and of significance to the pathogenesis or aetiology of the disease. In addition, this study highlighted a number of conceptual and practical difficulties in the study of antigenic mimicry and cross-reactivity in general.

1. INTRODUCTION

A. RHEUMATIC FEVER: OVERVIEW AND IMMUNOPATHOLOGY 2
1.A.1 Introduction 2
1.A.2 Incidence, Clinical Manifestations and Diagnosis 2
1.A.3 Pathogenic Concepts 4

B. ANTIGENIC MIMICRY, AUTOANTIBODIES AND AUTOIMMUNE DISEASE 6
1.B.1 Introduction 6
1.B.2 Antigenicity, Cross-reactivity & Antigenic Mimicry 6
1.B.3 Structural Properties of Cross-reactive Antigens 8
1.B.4 Autoantigens 10
1.B.5 Autoantibodies 11
1.B.5.1 Natural/Physiologic Autoantibodies 11
1.B.5.2 Origin of Autoantibodies 12
1.B.5.3 Pathogenic Autoantibodies 14
1.B.5.4 Polyclonal B cell Activation 15
1.B.6 Antigenic Mimicry and Autoimmune disease 16
1.B.7 Cross-reactivity: Serum and Monoclonal Antibodies 18

A. RHEUMATIC FEVER: OVERVIEW AND IMMUNOPATHOLOGY

1.A.1 Introduction

After more than 50 years of intensive investigation, the pathogenesis of rheumatic fever is still not understood. It is well-recognised that the group A streptococcus is the aetiologic agent, but the mechanism by which it induces pathology has not been elucidated.

Acute rheumatic fever has been described as, "a post-streptococcal disease characterised by nonsuppurative inflammation of various organs, most characteristically the heart" (Schaller and Szer, 1989). There are many excellent reviews on rheumatic fever and no point would be served in trying to emulate these (Zabriskie et al., 1971; Bisno, 1979, 1980; DiSciascio and Taranta, 1980; Zabriskie, 1982, 1985; Rotta, 1983; Senitzer and Freimer, 1984; Ayoub, 1984; Williams, 1985; Zabriskie and Gibofsky, 1986; Stollerman, 1988; Gibofsky and Zabriskie, 1988; Schaller and Szer, 1989).

This chapter presents a brief overview of the incidence, clinical manifestations and pathogenic concepts in rheumatic fever (section A), and then reviews some current concepts in autoimmunity - with particular reference to autoantibodies and antigenic mimicry, the hypothetical framework within which the investigations described in this thesis were performed (section B).

1.A.2 Incidence, Clinical Manifestations and Diagnosis

Although the incidence of rheumatic fever in most industrialised countries has declined markedly since 1950, the disease remains a major health problem in many third world countries, with an estimated 15-20 million new cases appearing each year.

The disease is certainly more common in impoverished living conditions, as reflected by reports of ongoing rheumatic fever in Soweto, South Africa (McLaren et al., 1975), in New Zealand (Stanhope, 1975), and in Egypt (Husby et al., 1976). Among Black South African school children the overall prevalence rate was shown to be 7 per 1000 (McLaren et al., 1975). However, recent resurgences of rheumatic fever in affluent communities in the USA (Hosier et al., 1987), indicate that improved socioeconomic and medical conditions alone, cannot account for the decline of the disease in industrialised countries. Neither can the use of antibiotics solely account for this decline, since it was already apparent before the introduction of penicillin.

There is considerable variation in the clinical manifestations of the disease - carditis, arthritis, chorea, erythema marginatum and subcutaneous nodules - which occur with varying degrees of severity. The first attack usually occurs in

children between the ages of 5 and 15 years of age, after repeated group A streptococcal pharyngeal infections.

The most serious consequence and characteristic feature, as well as the only permanently damaging manifestation, is carditis which may involve endocardium, myocardium and pericardium. About one third of first-attack patients have carditis, but the incidence of cardiac involvement increases with recurrent attacks (to about 50%). Arthritis is the most common manifestation, occurring in about three fourths of patients during the initial attack, whereas chorea (a neurologic manifestation involving caudate nucleii in the brain) occurs in about 8-23% of patients and has a longer latent period (one to six months) than the other manifestations (Feinstein and Spagnuolo, 1962; Massell et al., 1958). Manifestations affecting the skin are subcutaneous nodules and erythema marginatum, both usually only seen in patients with carditis (Feinstein and Spagnuolo, 1962; Massell et al., 1964).

The modified Jones Criteria (Table 1.1) are widely accepted as valuable guidelines in the diagnosis of rheumatic fever. There is no single diagnostic or laboratory test for the disease, and the various manifestations can be ambiguous and sometimes difficult to distinguish from those of other diseases.

TABLE 1.1 *(American Heart Association: Committee Report 1984)*

Jones Criteria (Revised) for Guidance in the Diagnosis of Rheumatic Fever

Major Manifestations	**Minor Manifestations**
	Clinical
Carditis	Previous rheumatic fever or rheumatic heart disease
Polyarthritis	Arthralgia
Chorea	Fever
Erythema marginatum	
Subcutaneous nodules	
	Laboratory
	Acute phase reactions: elevated erythrocyte sedimentation rate, C-reactive protein, leukocytosis
	Prolonged P-R interval

PLUS

Supporting evidence of preceding streptococcal infection (increased ASO or other streptococcal antibodies; positive throat culture for Group A streptococcus; recent scarlet fever).

The presence of two major criteria, or of one major and two minor criteria, indicates a high probability of the presence of rheumatic fever if supported by evidence of a preceding streptococcal infection. The absence of the latter should make the diagnosis doubtful, except in situations in which rheumatic fever is first discovered after a long latent period from the antecedent infection (e.g. Sydenham's chorea or low-grade carditis).

Two major criteria, or one major and one minor criterion, are highly suggestive, but not diagnostic, of rheumatic fever. In addition, it is important to establish the presence of an antecedent group A streptococcal infection (usually by demonstration of increased titres of streptococcal antibodies). (Chorea, as the sole manifestation, can usually be accepted as diagnostic).

1.A.3 Pathogenic Concepts

Gibofsky and Zabriskie (1988) list the following reasons for the slow progress in elucidating the pathogenesis of rheumatic fever: (1) the lack of a suitable animal or other experimental model; (2) the difficulty in deciphering which of the group A streptococcal products capable of causing tissue injury, are actually implicated in pathogenesis; (3) the long latency period between the initial streptococcal infection and the appearance of clinical and pathologic signs and symptoms, which restricts prospective studies.

The initial group A streptococcal infection of the throat is essential to pathogenesis. Although streptococci may be isolated from the skin or pyodermal lesions of patients with acute rheumatic fever, these are not associated with pathogenesis, nor have other groups of streptococci or other organisms been implicated in the disease. Furthermore, it appears that several group A streptococcal pharyngeal infections are needed before complications develop, since attacks of rheumatic fever rarely occur in children under 3 years of age (Rosenthal et al., 1968). Group A streptococcal infection of the upper respiratory tract appears therefore to hold important clues to the pathogenesis of rheumatic fever.

Hypotheses explaining this link of *S.pyogenes* to pathogenesis are listed by Schaller and Szer (1988): (1) rheumatic fever is caused by the persistence of *S.pyogenes* bacteria in the patient; (2) rheumatic fever is caused by the toxic intra-or extracellular products or components of *S.pyogenes*; (3) rheumatic fever is caused by a hypersensitivity or autoimmune reaction sparked off by group A streptococci; (4) rheumatic fever is a consequence of antigenic mimicry and partial tolerance between streptococcal and cardiac antigens.

There is no evidence to suggest that persistent group A streptococcal infections cause rheumatic fever; nor have the toxic products of the organism been directly implicated in pathogenesis; and hypersensitivity reactions exhibit progressively shorter latency periods than those seen in rheumatic fever. For such reasons (and others) theories of autoimmunity and antigenic mimicry between streptococcal and human antigens have appeared more credible.

The demonstration, in 60-80% of patients with acute rheumatic fever, of autoantibodies against cardiac tissue that cross-react with group A streptococcal antigens (Kaplan et al., 1961; Hess et al., 1964), as well as the deposition of IgG antibodies and complement in myocardial and valvular tissue

(evident by immunofluorescence) in some rheumatic fever patients (Kaplan and Svec, 1964), has been propounded as evidence of an autoimmune pathogenic mechanism. Difficulties with this theory are, (1) that the reactions are not entirely specific for heart tissue nor unique to rheumatic fever; (2) that it has not been established whether the cross-reactive antibodies and the depositions in the heart are the cause or result of pathology; (3) that the cross-reactive antigens, particularly the cardiac antigens, are still poorly defined and some of the findings have been contested.

The cross-reactivity between a human heart valve glycoprotein and *S.pyogenes* polysaccharides (Goldstein et al., 1967) has been contested by Kasp-Grochowska et al., 1972), although more recent evidence suggests that such cross-reactions may initiate recurrent attacks of rheumatic fever (Gowrishkar and Agarwal, 1980; Appleton, 1985). Regarding myosin as a cross-reactive autoantigen, the presence of autoantibodies to this protein in several other diseases (see Chapters 2 and 3), casts an element of doubt on its pathogenicity in rheumatic fever. A causative role for a cellular autoimmune mechanism in rheumatic fever has also not been proven (McLaughlin et al., 1972; Dale and Beachey, 1981) although more recent studies are quite convincing (Hutto and Ayoub, 1980; Kotb et al., 1989).

Part of the problem in defining causative factors in rheumatic fever, may be due to confounding genetic variables. The fact that only 1-3% of all patients with group A streptococcal pharyngitis develop rheumatic fever, seems to suggest a genetic predisposition or host factor(s) in such patients. However, HLA linkage has not been established (Murray et al., 1978; Falk et al., 1973; Ward et al., 1976). Studies of identical twins have shown a concordance rate of less than 20 %, which is lower than that found in twins with infectious diseases such as tuberculosis (Stollerman, 1975, 1986; Taranta, 1976). The most convincing evidence of a genetic factor for susceptibility to rheumatic fever, has been the detection of a B cell alloantigen (designated 883) in 75% of rheumatic fever patients compared to 16.5% of normal subjects (Pattarroyo et al., 1979). This antigen, defined by a monoclonal antibody (D8/17) is not associated with the human MHC system, and has an autosomal recessive mode of inheritance (Khanna et al., 1989).

Rheumatic fever may therefore involve a subtle "interplay between host, genetics and microbe" (Zabriskie, 1985). Such an interplay may manifest in molecular mimicry between antigens expressed by the group A streptococcus and human host genetic systems. How specific microbial antigens might initiate an autoimmune response in only certain hosts leading to cardiovascular disease, is an intriguing question presumably of relevance to other rheumatic and autoimmune disorders. The ensuing discussion reviews the role of antigenic mimicry and autoantibodies in autoimmune disease.

B. ANTIGENIC MIMICRY, AUTOANTIBODIES AND AUTOIMMUNE DISEASE

1.B.1 Introduction

The term "autoimmunity" refers to the reaction of the immune system with the host's own tissues. Autoimmunity could be the natural consequence of self-tolerance (for the immune system to "know itself" it must have "seen itself"), and according to present concepts self-reactive T cells and autoantibodies are integral components of the normal immune repertoire.

In some instances, autoimmunity can lead to disease. One way in which this can happen is through "antigenic mimicry", a term originally coined to refer to, "that particular situation in which microbes and mammalian tissues share antigenic determinants" (Zabriskie, 1982). This review is a discussion of some concepts in autoimmunity, and in particular of the role and nature of autoantibodies and autoantigens in provoking disease through the mechanism of antigenic mimicry (or cross-reactivity).

1.B.2 Antigenicity, Cross-reactivity & Antigenic Mimicry

Cross-reactivity has been defined as, "the ability to react with ligands other than the immunogen" (Bersofsky et al., 1989). Although one may speak of cross-reactive antibodies, more usually cross-reactivity is discussed from the point of view of the antigens. Cross-reactivity can only be defined with reference to a corresponding antibody or antiserum. Two antigens are said to be cross-reactive because they bind to the same antibody.

Cross-reactivity between two antigens does not imply any functional or evolutionary relationship between them. The presence of similar epitopes on two proteins may be entirely fortuitous. The scope for cross-reactivity is immense when one considers that an antibody can recognize a determinant as short as 4-7 amino acids (Atassi, 1975; Crawford et al., 1982). It has been calculated that, assuming all the amino acids are randomly distributed, a particular sequence of four amino acids would appear once in every 20^4 tetramers, a particular pentamer once in every 20^5 pentamers, and a particular sequence of six amino acids once in every 10^6 hexamers (Atassi, 1975).

Wilson et al. (1984) searched over 2500 amino acid sequences in a particular protein data base found 2469 hexamers, 186 septamers and 17 octamers with homologies. Cross-reactions defined particularly by monoclonal antibodies could be quite common between such sequences. Srinivasappa et al. (1986) examined the cross-reactivity with host tissues of over 600 mAbs to various viral polypeptides and found that 4% of these mAbs cross-reacted with determinants expressed on uninfected host tissues. These data demonstrate that cross-reactivity (antigenic mimicry) occurs quite frequently.

Besides such perfect homologies, two unidentical but similar epitopes may display cross-reactivity by binding the same antibody with different affinities (Lane and Koprowski, 1982). This increases the spectrum of potential cross-reactions and may explain the phenomenon of multiple organ reactivity reported with mouse and human monoclonal antibodies (Haspel et al., 1983; Satoh et al., 1983). Antibodies were shown to recognize either the identical molecule in different organs or else similar epitopes on unidentical molecules in different organs.

Conceivably, also similarities in the aggregate effect of the charged amino acid groups of two antigens, could make them cross-reactive. Such an interaction could occur heedless of the exact sequence of amino acids (homologous between their epitopes). Kabat (1986), investigating cross-reactivity between N-acetyl-neuraminic acid and denatured DNA, suggested that "similarities and differences in *charge distribution* could be responsible for immunological cross-reactions among what have been generally considered diverse and structurally unrelated substances".

In assessing the potential for cross-reactivity, account should also be taken of the effect of the interacting surface of the antibody. Contrary to static lock-and-key models of molecular recognition, antibody and antigen molecules have some flexibility to exist in different conformations, and can cooperate in such a way as to induce a better complementary fit. Such a process may involve side-chain movements of the antigen and possibly also of the backbone conformation (Getzoff et al., 1988). Strickland et al. (1987) reported a mutant antibody that had lost its original idiotope but expressed it again after binding to another anti-idiotype. This effect may have been induced by a conformational change in the antibody.

Another source of potential cross-reactivity in the antigen-antibody interaction lies in the polyreactive nature of antibodies (Van Regenmortel, 1989). The polyvalency of the antibody binding site may contribute to permitting the antibody to cross-react with various closely related epitopes or haptens (Talmage, 1959; Cameron and Erlanger, 1977).

Assay dependent variables can have an influence on the *in vitro* detection of cross-reactions. Van Regenmortel (1989) has argued that most studies purporting to measure "antigenicity *per se*" are instead actually measuring "cross-reactive antigenicity". Except for X-ray crystallography which visually portrays the spatial arrangements of the antibody-antigen interaction, most investigations are based on functional assays involving operationally-defined binding measurements. Such studies are dependent on assay conditions which can vary and thus have a determining influence on whether particular residues are interpreted as being part of a specific epitope. Such functionally-defined epitopes are usually comprised of only 3-8 residues whereas epitopes

structurally defined by crystallographic analysis, comprise on average 15 residues. The smaller average number of residues comprising functional epitopes thus exerts a major bias towards detection of cross-reactions.

Experimentally determined cross-reactions may have an artefactual component, and therefore caution is needed in interpreting their biological significance. For instance, although antigen is recognised *in vivo* in a denatured state, when it sticks to plastic in solid-phase assays it may fold or denature differently so as to create an artefactual set of (conformational) epitopes that cross-react spuriously.

Some cross-reactions can have survival value for the organism. Cross-reactivity in the form of antigenic mimicry between parasites and their hosts can confer on the parasite "a degree of molecular camouflage against the host's immune system" (Damian, 1987), thereby promoting its survival in the host. An example is the homology between *Plasmodium falciparum* antigens containing repetitive amino acid sequences that cross-react with the host protein thymosin-Á1 (a thymus peptide hormone implicated in T cell maturation). The similarity of the parasite's antigens to those of the host could lead to a weakening of the antibody response, or possibly even interference in the host's immune response (Dubois et al., 1988). Similarly, in rheumatic fever it is possible that antigenic mimicry between streptococcal M proteins and human heart antigens such as myosin and sarcolemmal membrane antigens, may have the effect of weakening the immune response to the streptococcal pathogen.

The fact that such antigens often do provoke an (auto)antibody (or T cell) response, is perhaps more surprising than elusion of the host's defence system due to the mimicry of autoantigens. How a microbial antigen can break the host's self-tolerance by mimicking a self-antigen, and trigger autoimmune disease, is a question that needs to be answered to understand how antigenic mimicry leads to autoimmune disease.

Discrimination between self and non-self is central to autoimmune disease, which represents a breakdown in such discernment. Antigenic mimicry could provoke such a breakdown.

1.B.3 Structural Properties of Cross-reactive Antigens

Are there structural features of antigens that make them more cross-reactive?

Antigens with regularly repeating structures and high epitope densities such as bacterial lipopolysaccharides (LPS) and DNA tend to be implicated in cross-reactions. Bacterial LPS is highly antigenic and elicits IgG and IgM monoclonal antibodies, whereas DNA is weakly immunogenic and generally elicits IgM monoclonals (Ghosh and Campbell, 1986). DNA often cross-reacts with

proteins, particularly cell surface proteins and with haptens, and cross-reactivity with cardiolipin is known to be due to the similarity in spacing between two phosphate groupings (Rauch et al., 1984). The high density of epitopes on both DNA and LPS is likely to generate low affinity antibodies. Such low affinity cross-reactions are readily detectable in sensitive laboratory assays and may be biologically irrelevant.

Similarly, some eukaryotic structural proteins with tandemly repeated sequences such as myosin (DeLozanne et al., 1985), intermediate filaments (Marchuk et al., 1984), nuclear lamins (Fisher et al., 1986) and tropomyosin (Stone and Smillie, 1978) have been implicated in cross-reactions. These are all coiled-coil proteins sharing a common structure comprised of tandem repeats of 7 amino acids, with hydrophobic residues at the first and fourth positions. Of these proteins, myosin and tropomyosin have been shown to cross-react with streptococcal M proteins implicated in rheumatic fever (Dale and Beachey, 1986; Fenderson et al., 1989).

Polymorphism is a second feature associated with increased cross-reactivity of antigens. Polymorphic antigens such as the histocompatibility antigens tend to elicit antibodies capable of binding similar but not identical antigens. A monoclonal antibody against HLA-B27 (the Class I MHC antigen associated with Reiter's disease and ankylosing arthritis) was shown to be cross-reactive with an antigen on *Yersinia pseudotuberculosis* (Chen et al., 1987). Similarly, some *Klebsiella* bacteria have been shown to cross-react with HLA-B27 (Ogasawara et al., 1986). How such cross-reactions trigger autoimmune arthritic lesions is not known; however this is only one of several possible mechanisms by which these lesions may be induced.

Ghosh and Campbell (1986) list the following features of antigens and antibodies which can make them more susceptible to irrelevant cross-reactions under experimental conditions (especially in solid phase binding assays): (1) antibodies of low affinity for the original immunogen have an increased potential for irrelevant cross-reactions; (2) antibodies of the IgM class, due to their multivalence, have a higher potential for nonspecific cross-reactivity; (3) the presence of Fc receptors on cell surfaces can bind the constant region of IgG antibodies nonspecifically; (4) antigens with a repeating structure or present at high concentrations in the assay, have an increased potential for nonspecific cross-reactivity. Genuine cross-reactions are experimentally best verified by kinetic analyses of the intrinsic affinities between antibodies and antigens, and by the reproducibility of the cross-reaction under identical conditions using pure antibody and identical amounts of the two antigens.

Genuine cross-reactions may be fortuitous and biologically irrelevant, or they may be "a reflection of functional or evolutionary relationships between the cross-reacting proteins" (Nigg et al., 1982). Antigenic mimicry may aid parasites and infective bacteria in eluding the host's immune system (Damian,

1987), and it is thought that such mimicry between *S.pyogenes* M proteins and human antigens assists the bacteria in eluding phagocytosis in rheumatic fever. In some instances, cross-reactivity may lead to autoimmune disease with production of autoantibodies (and possibly autoreactive T cells). The ensuing discussion examines the role of autoantigens and autoantibodies in provoking (or indeed preventing) autoimmune disease.

1.B.4 Autoantigens

Autoantigens are the self-antigens to which autoantibodies bind. They are not restricted to any particular macromolecular species, but may be proteins, nucleic acids, carbohydrates, glycoproteins, lipoproteins, phospholipids or steroids. They tend to be phylogenetically conserved rather than autospecific, and can be grouped into ubiquitous or organ specific autoantigens. Examples of ubiquitous autoantigens are nuclear antigens such as ribonucleoproteins and nucleic acids that are present in various eukaryotic cell types as well as in bacteria. Organ (or cell) specific autoantigens are acetylcholine receptors, insulin and less well-defined determinants on platelets and pancreatic B cells. Some highly conserved autoantigens control vital cellular functions such as DNA repair and RNA splicing (Tan, 1989), but the autoantibodies to these antigens do not appear to disrupt cellular function.

Foreign proteins can also elicit autoantibodies. For example, Nisonoff et al. (1967) showed that rabbits immunized with mouse cytochrome c produced antibodies not only to the "foreign" murine epitopes, but also contained autoantibodies to rabbit cytochrome c. The highly conserved nature of autoantigens is borne out by similar experiments in which rabbit antisera to cytochrome c were shown to bind the same stretch of residues in all mammalian cytochrome c's (Jemmerson et al., 1985).

By what mechanisms might autoantigens trigger an immune response? Bach (1989) described five possible mechanisms: (1) sequestered autoantigens or those appearing late in development may be recognised as foreign because they were not exposed to the immune system during embryonic life for the self-tolerance to be established; (2) autoantigens may become immunogenic through modification by chemical or infectious agents; (3) cross-reactions with foreign determinants may permit T cell help for production of antibodies against identical or similar autoantigens; (4) autoantigen modification may be endogenous (as in the case of abnormal IgG glycosylation patterns of RA patients); (5) autoantigens may be rendered immunogenic through their association with aberrantly expressed class II MHC products.

Weckerle (reported in Cohen, 1989) recently defined three characteristics of pathogenic autoantigens pertaining specifically to experimental autoimmune encephalomyelitis (EAE): (1) the autoantigen should be presentable by tissue cells at the site of the disease (2) the autoantigen elicits a strong T-cell

response under the control of Ir (MHC) genes; (3) autoantigens can evoke a cytotoxic T-cell response of either CD4 or CD8 phenotype.

The pathogenicity of a particular autoantigen may not always be immediately evident. Conceivably, an immunogen might cause the release of a sequestered autoantigen which could evoke a chronic autoimmune response - what Oldstone (1987) called a "hit-and-run event". A released "sequestered antigen" may elicit autoantibodies and mediate other forms of immunological assault on the tissue, without itself having been the initial immunologic target. Zabriskie (1982) propounded such an hypothesis for rheumatic fever. He postulated that "crypto-antigens" could be uncovered following an inflammatory insult to the heart by streptococcal antigens, initiating a "...vicious cycle of damage...(that) would generate a chronic autoimmune process, perhaps exacerbated by intercurrent microbial infections".

The presence of pathogenic autoantigens prompts the question as to how natural or innate tolerance of autoantigens is achieved and maintained. For tolerance to be achieved under physiological conditions, it is thought that an autoantigen must at some stage have been in contact with lymphocytes and should therefore be potentially immunogenic. It is plausible that B and T cells sensitized to the autoantigen could be held in check by suppressor T cells that have also "seen" the antigen (Harris et al., 1982; Cairns et al., 1986). There is however some controversy about such a role for suppressor T cells and even about their existence (Sercarz, 1987). Coutinho (reported in Cohen, 1989) has proposed an alternative hypothesis whereby self-tolerance is maintained paradoxically by self-reactivity or "self-assertion": by interconnected autoantibodies and lymphocytes that recognize each other and autoantigens and thereby maintain a dynamic equilibrium.

1.B.5 Autoantibodies

1.B.5.1 Natural/Physiologic Autoantibodies

Avrameas (1986) has argued that it is through recognition of autoantigens that the immune system derives its ability to recognize and respond to foreign non-self antigens. Whereas it was previously thought that recognition of self would lead to self-destruction through autoimmune disease (Burnet, 1959), it is now certain that such recognition is an integral part of normal immune function. Natural autoantibodies are thought to be a healthy component of the normal immune repertoire.

Cohen (1984) has termed this form of immune self-recognition "physiologic autoimmunity" to distinguish it from "pernicious autoimmunity", or pathologic autoimmunity in which recognition of self is destructive. Two examples of

physiologic autoimmunity essential to the normal function of the immune system are the following.

1. *Recognition of self antigens of the MHC.* This system differs from other forms of signal transfer between cells (such as those regulating the endocrine system) in that it is a form of true adaptive immunity. The receptors that recognise self MHC are clonally distributed among classes of lymphocytes, and subpopulations of T cells which recognize an antigen in association with a particular MHC allelic product as a self signal for cell cooperation. Furthermore, as a signal for cell cooperation, the MHC responds to a particular environment of differentiated lymphocytes with an adaptive flexibility that is different to physiologic recognition systems (Cohen , 1984).

2. *The anti-idiotypic network.* In this system, idiotypic antibodies can be recognised as self-antigens, and their expression regulated, by anti-idiotypic antibodies (Eichmann, 1978; Binz et al., 1976). The anti-idiotypic response thus entails self-recognition that can be considered as a form of physiologic autoimmunity conducive to the healthy function of the immune system (Cohen, 1984).

Natural autoantibodies could be considered another example of physiologic autoimmunity. Typically these antibodies bind highly conserved self-antigens such as albumen, nucleic acids, cytoplasmic filaments, cytochrome C, collagen and actin (Guilbert et al., 1982; Avrameas et al., 1983). They are mostly IgM antibodies, have a low affinity for autoantigens and display widespread cross-reactivity even with bacterial antigens and haptens (Avrameas et al., 1983).

Their presence in hybridomas derived even from normal, newborn, germ-free mice suggests that natural autoantibodies may be fundamental to the normal function of the immune system. Although they may possibly be the result of V-gene rearrangements, random mutations and junctional imprecisions (Schwartz, 1986), their evolutionary conservation suggests a biological role for them. Hypotheses explaining their function include the following: (1) they act as a first line of defence against invading pathogens (Avrameas et al., 1983); (2) they may be involved in the disposal of catabolic products from the organism (Grabar et al., 1983); (3) they may be the precursors of antibodies to exogenous antigens - their cross-reactivity with these exogenous antigens may give the immune system a potential advantage in eliminating them (Naparstek et al., 1986) (4) they may blind the immune system to self antigens, thereby preventing autoimmune disease that might occur through mimicry with invasive bacterial epitopes (Cohen and Cooke, 1986); (5) they may play a regulatory role in the humoral immune response (Mahana et al., 1988).

1.B.5.2 Origin of Autoantibodies

What is the source of these natural autoantibodies? Possibly through random V gene rearrangements producing the immensely diverse antibody repertoire, some variable regions are created capable of binding autoantigens. The presence of autoantibody-producing B cells indicates that such recombinations are expressed and form part of the actual V gene repertoire.

It appears that at least some physiologic autoantibodies are encoded by germline V genes . There do not however appear to be abnormalities or disease-specific V genes in the antibody repertoire, accounting for autoantibodies. Analyses of over 100 autoantibodies deriving from different V gene families has not revealed any particular bias. In addition, analysis of V gene sequences of autoantibodies to determine whether these antibodies arise by mutation, indicates that they can arise from unmutated germline genes (Reininger et al., 1987)

The $CD5^+$ (Leu-1) subset of human B cells (and their $Ly\text{-}1^+$ B murine counterparts) appear committed to the production of polyreactive antibodies that bear many features of autoantibodies (Casali et al., 1987; Casali et al., 1988; Casali and Notkins, 1989). This is not the sole source of autoantibodies since it is known that $Ly\text{-}1^-$ and $CD5^-$ cells also produce them. However, the polyreactive antibodies produced by $CD5^+/Ly\text{-}1^+$ cells are similar to autoantibodies in being mostly IgM, and in binding with low intrinsic affinity to a range of self and exogenous antigens including ssDNA and rheumatoid factor (Casali et al., 1987, 1988). Although, the antibodies produced by $CD5^+/Ly\text{-}1^+$ cells do bind autoantigens, this is only one of their functions (Casali and Notkins, 1989).

The association of $CD5^+/Ly\text{-}1^+$ cells with pathogenic autoantibodies and autoimmune disease, is based on the striking increase in their numbers in autoimmune disorders such as rheumatoid arthritis, in humans and in lupus-prone NZB mice. In RA these cells constitute 27-52 % of the circulating B cells (Hardy et al., 1987; Burastero et al., 1988) and in lupus-prone NZB mice they are increased 5-to 10 fold (Hayakawa et al., 1984). However, the increase in $CD5^+$ cells in RA could not be correlated with clinical disease activity (Plater-Zyberk, et al., 1985). Furthermore, there is no increase in the proportion of $CD5^+$ cells in SLE and, in these patients, high affinity IgG anti-ssDNA antibodies are produced by $CD5^-$ cells. The polyreactive antibodies produced by their $CD5^+$ cells are similar to those found in healthy subjects. It would seem that polyreactive low affinity autoantibodies produced by $CD5^+$ cells are functionally different to the monoreactive, high affinity autoantibodies characteristic of various autoimmune diseases. Possibly, pathogenic autoantibodies derive from lymphocytes that have been positively selected through somatic point mutations, and are a result of an antigen-driven process. It seems in any case unlikely that $CD5^+$\$Ly\text{-}1^+$ cells by themselves cause the

development of autoimmune disease, but they probably do play a role as a first line of defence in a primary, low affinity, IgM response against infectious pathogens.

In summary, $CD5^+$ \Ly-1^+ cells are one source of autoantibodies but not the exclusive source. It has been suggested that they could be the major source of physiologic natural autoantibodies whereas pathogenic autoantibodies could derive from mature $CD5^-$/ly-1^- cells (Bach, 1986), but there is also evidence of pathogenic autoantibodies deriving from Ly-1^+ cells (Stall et al., 1986).

1.B.5.3 Pathogenic Autoantibodies

Pathogenic autoantibodies are usually IgG, of higher affinity and frequently express private idiotypes (Tron et al., 1982, 1983), in contrast to physiologic autoantibodies which are IgM, polyspecific, encoded by germline genes and more often express public idiotypes. Whether the postulated difference in pathogenicity between the two types of autoantibody is a function of differing affinities or specificities, and whether autoantigen triggers formation of pathogenic autoantibodies, are unanswered questions.

Autoantibodies may be produced by polyclonal B cell activation. In systemic autoimmune diseases, such B cell activation occurs frequently, although in organ specific autoimmune diseases autoreactive clones to specific antigens proliferate preferentially (Klinman and Steinberg, 1987; Dziarski, 1988). It is known that *in vitro* polyclonal B cell activation does lead to formation of autoantibodies such as anti-DNA antibodies, rheumatoid factor and anti-erythrocyte antibodies (Schwartz and Datta, 1989). B cells could possibly be activated through mitogenic stimulation, through T cell lymphokines or may be inherently different to other B cells. Bacteria and parasites can produce mitogens, and the autoantibodies produced in trypanosomiasis (Kazyumba et al., 1986) and malaria (Playfair, 1982) are probably the result of B cell polyclonal activation.

The correlation of autoantibodies with autoimmune diseases does not imply that they are pathogenic. Indeed, as Cohen and Cooke (1986) argue they may play a protective role. Possibly, different mechanisms of B cell activation may produce either physiologic or pathogenic autoantibodies. It has been suggested that polyclonal activation of B cells might increase production of physiologic autoantibodies, whereas T-cell dependent activation may be necessary for generation of pathogenic IgG autoantibodies (Schwartz and Datta, 1989).

Do autoantibodies associated with particular autoimmune disorders, actually cause the disease? Schwartz and Datta (1989) have suggested five methods (in descending order of experimental rigor) for determining the pathogenicity of autoantibodies: (1) reproduction of the lesion through administration of the autoantibody to healthy control subjects (2) production of lesions by

immunization with the purified autoantigen (3) simulation of lesions *in vitro* (4) isolation from the disease lesions of the relevant autoantibody (5) correlation of autoantibody levels with clinical disease activity.

Induction of autoimmune lesions in animals through injection of the relevant human autoantibodies, has been demonstrated in the case of pemphigous vulgaris in mice (Anhalt et al., 1982) and in Goodpasture's syndrome where autoantibodies specific for glomerular capillary basement membrane were shown to bind glomerular membranes in monkeys in identical fashion to the human lesions (Wilson and Dixon, 1973). Induction of autoimmune disease through injection of the autoantigen, however, applies only to organ specific diseases. It is well-established now that immunization of mice with thyroglobulin can produce inflammatory lesions in the thyroid, that resemble those seen in Hashimoto's thyroiditis. Similarly, immunization with the autoantigen myelin can produce encephalomyelitis in animals. On the other hand, it has not been possible to induce non-organ specific (systemic) autoimmune diseases (e.g. SLE) in animals by immunization with any of the implicated autoantigens. Possibly the correct autoantigens have not been tried or the animals used were not susceptible to the disease; or else the autoantibodies may be non-pathogenic, arising from polyclonal B cell activation consequent upon some prior pathogenic event.

The presence of autoantibodies in disease lesions can be an important clue to their pathogenicity. In acute rheumatic fever there is mass deposition of immunoglobulin along sarcolemmal membrane, but there is no evidence that these are autoantibodies. In contrast, the immunoglobulin isolated from kidneys of patients with lupus glomerulonephritis are autoantibodies. Although some autoantibodies correlate with development of clinical disease (e.g. anti-DNA antibodies in SLE), others, though regularly associated with the disease (e.g. rheumatoid factor in rheumatoid arthritis), cannot be shown to cause lesions.

Some of the mechanisms by which autoantibodies operate to provoke autoimmune disease are through opsonization, immune complex formation, complement-dependent cell cytotoxicity, receptor blockade or stimulation.

1.B.5.4 Polyclonal B cell Activation

The control exerted by the immune system through tolerance and suppression on B cells, is not as stringent as that exerted on T cells. Nonetheless, despite the nonspecific expansion of B cells, autoantibodies do display preferential specificities in many autoimmune diseases. A more truthful representation of autoantibody production might therefore combine models of polyclonal B cell activation and induction of such antibodies by autoantigen.

Dziarski (1988) has proposed such a combined model: individuals prone to autoimmune disease may have B cells intrinsically more sensitive to polyclonal activators (such as mitogens) and accessory signals (such as lymphokines); these activators may stimulate the cells to differentiate into polyclonal antibody-(and autoantibody) secreting cells; finally, autoantigens and lymphokines (possibly more readily "available" in autoimmune-prone individuals) may expand these clones by an antigen-driven selection process, at the same time causing a switch to IgG and IgA production. More simply, Ishigatsubo et al. (1988) have suggested that possibly the effects of polyclonal B cell activation "create an environment in which autoantigens are better able to magnify and perpetuate the production of specific autoantibodies". Polyclonal activation may be a primitive form of immunity that immediately produces a low affinity cross-reactive antibody before maturation of specific, high-affinity antibodies (Dziarski, 1988).

Whatever the mechanism, it is puzzling that polyclonal expansion of B cells should lead to development of nephritis in one SLE patient, and to thrombocytopenia in another; or, perhaps analogously, to carditis in one rheumatic fever patient and chorea (without any cardiac involvement) in another. Why do not all SLE patients produce anti-Sm antibodies; why do only 60-80% of ARF patients have antibody to heart antigens? Possibly such variable manifestations reflect an interplay between genetic and environmental factors in these diseases.

1.B.6 Antigenic Mimicry and Autoimmune disease

Antigenic mimicry can provoke an autoimmune response only when the foreign antigen is at once sufficiently similar to the host (auto)antigen to be cross-reactive, and yet dissimilar enough to break the host's immunologic self-tolerance.

This suggests a more complex relationship between foreign and host antigen than can be gleaned from their amino acid sequences. For instance, it has been suggested that the cross-reactive foreign epitopes might consist of a "self" (similar) domain binding the B cell, and a "foreign" (dissimilar) domain that binds a T cell receptor and thereby subverts tolerance (Schwartz and Datta, 1989). Another suggestion, is that a foreign antigen may mimic conformations of autoantigens without mimicking their peptide sequences (Cohen, 1989).

Another example of a complex interaction between foreign and self antigen was shown by Stetler and Jacob (1985). Rabbits immunized with purified RNA polymerase enzyme developed not only antibodies to this enzyme but also distinct non-crossreactive population of antibodies reacting with nucleic acids (including DNA). The lack of shared epitopes between RNA polymerase and

nucleic acids, suggests that the immunogenicity of the latter derives from physical association with the former.

Antigenic mimicry can induce an autoimmune response without provoking disease. For example, a 10-residue peptide of myelin basic protein was demonstrated to share homology with a six-residue section of hepatitis B virus polymerase, and antibodies raised in rabbits to the latter viral protein cross-reacted with myelin basic protein, but the animals developed no signs of the disease (Fujinami and Oldstone, 1985).

Antigenic mimicry may require only a "hit-and-run" event by an immunogen (such as a virus) to induce a cross-reaction that perpetuates immunologically mediated tissue injury (Oldstone and Notkins, 1986). After clearance of the foreign antigen, components of the immune system could continue to assault host tissue, leading to release of more host antigens and production of antibodies, and so on. Alternatively such a "hit-and-run" event may release sequestered immunologically privileged antigens that could induce and perpetuate an autoimmune response in the host. Another scenario of a chronic or progressive autoimmune response, could occur with viruses or parasites that cyclically or continuously express cross-reactive (virulent) antigens while persisting in host tissues.

By way of contrast, an example of antigenic mimicry in which there is a high degree of sequence homology between foreign and self antigens, is found between host and pathogen heat shock proteins (HSP). It has been shown that a population of human T cells specific for the 65 kD heat shock protein of *Myocbacterium leprae* could also recognize the kindred human HSP which is possibly preferentially expressed in stressed monocytes-macrophages (report by Polla and Young, 1989). Such stressed human cells could upregulate their HSPs which might become available for recognition by T cells or antibodies, and induce an autoimmune response. Homology of the pathogen's HSP with that of the host could have evolutionary significance in rendering the pathogen less vulnerable to environmental stresses inside the host cell (Polla and Young, 1989).

Are there then any particular characteristics that pathogenic autoantigens have in common? One enigma concerns the dominance of particular autoantigens (e.g. HSP65, myelin basic protein and thyroglobulin). Why in the pancreas are only the B cells of the islets attacked; why of all the multifarious antigens in the thyroid, are only thyroid peroxidase, thyroid stimulating hormone and thyroglobulin implicated in disease? This bias towards particular autoantigens illustrates what Cohen (1989) refers to as, "the unexpected regularity of autoimmunity", and it hints at a more complex interaction between foreign antigen and components of the immune system, that cannot be gleaned from structural considerations of the antigens alone.

One such immunodominant autoantigen may be myosin. Structural features of this muscle protein, and some related molecules, may play a role in determining their dominance as autoantigens. Molecules like myosin, tropomyosin, nuclear lamins and intermediate filaments, all have tandemly repeated structures which may be highly immunogenic and thus be targets of an autoimmune response (Dropcho et al., 1987).

Myosin has been implicated as a cross-reactive autoantigen in rheumatic fever (Krisher and Cunningham, 1985; Dale and Beachey, 1985; Cunningham et al, 1986; Dale and Beachey, 1986), and as a non-crossreactive autoantigen in Coxsackie B3-induced autoimmune myocarditis (Alvarez et al., 1987; Rose et. al., 1988). In addition, antibodies to myosin have been found in patients following cardiac surgery (De Scheerder, 1985).

Other dominant autoantigens include keratin (Cunningham et al., 1984; Fenderson et al., 1989) which is also found as an autoantigen in rheumatic fever; vimentin which cross-reacts with measles phosphoprotein (Fujinami et al., 1983) and also shares homology with vaccinia virus haemagglutinin (Dales et al., 1983); and heat shock proteins (Sheshberadaran and Norrby, 1984).

Whether these autoantigens themselves induce autoimmune disease or are merely innocent targets of an autoimmune response elicited by some other mechanism, is difficult to establish. Whereas myosin has not been shown to be capable of causing rheumatic fever, some studies have shown that immunization with myosin, of mice genetically predisposed to post Coxsackie B3 myocarditis, induced a severe myocarditis similar in appearance to the natural disease (Neu et al., 1987).

1.B.7 Cross-reactivity: Serum and Monoclonal Antibodies

Finally, since cross-reactions are defined with respect to a particular antibody or antiserum (Berzofsky et al., 1989; Van Regenmortel, 1989), characteristics of the antibodies defining the reaction should be examined. Berzofsky and Schechter (1981) defined two forms of cross-reactivity. Type I or "true crossreactivity" occurs when two ligands react at the same site on the same antibody molecule but with different affinities. The example mentioned is the cross-reactive binding of the haptens dinitrophenyl (DNP) and trinitrophenyl (TNP) to the anti-DNP antibodies, but with different affinities (Little and Eisen, 1969). Type II cross-reactivity ("partial cross-reactivity or shared reactivity") takes account of heterogeneous antibody populations (i.e. most conventional sera) where the cross-reactive ligands can react with all or only a subpopulation of the antibodies present. Monoclonal antibodies usually display Type I cross-reactivity.

In the experimental analysis of cross-reactivity, monoclonal antibodies have the advantage that the molecules in a sample are homogeneous and all have the

same variable region structure. This homogeneity can permit a more sophisticated analysis of specificity not possible with polyclonal sera. They can be used to detect both similarities and distinctions between related antigens. Whereas a homogeneous sample of (monoclonal) antibodies may reveal similarities between ligands, these may be concealed by the competing antibody reactivities in polyclonal sera. For example, using monoclonal antibodies Pierres et al. (1981) could show that the I-A and I-E antigens in the mouse MHC shared determinants, whereas this cross-reaction could not be shown using polyclonal sera.

Similarly, mAbs have been used to distinguish closely related specificities in class I and class II MHC that could not be defined using polyclonal sera (Pierres et al., 1981). In trying to absorb out irrelevant reactivities from polyclonal sera, there is the risk of at the same time losing reactivity. This could not occur in a homogeneous antibody sample, where all molecules have the same reactivity.

Fine specificity analysis of epitope clusters can be achieved by using groups of mAbs in competitive binding assays. Such an analysis using mAbs was made of the epitope clusters on the CD4 molecule of T cells (Sattentau et al., 1986). However, this fine specificity of mAbs can in some instances be a disadvantage. mAbs will display cross-reactivity not only between the numerous antigens of identical chemical structure but, can also cross-react with determinants whose associated chemical properties (e.g. surface charge, conformation) permit such binding (Ghosh and Campbell, 1986).

Furthermore, unwanted cross-reactions obtained with mAbs cannot be abolished by absorption without removing all reactivity (Berzofsky et al., 1989). Irrelevant cross-reactions can often be absorbed from polyclonal sera without adversely affecting relevant binding. Indeed, the removal of such (competing) reactivities from polyclonal sera could conceivably enhance their specificity. Finally, polyclonal sera can bind multivalent antigens more effectively (where this is important) than might monoclonal antibodies, which may display a weaker univalent interaction with the antigen.

It has been suggested that the most useful antibody reagent for detection of cross-reactions may be a mixture of monoclonal antibodies judiciously chosen according to their known (and often overlapping) specificities (Berzofsky et al., 1989). Such concoctions are however not often available.

In conclusion, the differing results obtained through the use of either polyclonal sera or monoclonal antibodies in studies of cross-reactivity/antigenic mimicry, bear out Van Regenmortel's (1989) contention that such assays are based on operationally-defined binding measurements and that the conditions of each particular assay will have a large influence on whether cross-reactivity/antigenic mimicry is detected.

2. ANTIBODIES TO CARDIAC, NUCLEAR AND STREPTOCOCCAL ANTIGENS

2.1 INTRODUCTION 21
2.1.1 Myosin ELISA 22
2.1.2 Actin ELISA 23
2.1.3 Collagen Type II ELISA 24
2.1.4 Heart Extract ELISA 24
2.1.5 Group A Streptococcus ELISA 25
2.1.6 Streptococcal M Protein ELISA 25
2.1.7 ASO and anti DNAse antibodies 25

2.2 RESULTS AND DISCUSSIONS 27
2.2.1 Study Design and Statistical Analyses. 27
2.2.1.1 Study Design 27
2.2.1.2 Patient Será 27
2.2.1.3 Rabbit Hyperimmune and Nonimmune Control Sera 30
2.2.1.4 Patient Sera: Immunoglobulin Concentrations 30
2.2.2 Cardiac Autoantigens 32
2.2.2.1 Heart Extract ELISA 32
2.2.2.2 Myosin ELISA 34
2.2.2.3 Actin ELISA 36
2.2.2.4 Phosphorylase b ELISA 38
2.2.2.5 Anticardiolipin Antibodies (ACA) 40
2.2.2.6 Collagen ELISA 40
2.2.3 Group A streptococcal Antigens 42
2.2.3.1 ASO and anti-DNAse B Titres 42
2.2.3.2 Group A Streptococcus ELISA 42
2.2.3.3 M Protein ELISAs 45
2.2.4 Nuclear Autoantigens 48
2.2.4.1 Antinuclear Antibodies (ANA) 48
2.2.4.2 Extractable Nuclear Antigens (ENA) 52
2.2.4.3 Anti-DNA Antibodies 53
2.2.4.4 Immunocytochemistry: Biotin-Streptavidin-Peroxidase and Immunofluorescence 53

2.3 CONCLUSIONS 60

2.1 INTRODUCTION

The enzyme-linked immunosorbent assay (ELISA) and other techniques were used in this study to measure antibodies in rheumatic fever patient and control sera against cardiac and streptococcal antigens that have been implicated in antigenic mimicry in the pathogenesis of rheumatic fever. The findings of previous investigations using similar ELISA techniques for measuring antibodies to several of these antigens are reviewed in this section.

The ELISA technique has been used by several investigators to measure autoantibodies in both healthy individuals and in various diseases (Guilbert et al., 1982; Dighiero et al, 1982., 1985; Avrameas et al., 1981; Matsiota et al., 1987). The technique is sensitive and suited to screening large numbers of sera, but there are some potential pitfalls that should be considered in interpreting results. Three of these are the following.

1. Due to the sensitivity of the technique, it has been argued that irrelevant binding properties of the antibodies may be exaggerated (RS Schwartz, 1986). Controls and optimal conditions for all stages of the assay should therefore be determined, and the antigen should preferably be well-defined (Gripenberg and Kurki, 1986). This may present a problem when using ill-defined autoantigens.

2. Even highly purified antigens may "behave" artefactually depending on the assay conditions. Denaturation may occur through binding of the antigen to plastic or through excessive detergent concentrations (in wash buffers for instance). Antigenic determinants may be lost, or else the antigen may exhibit new binding sites. Such artifacts can lead to a confusing variability in specificity. For instance, Guilbert et al. (1982) found that anti-cytochrome C and anti-collagen antibodies were not inhibited at all by their respective antigens, except when these antigens were immobilized on plastic.

3. The specificities of pathologically or experimentally induced antibodies and natural autoantibodies may differ. Autoimmune disease may induce autoantibodies of a different specificity to the natural autoantibodies against the same antigen. For example, Guilbert et al. (1982) discovered differences in the specificities and avidities of induced and natural anti-actin autoantibodies. Induced antibodies tended to react more strongly or at a higher rate than natural antibodies with antigen coated onto plates. Knowledge of such effects could be important in investigations aimed at trying to determine the pathogenicity of particular autoantibodies.

Despite these shortcomings however the ELISA is well suited for screening large numbers of sera against both purified antigens and crude antigenic extracts, and was used in this study to examine and compare antibody levels to various cardiac autoantigens and streptococcal products among different groups of rheumatic fever and control subjects.

2.1.1 Myosin ELISA

Dale and Beachey (1985b) found that ARF sera at a dilution of 1:400 in ELISA contained higher mean titres of antibodies to myosin (from rabbit heart), than either controls or AGN patients. Patients with carditis had the highest titres of anti-myosin antibodies. In addition, it was shown that streptococcal pep M5 rabbit immune sera cross-reacted with myosin in this ELISA, and could be inhibited by pep M6 and pep M19 but not by pep M24, indicating that these three streptococcal M protein all shared epitopes with myosin. Immunoblotting studies showed that human cardiac myosin also contained epitopes that crossreacted with pep M5.

Similarly, Cunningham et al. (1988) showed that ARF serum antibodies affinity-purified against myosin cross-reacted in ELISA with pep M5 and whole group A streptococci, as well as with DNA and crude extracts of heart. Interestingly, these cross-reactive antibodies were also present in normal sera, although at a much lower concentration, suggesting that they may occur as natural autoantibodies. The finding of cross-reactivity between myosin and M type 5 *S.pyogenes* antigens was confirmed in ELISA using murine mAbs evoked by these streptococcal antigens (Cunningham and Swerlick, 1986), and using human monoclonal antibodies (Cunningham et al., 1988). Dale and Beachey (1986b) used rabbit immune sera to pep M5 in ELISA inhibition experiments against myosin, to localise the myosin-reactive epitopes on pep M5.

Antibodies to myosin are not unique to rheumatic fever, but are also found in sera of patients with polymyositis (Wada et al., 1983) and coxsackievirus B-induced myocarditis (Neu et al., 1987), as well as in about 30% of patients who have had cardiac surgery (De Scheerder et al., 1985). Patients with acute myocardial infarction have been found to have antibodies to tropomyosin (Cummins et al., 1981), an associated molecule which has also been found to share reactivity with *S.pyogenes* M5 antigens and has been considered to be of relevance to rheumatic fever (Fenderson et al., 1989).

Anti-myosin antibodies are thus not unique to rheumatic fever but occur in other diseases, and have also been found as natural autoantibodies in healthy individuals (Dighiero et al., 1983). Nonetheless, the cross-reactivity this molecule displays with group A streptococcal antigens associated with rheumatic fever, suggest that it may be implicated in antigenic mimicry with *S.pyogenes*.

2.1.2 Actin ELISA

Antibodies to actin have been found both as natural autoantibodies in normal sera (Avrameas et al., 1981; Guilbert et al., 1982) and in diseases such as chronic active hepatitis (Gabbiani et al., 1973; Bretherton et al., 1983), myasthenia gravis (Williams et al., 1986), alcoholic cirrhosis (Cunningham et al., 1985) and Waldenström's macroglobulinemia (Avrameas et al., 1981). In addition, De Scheerder et al. (1985) demonstrated anti-actin antibodies in 25% of patients who had undergone cardiac surgery.

As a major muscle protein of heart, actin may be relevant as an autoantigen in rheumatic fever, but evidence for this is only indirect. Its mol.wt. (42 kD) corresponds to that of a constituent in immunoblotted crude heart extracts which react with rabbit antisera and murine mAbs to group A streptococcal M5 protein (Cunningham et al. 1984; Sargent et al. 1987) (see Table 3.1). Furthermore, some murine and human mAbs evoked by M5 protein, have been found to cross-react with actin (Cunningham and Swerlick, 1986; Fenderson et al., 1989; Cunningham et al., 1988). These same mAbs also reacted with myosin.

Human sera have been found to react only weakly with actin in ELISA (Kurki, 1978; Guilbert et al., 1982). It has been suggested that the human antibodies react mainly with native actin, and that this is denatured in ELISA (Gripenberg and Kurki, 1986). In rabbits, on the other hand, it was found that anti-actin antibodies could be evoked only by immunization with the denatured molecule, suggesting that in this species the immunogenic epitopes are hidden in the native protein (Lazarides and Weber, 1974). Human and rabbit sera thus appear to react quite differently to actin.

Kurki (1978) found that human sera reacted similarly with human or bovine actin, and that there did not appear to be differences in reactivity to monomeric (G) or polymeric (F) actin. In chronic active hepatitis and in alcoholic cirrhosis, however, anti-actin antibodies seem to be directed to G-actin (Cunningham et al., 1985; Bretherton et al., 1983).

Concerning the potential pathogenicity of anti-actin antibodies, it has been shown that cytoplasmic intermediate filaments (comprised of actin) are capable of activating complement (Linder et al., 1979). It has also been suggested that actin may be expressed on the cell surface (Owen et al., 1978) and that tissue damage may be caused by antibody-mediated complement lysis (Toh, 1979).

These studies suggest that actin may be a relevant autoantigen in rheumatic fever.

2.1.3 Collagen Type II ELISA

It is now well-established that some patients with rheumatic disorders have autoimmunity to collagen. Both humoral and cellular reactivity to collagen have been found (Steffan et al., 1963; Andriopoulos et al., 1976; Stuart et al., 1983). It is also known that antibodies to collagen form part of the natural autoantibody repertoire (Guilbert et al., 1982).

Antibodies to type II collagen from patients with rheumatoid arthritis have in some cases been shown to be arthritogenic. When injected into mice, these purified antibodies were capable of inducing arthritis (Wooley et al., 1984). It has not been resolved whether the antibodies to type II collagen which appear in some RA patients, merely reflect a change in the immunologic response of the host, or whether they precede the onset of clinical disease and are pathogenic (Möttönen et al., 1988).

In rheumatic fever, polyarthritis has been shown to occur in about 75% of patients during the initial attack (Schaller and Szer, 1989). Whether these patients have raised levels of antibody to collagen is not known. Both murine and human monoclonal antibodies reactive with cardiac and streptococcal M5 antigens showed no cross-reactivity with collagen (Cunningham and Swerlick, 1986; Cunningham et al., 1988).

The frequency of anti-collagen antibodies in human sera is now thought to be considerably lower than was originally estimated. The frequency of anti-type II collagen antibodies in RA patients was reported at anywhere between 3-50%, but is now generally considered to be closer to 3%. In RA, antibodies to type II collagen are predominantly of the IgM class (Clague et al., 1981). Although they occur at the highest frequency in active and destructive forms of RA, it has not so far been possible to show a correlation between these antibodies and the clinical stage of the disease (Clague et al., 1981) or to ascribe them a pathogenic role (Möttönen, 1988). Since rheumatic fever is often viewed as a connective tissue disease, antibodies to collagen may be relevant to the aetiology of the disease.

2.1.4 Heart Extract ELISA

ELISAs for the measurement of antibody to crude extracts of human heart have been used for screening monoclonal antibodies reactive with streptococcal antigens (Cunningham and Russell, 1983; Cunningham et al. 1984).

Recently, Shastry et al. (1988) used sodium deoxycholate extracts of human heart as solid-phase antigen in an ELISA and showed elevated titres of IgG and IgM heart-reactive antibodies in sera of ARF and RHD patients.

2.1.5 Group A Streptococcus ELISA

Cunningham and Russell (1983) screened murine monoclonal antibodies against whole group A streptococci using an ELISA based on methods described by Voller et al. (1980). The method entails fixation of whole bacteria as a solid phase antigen to polyvinyl chloride microplates using glutaraldehyde, in a peroxidase-conjugated second antibody system.

Cunningham et al. (1988) screened human mAbs against group A streptococci using this ELISA, and also used this method to compare the reactivity of a panel of heart-reactive murine mAbs, against different groups of streptococci (Cunningham et al., 1984).

2.1.6 Streptococcal M Protein ELISA

Dale et al. (1980, 1982) and Beachey et al. (1981) developed a sensitive ELISA for measuring antibodies to various streptococcal M proteins, based on the original protocol of Russel et al. (1976).

M proteins were coated onto polystyrene tubes, and binding of test antibody detected using an alkaline phosphatase conjugated second antibody and p-nitrophenyl phosphate substrate solution. Most ELISAs for M protein antibodies have followed this protocol.

Bisno et al. (1982) used this ELISA to measure type specific antibodies to pep M types 5, 6 and 24 in Chilean ARF patients and found that 32% had a positive titre to M5 compared to only 3% of normal age-matched control subjects. 38% of ARF patients and 29% of controls had antibodies to M type 6 protein, whereas neither patients nor controls had antibodies to M24. (There was no correlation in individual sera between anti-M5 and anti-M6 antibodies as measured by this ELISA). In this Chilean population of ARF patients, M5 was therefore an important "rheumatogenic" streptococcal serotype. Type 5 has also been found to be the major rheumatogenic serotype in England and the United States (Bisno, 1980). The importance of type 5 M protein as a rheumatogenic serotype in South Africa has not been reported.

2.1.7 ASO and anti DNAse antibodies

Although elevated streptococcal antibody titres are not diagnostic of rheumatic fever, the absence of a group A streptococcal infection makes the diagnosis of rheumatic fever based on the Jones criteria doubtful, except where the disease is discovered only long after the antecedent streptococcal infection as in Sydenham's chorea or low-level carditis (Bisno, 1979).

The ASO test is the most widely used of the streptococcal antibody tests. It is however susceptible to false positive titres associated with liver disease, growth of certain bacteria in the serum, and oxidation of the streptolysin O

reagent. The DNAse B test is not subject to such false positive titres, and is often considered the best single test for the serological detection of recent group A streptococcal infection. Normal values for both tests can vary with age of the subject, season of the year and geographical area.

Stollerman et al. (1956) found that in serum samples taken within 2 months of onset of the disease, 80 percent of patients with ARF had ASO titres of greater than 200 Todd units/ml. If three different anti-streptococcal antibody tests were performed, elevated titres of anti-streptococcal antibody could be found in more than 95 percent of ARF patients.

Anti-cardiolipin antibodies (ACA) have been found both as natural autoantibodies (Yadin et al., 1989) and have been associated with SLE and various connective tissue disorders as well as with arterial and venous thrombosis and ischaemic heart disease (Asherson and Harris, 1986; Colaco and Male, 1985; Hamsten et al., 1986). Whereas Hamsten et al. (1986) concluded that elevated ACA are a marker for recurrent cardiovascular conditions rather than a risk factor, Morton et al. (1986) suggested that they may be the result of an immunological response to myocardial necrosis. Klemp et al. (1988) suggested that myocardial ischaemia can also induce an immunological response leading to production of ACA.

Since ACA are known to be raised in autoimmune conditions such as SLE and other connective tissue disorders, as well as in certain pathologies involving damage to the heart, measurement of these autoantibodies seemed appropriate to the investigation of autoantibodies in rheumatic fever. Zabriskie (1967) showed that anti-heart antibodies cross-reactive with certain streptococcal strains in rheumatic fever patients, were specifically reactive with cardiolipin.

2.2 RESULTS AND DISCUSSIONS

2.2.1 STUDY DESIGN AND STATISTICAL ANALYSES.

2.2.1.1 Study Design

Sera from seven groups of 20 subjects each were investigated in the assays described here. The objectives were, to compare autoantibody profiles in rheumatic fever and control groups of subjects as well as to compare levels of antibodies to various streptococcal antigens among these groups. An additional objective was to identify rheumatic fever sera with high titres of antibodies to both heart and streptococcal antigens, for the purpose of screening human heart cDNA expression libraries for cross-reactive antigens (Chapter 5).

The assays used were ELISAs, tissue immunofluorescence and immunoperoxidase staining and immunoelectrophoresis.

All sera were tested in duplicate in the ELISAs, and at three ten-fold dilutions. Both the "no-antigen" and the "no-serum" ELISA blanks were subtracted from each result. This is a very rigorous control for nonspecific binding, and explains why the reported optical density readings are often low and sometimes negative.

Results are discussed here only in terms of statistical P values (Table 2.1;see page 29) without stating the confidence intervals. These can however be gleaned from the box plot graphs and from Appendix A, which presents the statistical parameters of all results, as well as a synopsis of the statistical methods applied.

2.2.1.2 Patient Sera

Serum was prepared by standard procedures from blood freshly drawn from patients in the study groups, aliquoted and stored at -20°C. Sera from 20 patients in each group were studied. Ethical approval to conduct this research was obtained from the Ethics and Research Committee of the University of Cape Town Medical School, and with the consent of all subjects and their parents where applicable. The criteria for admission of patients to the study are presented in Appendix C.

Group 1: **Acute Rheumatic Fever (ARF).**
Sera from children with acute rheumatic fever were obtained within two weeks of the onset of acute symptoms, and all had evidence of clinical carditis. The diagnosis of acute rheumatic fever was made according to the revised Jones Criteria (American

Heart Association, 1984) (see Table 1.1). One patient had chorea and six had polyarthritis. Clinical and demographic particulars of the ARF patients are summarised in Appendix B. The children were all seen at Red Cross War Memorial Children's Hospital in Cape Town. Their mean age was 9.1 years (SD 3.6).

Group 2: **Acute Glomerulonephritis (AGN).**
Sera were obtained from children seen at Red Cross War Memorial Children's Hospital who had a clinical diagnosis of acute glomerulonephritis. This consisted of an acute illness with oedema and haematuria of less than a week's duration. Most had oligaemia and hypertension and all had abnormal urea and creatinine levels, and did not have signs of the nephrotic syndrome or other renal pathology. Cultures for streptococcal infection were not obtained on most of these children. Mean age was 7.1 years (SD 3.6).

Group 3: **Chronic Rheumatic Fever - Children (CRF-c).**
Serum was obtained from children attending the Rheumatic Fever Clinic at Red Cross War Memorial Children's Hospital. All had rheumatic valvular heart disease and none had any evidence of acute illness or had had previous cardiac surgery. Mean age was 11.7 years (SD 3.2).

Group 4: **Chronic Rheumatic Fever - Adults (CRF-a).**
Serum was obtained from patients assessed at the Cardiac Clinic, Groote Schuur Hospital, by clinical and historical criteria to have rheumatic valvular heart disease. None had undergone cardiac surgery or had signs of acute illness. Mean age was 38.2 years (SD 9.7).

Group 5: **Ischaemic Heart Disease (IHD).**
Sera were obtained from patients attending the Cardiac Clinic at Groote Schuur Hospital, who were assessed to have ischaemic heart disease. None had cardiac surgery or were in the immediate convalescent phase following myocardial infarction. Mean age was 56.0 years (SD 8.8).

Group 6: **Child Control (CONT-c).**
Sera were obtained during a study on the epidemiology of asthma from healthy (non-asthmatic) Black rural schoolchildren. These children came from the Transkei in South Africa, and none had any history of rheumatic fever or cardiac disease, or signs of heart disease on clinical examination. Subjects in this group thus came from a different area to the other subjects in the study group.

TABLE 2.1 Statistically significant differences in the comparisons between groups

Statistical P values of significant differences in comparisons between sets of data from the seven groups of subjects. See Appendix A for confidence intervals and univariate descriptive statistics of each group of subjects for the various assays.

	COMPARISONS ON ADULTS			COMPARISONS ON CHILDREN						COMPARISONS ON CHILDREN VS ADULT CONTROLS			
	Control group vs		I.H.D. vs	Control group vs			A.R.F. vs	C.R.F. vs	C.R.F. vs	Adult Control Group vs			
	C.R.F.	I.H.D.	C.R.F.	A.R.F.	C.R.F.	AGN	AGN	AGN	A.R.F.	A.R.F.	A.G.N.	C.R.F.	Cont-C
DNASE B					0.0001	0.0043	0.0179	0.0649*	0.0003	0.0002	0.0005	0.0038	0.0001
IgG	0.0007		0.0011	0.0011					0.0286	0.0003	0.0017	0.0002	0.0094
IgA	0.0032	0.0082		0.0377	0.0002		0.0089	0.0001				0.0168	
IgM													
Protein M1	0.0051		0.0113		0.0442*			0.0204	0.0220	0.0023	0.0020		0.0049
Protein M3	0.0004		0.0002		0.0182			0.0130		0.0265	0.0019	0.0292	0.0049
Protein M5	0.0043									0.0284	0.0037		0.0090
Protein M6	0.0458*								0.0442*	0.0084	0.0177		0.0195
Protein M12	0.0004	0.0276	0.0019		0.0255			0.0077		0.0107	0.0004		0.0016
Protein M18	0.0013	0.0347	0.0011		0.0153			0.0130	0.0252	0.0060	0.0005		0.0042
Protein M19	0.0004	0.0260	0.0055		0.0312			0.0108	0.0242	0.0055	0.0012		0.0030
Protein M24	0.0198		0.0220			0.0210							
Heart 100	0.0011		0.0077	0.0029	0.0002		0.0411*	0.0253					0.0083
Heart 1000			0.0196		0.0042								0.0101
Heart 10000													
Myosin 100	0.0033		0.0019										
Myosin 1000													
Myosin 10000													
Actin 10			0.0148			0.0443*	0.0552*	0.0076		0.0039	0.0005	0.0021	0.0395*
Actin 100			0.0070	0.0057		0.0049		0.0125	0.0547	0.0018	0.0026		
Actin 1000	0.0527*		0.0087	0.0020		0.0002		0.0243		0.0402*	0.0047		
Phos B100	0.0055		0.0149		0.0002		0.0225	0.0007			0.0071		0.0073
Phos B1000	0.0039		0.0022	0.0241	0.0056		0.0192	0.0059			0.0139		0.0137
Phos B10000													
Strep 100			0.0420*							0.0139	0.0171		0.0294
Strep 1000	0.0458*		0.0205		0.0476*				0.0442	0.0049	0.0147		0.0107
Strep 10000			0.0133						0.0147	0.0139			
ASO		0.0001		0.0090	0.0404*		0.0012	0.0418*	0.0001	0.0001	0.0020		0.0014
Collagen II	0.0033		0.0133										
Anti-cardio-lipin IgG	0.0516*	0.0594*		0.0262						0.0265			
Anti-cardio-lipin IgM	0.0462*						0.0422*						
Anti-nuclear antigen	0.000		0.003	0.000	0.012	0.044*							
Immunocyto-chemistry		0.030	0.003	0.000	0.000	0.003	0.011	0.007					

* marginal statistical result at 5% significance level

Table 2.1 Statistical P values

Impetigo is a common problem in this area and risk of streptococcal infection is increased.

Group 7: **Adult Control (CONT-a).**
Serum was obtained from healthy laboratory and hospital staff at the Red Cross War Memorial Children's Hospital. None had any history of rheumatic fever or rheumatic heart disease, or any other form of heart disease or illness affecting the immune system. Mean age was 34.5 years (SD 5.6).

2.2.1.3 Rabbit Hyperimmune and Nonimmune Control Sera

Rabbit hyperimmune sera for use as positive method controls, were obtained either commercially or were raised in the laboratory according to procedures described in Materials and Methods (section 7.A.1). A pool of five non-immune rabbit sera was used as a negative control.

2.2.1.4 Patient Sera: Immunoglobulin Concentrations

Immunoglobulin concentrations were measured using a Behring Nephelometer Analyzer (Software Version N1.4).

The ARF and adult CRF patients showed raised levels of IgG compared to normal children and adults (ARF, $p=0.0011$; CRF-a, $p=0.0007$). IgA levels were significantly raised in all rheumatic fever sera compared to normal child and adult controls (ARF: $p=0.0372$; CRF-c: $p=0.0002$; CRF-a: $p=0.0032$). IgA was also increased in IHD patients ($p=0.0082$). There were no statistically significant differences in IgM concentrations in any of the groups. These results are graphically presented in Fig 2.1.

Raised levels of IgG and IgA in rheumatic carditis are known features of rheumatic fever (Benatar et al., 1988). Raised IgA may be partly due to pharyngeal infection by *S.pyogenes*. In Chagas disease, an autoimmune disease also involving the heart, increased concentrations of IgA are known to occur (Sa Ferreira, et al., 1983). IgA deficiency on the other hand (which is fairly common in the normal population) occurs at a disproportionately high frequency in autoimmune diseases such as rheumatoid arthritis (Bluestone et al., 1970), SLE (Cassidy et al., 1969) and Sjögren's syndrome (Kätkä et al., 1988). Interestingly, healthy subjects with IgA deficiencies have been reported to have high a incidence of certain autoantibodies (Yewdall et al., 1983).

Since immunoglobulin concentrations can vary with age, sex, heredity and environment, in addition to being susceptible to technical errors in the assays (Rose and Friedman, 1980), interpretation of these data are problematical. Generally, however, raised immunoglobulins (especially IgG) are indicative of recent or recurrent infection.

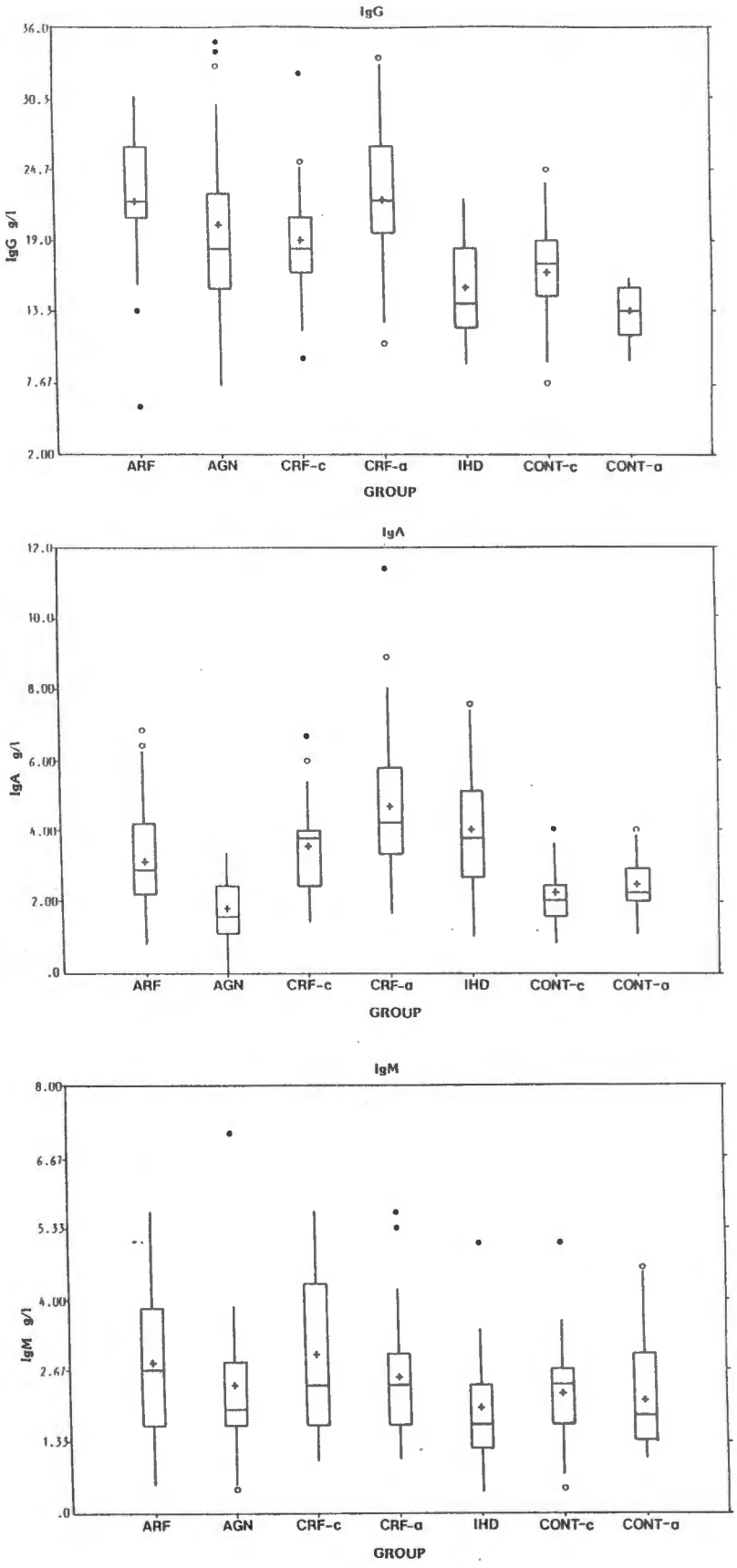


Fig. 2.1 Serum immunoglobulin concentrations in the seven groups of patients and normal subjects.
Serum concentrations (g/l) of IgG, IgM and IgA were measured by nephelometry in 20 individuals in each of the seven groups of patients and control subjects (section 2.2.1.4).
Results of statistical comparisons between groups are presented in Table 2.1, and descriptive statistics for each group are given in Appendix A. The figure shows box plot graphs depicting the statistical mean (+), the median (horizontal bar), the interquartile range (IQR: the boxed area comprising values falling within the 75th-and 25th percentiles) and outlying values. (See Appendix A for annotated description of the box plot and Abbreviations for serum "group" designations).

2.2.2 CARDIAC AUTOANTIGENS

2.2.2.1 Heart Extract ELISA

Human heart detergent (CHAPS) extract (section 7.A.11) was coated onto flat bottom PVC plates at a concentration of 5μg/ml. Sera were tested for reactivity to this antigenic extract as described in Materials and Methods (section 7.A.6).

As a method control, a rabbit antiserum raised to the heart extract was used. (It reacted more than three times as strongly in the ELISA as the pool of five nonimmune rabbit sera). To control for interassay variability, each assay also included a particular ARF serum with high reactivity to heart in ELISA and by immunocytochemistry (section 7.A.18).

It was shown that, at a serum dilution of 1:100, the CRF adult patients contained raised titres of heart-reactive antibodies (HRA), compared to the other adult groups (Table 2.1. CRF-a; CONT-a, $p=0.0011$; CRF-a:IHD, $p=0.0072$). Similarly, both child rheumatic fever groups had raised HRA at a 1:100 serum dilution, compared to the non-rheumatic child groups (ARF:CONT-c, $p=0.0029$; ARF:AGN, $p=0.0411$; CRF-c:CONT-c, $p=0.0002$; CRF-c:AGN, $p=0.0253$). These trends are graphically presented in Fig 2.2, in which the markedly higher HRA mean titre of the adult CRF patients can be seen.

This ELISA thus presents evidence, in rheumatic fever patients, of raised HRA to detergent extracts of human heart. These results confirm the findings of Shastry et al. (1988) who found that both ARF and CRF patients (various subgroups) had raised HRA (IgG and IgM), compared to AGN patients and controls. The present study measured only IgG antibodies, since pathogenic antibodies are generally considered to be of this class (Avrameas, 1986). Shastry et al. (1988) showed that the incidence of IgG HRA in rheumatic fever patients was higher than IgM. Their assay used sodium deoxycholate extracts of human heart, and was able to detect significant differences in HRA titre between rheumatic and control subjects in dilutions of up to 1:36000 compared to only 1:1000 in the present assay. This contrasts with the dilution of between 1:100 and 1:400 used by Zabriskie and Friedman (1983) for detection in ELISA of HRA to a purified 43 kD antigen in saline extracts of bovine heart.

Interestingly, Shastry et al. (1988) found that HRA persisted in all groups of rheumatic patients irrespective of whether they were on penicillin prophylaxis, in clinical remission or in a quiescent state with a history of the disease of 2-14 years standing.

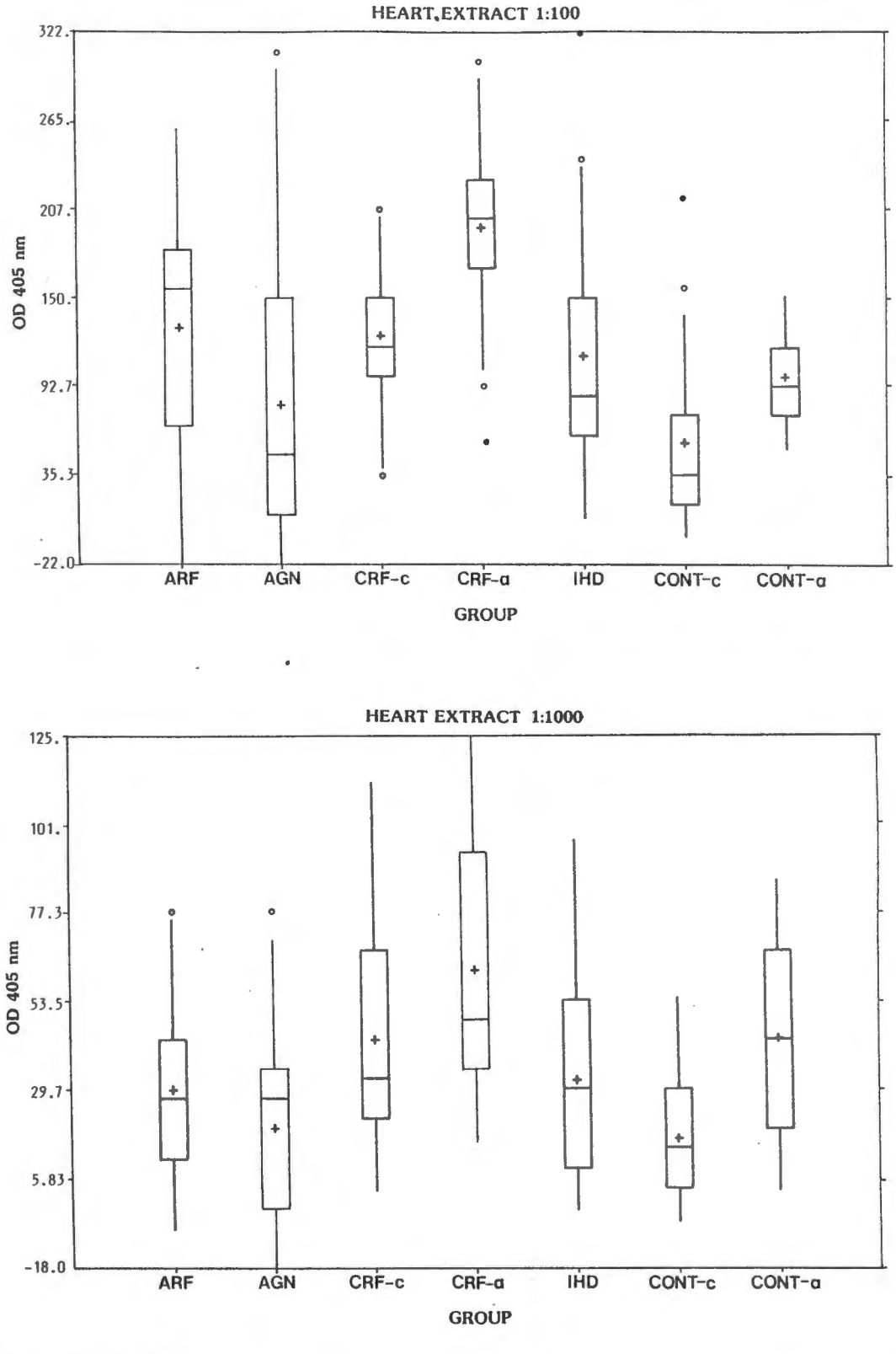


Fig. 2.2 Heart extract ELISA

The reactivities of the 20 sera in each of the seven groups of subjects (section 2.2.1.2) were tested in ELISA against crude extracts of human heart (sections 7.A.6) at dilutions of 1:100, 1:1000 and 1:10000. The figure summarises and compares the statistical mean, median and interquartile range (and outlying values) of the optical density readings (OD 405 nm) of each of the groups of 20 sera at dilutions of 1:100 and 1:1000.

(See also Table 2.1 for statistical P values of comparisons between groups, and Appendix A for univariate descriptive statistics for each group of sera).

In addition, they found both IgG and IgM HRA in sera of normal individuals ranging in age from 8-35 years. They speculated that these were natural autoantibodies, and cite the report of Leuker et al. (1975) who found an increase in activated B lymphocytes in acute and chronic rheumatic fever, which may be associated with the production of autoantibodies. In this study, HRA were also found in normal children and adults as well as in the non-rheumatic disease control groups (AGN, IHD), as seen in Fig. 2.2.

The relevance of HRA in the pathogenesis of rheumatic heart disease, and the significance of these antibodies in control subjects, has not been elucidated. The high mean titre in the adult CRF groups suggests that these antibodies may play a role in ongoing chronic disease, and it has been suggested that they may be implicated in the formation of local immune complexes thereby causing cardiac damage (Yoshinoya et al., 1980; Shastry et al., 1988).

2.2.2.2 Myosin ELISA

Serum antibodies to bovine muscle myosin (Sigma) were measured in ELISA as described in Materials and Methods (section 7.A.6). A commercially-available rabbit antiserum to whole myosin (Sigma) was used as a method control. At a dilution of 1:100, it reacted consistently with a reading greater than three times that of the pool of five nonimmune rabbit sera.

Among the adult groups, only adult CRF patients had significantly raised titres of anti-myosin antibodies (AMA) (Table 2.1: CRF-a:CONT-A, $p=0.033$; CRF-a:IHD, $p=0.0019$). Although none of the child rheumatic fever groups had raised AMA by comparison with the child control group, the mean AMA titre of the ARF group (OD_{405} = 167.7, SD 129.2) was virtually equivalent to that of the adult CRF group (OD_{405} = 177.2, SD 81.3). This can be seen in Fig.2.3. Statistical correlations between AMA and antibodies to other streptococcal and cardiac antigens, showed a possible correlation only in the CRF adult group, between AMA and heart reactive antibody ($r=0.793$).

These results can be compared to the study of Dale and Beachey (1985b) who found that ARF patients had raised AMA in ELISA at serum dilutions of 1:400, compared to AGN and normal controls. Patients with carditis had the highest AMA. Cunningham et al. (1988) affinity purified AMA from ARF sera and showed that they cross-reacted with group A streptococci, M type 5, pep M5 protein as well as with heart extracts.

The finding in this study of antibodies to myosin in normal subjects suggests that they occur as natural autoantibodies in humans. Whereas autoantibodies to actin occur at high frequency (Dighiero et al. 1982; 1983), the incidence of natural autoantibodies to myosin is much lower (Dighiero et al., 1983). Fairfax et al. (1977) found that among fifty-five patients with various cardiac and skeletal muscle disorders, only one serum had anti-myosin activity.

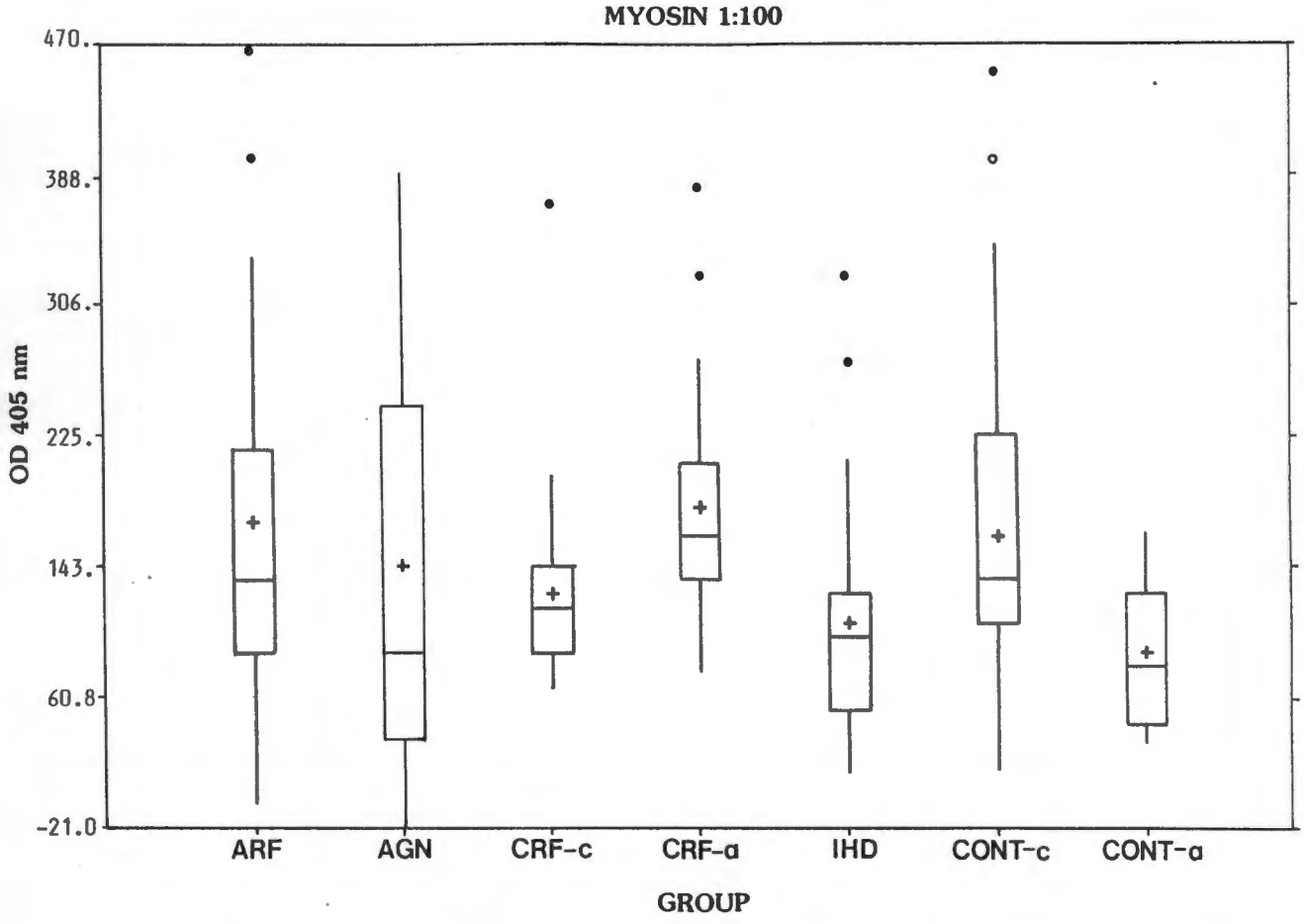


Fig. 2.3 Myosin ELISA

The reactivities of the 20 sera in each of the seven groups of subjects (section 2.2.1.2) were tested in ELISA against bovine muscle myosin (Sigma) (section 7.A.6) at dilutions of 1:100, 1:1000 and 1:10000. The figure summarises and compares statistical mean, median and interquartile range (and outlying values) of the optical density readings (OD 405 nm) of each of the groups of 20 sera at a dilutions of 1:100.

(See also Table 2.1 and Appendix A ~~for statistical~~ for comparative and univariate statistics).

One of the reasons adduced for the low incidence of AMA, is that damaged cardiac muscle has been found to bind and absorb out this antibody when the sarcolemma is disrupted (Khaw et al., 1979). It is possible that in rheumatic fever antibodies to myosin may be absorbed out of the serum resulting in lowered titres of circulating AMA. It is also known that the muscle and cytoplasmic myosins differ in their chemical and physical properties, and this may result in a variety of different autoantibodies (Pollard and Weihing, 1974). Thus, an individual AMA assay may detect only subpopulations of myosin autoantibodies.

This study presents some evidence of anti-myosin antibodies in rheumatic fever sera, which is in agreement with the findings of other investigators. Myosin appears to be an important autoantigen implicated in antigenic mimicry with *S. pyogenes*. Cross reactivity between myosin and group A streptococcal antigens was investigated by immunoblotting and is reported in chapter 3.

2.2.2.3 Actin ELISA

An ELISA was developed for measuring IgG antibodies to actin (section 7.A.4). A commercially-available rabbit anti-actin hyperimmune serum (Biomakor) was used as a method control, and reacted at a dilution of 1:5000 with the same intensity as a 1:100 dilution of a pool of 5 normal rabbit sera.

As a positive control, twenty sera from patients with chronic active hepatitis (CAH) (mean age 28 years, SD 5.4) were tested in this ELISA since it has been shown that about 30% of these sera contain anti-actin antibodies (Bottazzo et al., 1976). Their mean reactivity at a dilution of 1:100 ($OD_{405} = 0.142$; SD, 0.026) was 2.5-3 times greater than that of both normal control groups of sera (Appendix A).

ARF and AGN sera showed elevated levels of anti-actin antibodies (AAA) compared to child controls, at dilutions of both 1:100 and 1:1000 (ARFs $p = 0.0057$ and $p = 0.002$; AGN; $p = 0{,}0049$ and $p = 0.0002$). Fig. 2.4 shows box plots comparing AAA in groups of sera at dilutions of 1:100 and 1:1000.

Antibodies to actin have been found both as natural autoantibodies in healthy individuals, as well as in patients with diseases such as CAH. They are frequently elevated in patients following cardiac surgery (section 2.1.2). De Scheerder (1985) found a correlation between IgG AAA (as well as anti-myosin antibodies) and a cross-striational immunofluorescence pattern on frozen sections of monkey heart. (The results of the actin ELISA and immunocytochemistry could not be statistically compared in this study because of differences in the data systems, but it was noted that one of the ARF sera (patient No. 53) with the highest AAA titres, also had a high anti-myosin antibody titre and reacted intensely with heart tissue in the biotin-streptavidin-peroxidase assay).

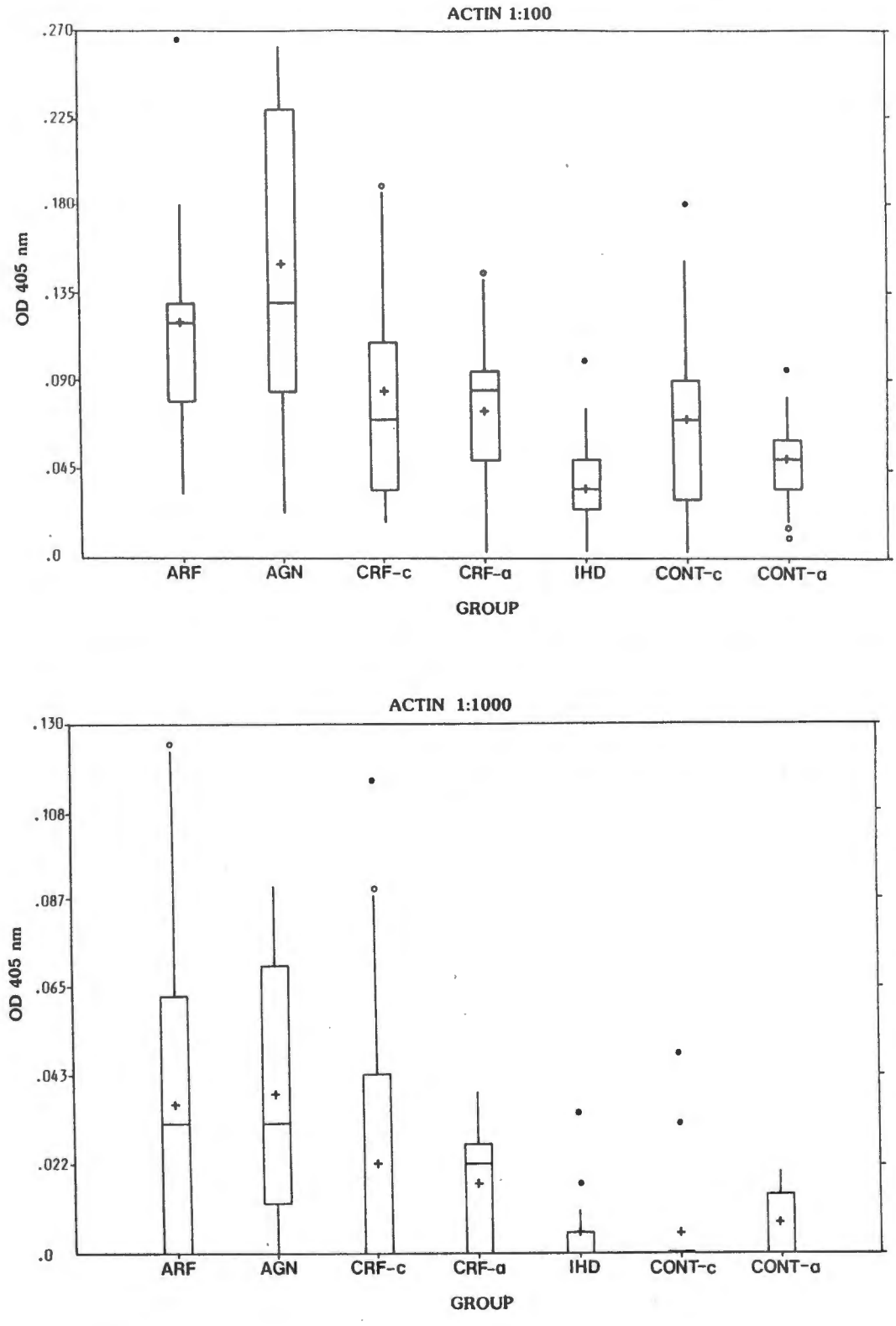


Fig. 2.4 Actin ELISA

The reactivities of the 20 sera in each of the seven groups of subjects (section 2.2.1.2) were tested in ELISA against rabbit cardiac actin (Sigma) (section 7.A.4) at dilutions of 1:10, 1:100 and 1:1000. The figure depicts the results for the serum dilutions at 1:100 and 1:1000.
(See legends to Figs. 2.1-2.3 for descriptions of statistical parameters depicted in figure. See Table 2.1 and Appendix A).

The presence of AAA in ARF sera has not previously been reported. However Fenderson et al. (1989) found that mouse monoclonal antibodies evoked by streptococcal M5 membranes (and reactive with tropomyosin) were cross-reactive in immunoblotting with actin. Also, two human mAbs developed by Cunningham et al. (1988) were cross-reactive with heart and group A streptococcal antigens, and were inhibited in immunoblotting assays by actin (T7.1 and T6; see Table 3.1).

Actin has therefore been indirectly implicated as an autoantigen in rheumatic fever. The present study shows evidence for raised titres of AAA in the ARF (and AGN) sera, but the reactivity was not intense. This finding may be due to low affinity antibodies associated with acute infection.

2.2.2.4 Phosphorylase b ELISA

Preliminary investigations showed evidence by immunoblotting that ARF patients have antibodies to muscle phosphorylase b. Dale and co-workers (Veterans Administration, Memphis, Tennessee USA) found that approximately 60% of ARF sera tested by ELISA had significant levels of anti-phosphorylase b antibodies (personal communication to DW Beatty, 1987).

In this study, autoantibodies to phosphorylase b (Sigma) were measured by coating it onto flat-bottom PVC plates at a concentration of 20μg/ml, and performing the ELISA as described in Materials and Methods (section 7.A.6). The rabbit antiserum raised to the heart extract (section 7.A.1) was found to react strongly with this protein (both in immunoblots and ELISA), and was therefore used as a method control. An ARF serum reacting strongly with the enzyme was included on each plate as an interassay control.

All rheumatic fever groups of sera were shown to have significantly elevated levels of anti-phosphorylase b antibodies (APA) when compared to adult and child control groups. In ARF patients, APA were elevated at a serum dilution of 1:1000 by comparison with normal children ($p = 0.0241$). In adult CRF patients, APA were significantly raised compared to normal adults at dilutions of both 1:100 and 1:1000 ($p = 0.0055$ and $p = 0.0039$, respectively). Similarly, at these dilutions, the child CRF group had raised APA compared to controls ($p = 0.0002$ and $P = 0.0056$, respectively). APA in AGN patients were significantly lower than in any of the rheumatic groups. These results are graphically presented by box plots in Fig. 2.5

Statistical correlation analyses between APA and antibodies to other tested cardiac and streptococcal antigens, showed that in all patient groups with cardiac disease, there was some correlation between raised APA and raised heart-reactive antibodies (HRA) (ARF, $r = 0.77$; CRF-c, $r = 0.70$; CRF-a, $r = 0.73$; IHD, $r = 0.71$).

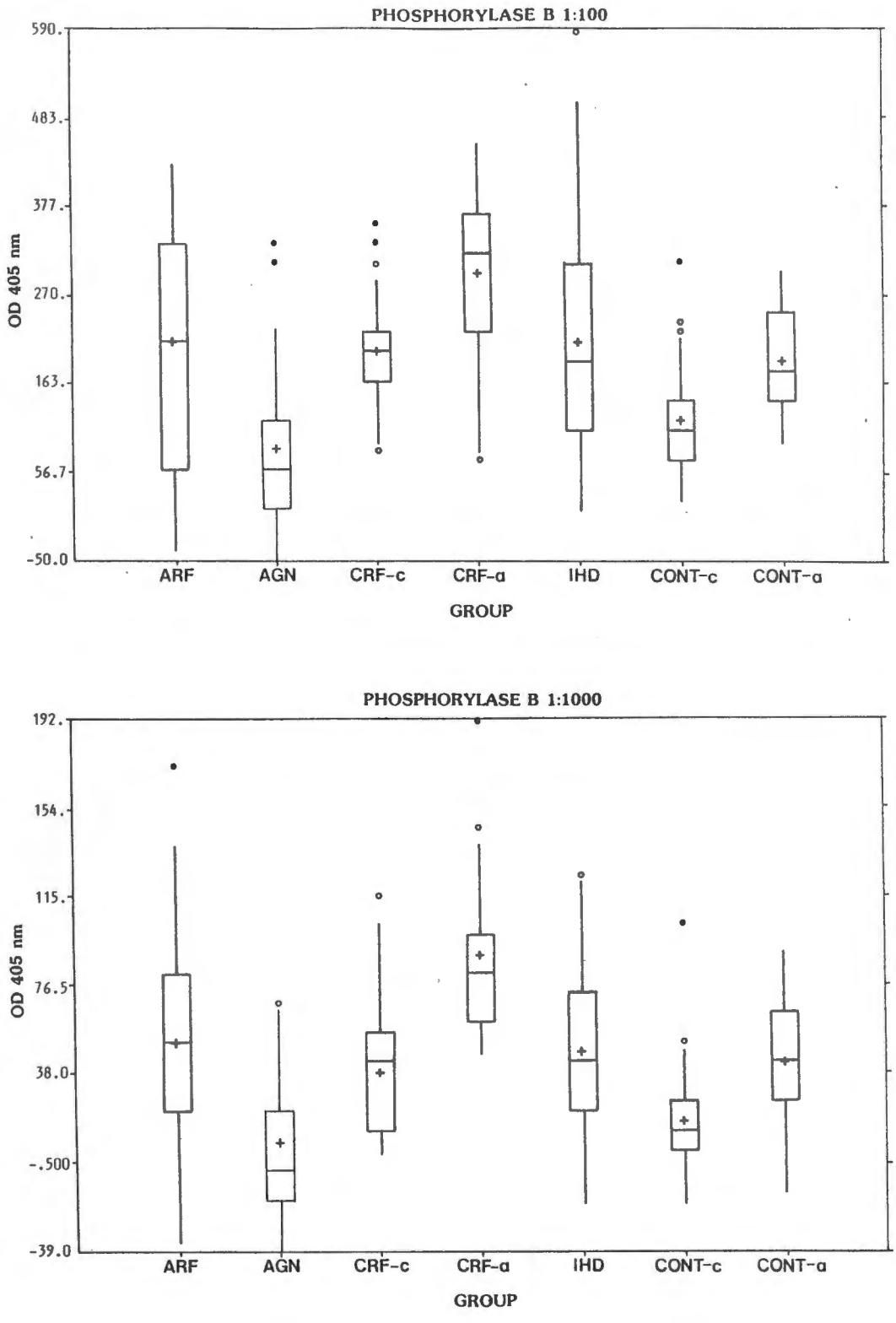


Fig. 2.5 Phosphorylase b ELISA

The reactivities of the 20 sera in each of the seven groups of subjects (section 2.2.1.2) were tested in ELISA against phosphorylase b (Sigma) (section 7.A.6) at dilutions of 1:100, 1:1000 and 1:10000. The figure depicts the results for the serum dilutions at 1:100 and 1:1000.
(See legends to Figs. 2.1-2.3 for description of statistical parameters depicted, and see Table 2.1 and Appendix A for comparative and univariate statistics).

Phosphorylase b may possibly therefore be an important cardiac autoantigen. Its pathogenic relevance is brought into question by the presence of APA in both normal child and adult sera, although in significantly lower titres (Fig.2.5 and Table 2.1). No correlations among groups of sera could be found between APA and antibodies to streptococcal antigens. However, comparing individual sera, it was noticed in this study that the two ARF sera with the highest APA titres also had among the highest titres of antibodies to all the streptococcal M proteins. Dale and co-workers were able to show, using antisera to pep M5, cross-reactivity between phosphorylase b and pep M5, M6 and M19 proteins (personal communication to D. W. Beatty, 1987).

2.2.2.5 Anticardiolipin Antibodies (ACA)

IgG and IgM antibodies to cardiolipin were measured by an ELISA technique first described by Lockshin et al. (1985) and modified by Klemp at al. (1988). Briefly, cardiolipin (Sigma) dissolved in ethanol was air-dried under nitrogen onto microtitre plates (Cooke system), incubated with serum and positive binding was detected using alkaline phosphatase conjugated second antibodies.

Normal ranges for anti-cardiolipin IgG (ACAG) and anti-cardiolipin IgM (ACAM) were established by Klemp et al. (1988) by standardizing sera from over one hundred controls (average age 30.83 years) to a single positive serum sample. Levels above 34 units for ACAG, and above 37 units for ACAM were regarded as elevated.

By comparison with these levels, only four sera in the present study showed elevated ACA levels: three child controls and one IHD. Comparisons of ACA levels between groups, showed that the control child group had significantly raised levels of ACAG compared to the ARF group ($p = 0.0262$). Similarly, the adult controls have marginally raised ACAM compared to the adult CRF patients ($p = 0.0462$).

The elevated ACA levels in the adult control group in this study, may be in agreement with the findings of Manoussakis et al. (1987) who found evidence of raised ACA in normal elderly individuals. However, Klemp et al. (1988) could find no evidence of such age-related trend, and the high ACA levels in the child control group in the present study, supports this view.

The results in this study do not indicate a role for anti-cardiolipin antibodies in the pathogenesis of rheumatic fever.

2.2.2.6 Collagen ELISA

The ELISA method of Boissier et al. (1987) was used (with modifications) to measure antibodies to type II collagen. Bovine type II collagen (Southern

Biotechnology Associates) was coated onto Nunc Immuno Maxisorp plates overnight at 4°C at a concentration of 10μg/ml (section 7.A.5). Sera were reacted with the collagen for 2 hours at room temperature at a dilution 1:100, and binding detected using an Fab'$_2$ goat anti-human IgM (μ) peroxidase-conjugated second antibody (Zymed).

A goat antiserum to bovine type II collagen (Southern Biotechnology Associates) was used as a method control. In addition, twenty rheumatoid factor positive sera from patients with rheumatoid arthritis were tested in this ELISA, and showed a mean reactivity four to five times greater than that of the normal adult group (mean $OD_{405} = 0.11$; SD, 0.02). To control for interassay variability, each plate also included two human sera with high titres of anti-collagen type II antibodies (a gift from Dr. PJL Holt, Department of Rheumatology, University of Manchester, U.K.).

There was insufficient serum from the child control group to include these in the collagen ELISAs. Statistical comparisons were therefore made against the normal adult group and against a new control group of child surgical patients. (Not one of the main study groups. Mean age 5.2, SD 2.1).

Only the CRF adult group showed raised IgM antibodies to collagen, compared to both the adult normal and IHD groups ($p = 0.0033$ and $p = 0.0133$, respectively). None of the child groups showed elevated titres when compared against either the normal adult group, or against the child surgical control patients. Results of these ELISAs were in general very low.

Antibodies to type II collagen have been reported to be predominantly of the IgM class (Clague et al., 1981), but other investigators have found IgG antibodies (Watson et al., 1986; Möttönen et al., 1988). In rheumatoid arthritis, Möttönen et al. (1988) could find no association between clinical onset of the disease and anti-collagen antibodies, which often appeared years before the disease. In the present study, the presence of anti-collagen antibodies in only the adult CRF group, would similarly seem to preclude a pathogenic role for these antibodies.

Although in rheumatoid arthritis both IgG and IgM antibodies to collagen are usually raised in the same patient (Clague et al., 1981), it is possible that IgG anti-collagen antibodies may show a different pattern of reactivity in rheumatic fever.

2.2.3 GROUP A STREPTOCOCCAL ANTIGENS

2.2.3.1 ASO and anti-DNAse B Titres

The presence of antibodies to group A streptococcal products is a part of the Jones Criteria for diagnosing rheumatic fever (Chapter 1). Antibodies to streptolysin O (ASO) and to DNAse B (anti-DNAse B) were measured using commercially available kits (Cooper Biochemical, USA; Wampole Laboratories, USA).

It was shown that ARF patients had significantly raised ASO titres compared to normal children ($p = 0.009$). ASO titres of the child and adult CRF groups were not raised since their rheumatic streptococcal infection was not recent. The significantly raised ASO in IHD patients ($p = 0.0001$), is inexplicable.

Anti-DNAse B antibody levels followed a different trend. In particular the normal child group had a mean antibody titre and range virtually identical to the ARF group (Appendix A. ARF: mean = 1451.4, IQR 1440; CONT-c: mean = 1385.3, IQR 1860). This is clearly seen in Fig 2.6 Statistical comparisons against this control group masked the high titres of antibody evident in the other child groups (AGN and CRF-c).

The differences in antibodies to streptococcal antigens shown by the ASO and DNAse B assay among the groups, is partly due to the geographical selection bias of the normal child group. These Black rural children came from an area where impetigo, an *S.pyogenes* skin infection, is common. It is known that low levels of ASO are found in streptococcal skin infections, and this may explain why anti-DNase B titres are higher than ASO in this group.

2.2.3.2 Group A Streptococcus ELISA

An ELISA method for measuring antibodies to whole group A streptococci was adapted from Cunningham and Russell (1983) who used it for screening murine hybridomas evoked by type M5 streptococcal membranes (see Chapter 4).

Group A streptococci were cultured from the throat-swab of one of the patients with rheumatic carditis, and fixed using glutaraldehyde to flat bottom PVC microtitre plates. Antibodies to whole streptococci were measured as described in Materials and Methods (section 7.A.3). A rabbit hyperimmune serum raised to this culture (section 7.A.1), as well as a commercially available rabbit typing serum to group A streptococci (Wellcome Diagnostics), were used as positive method controls. To control for interassay variability, an ARF and an AGN serum of high titre were included on each plate.

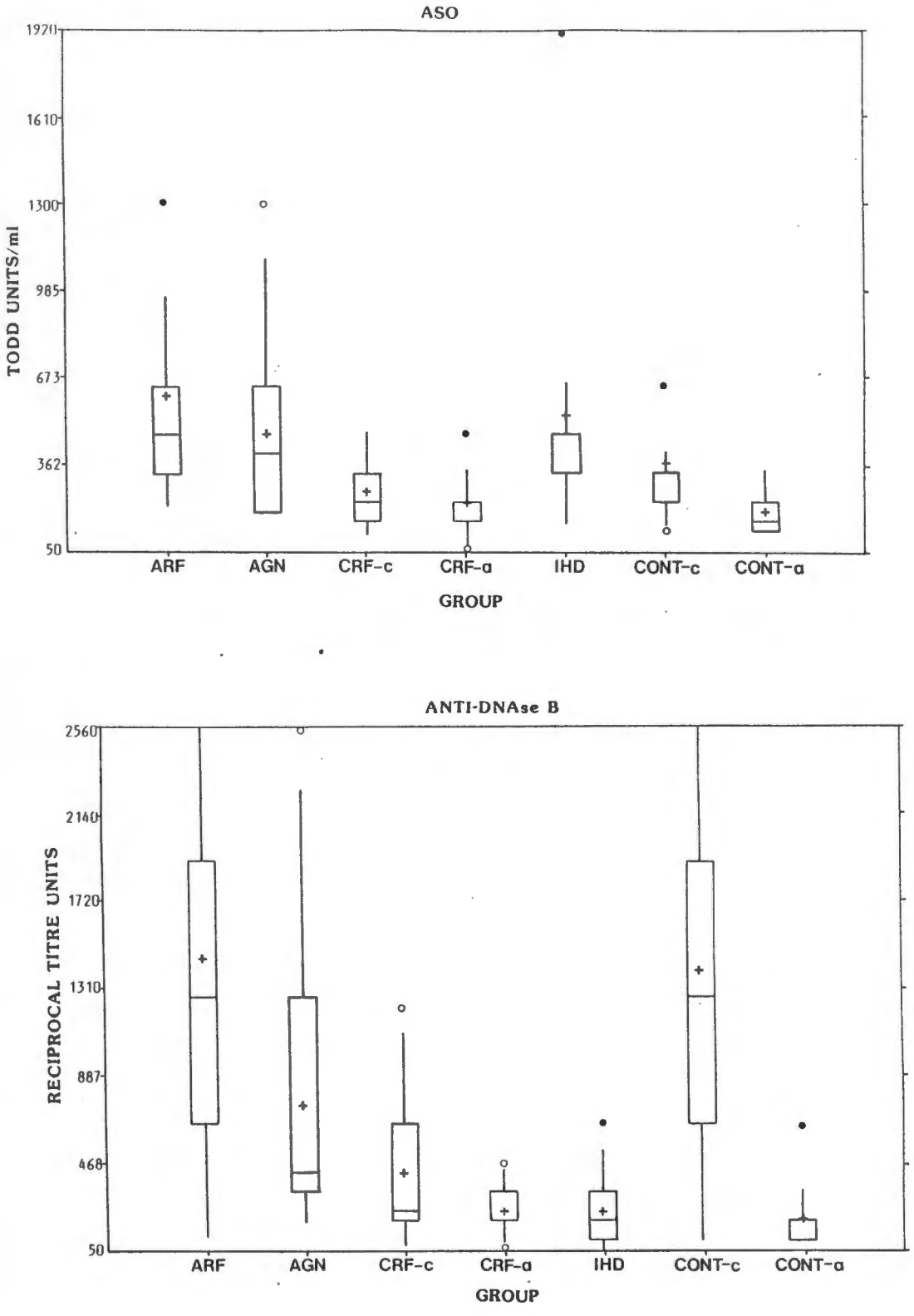


Fig. 2.6 Antistreptolysin O and anti-DNAse B streptococcal antigen titres

In all 20 sera in the seven groups of patients, antibodies to the group A streptococcal products streptolysin O (ASO) and DNAse B (anti-DNAse B) were measured by standard procedures using reagents purchased from Cooper Biochemical (USA) and Wampole Laboratories (USA). Anti-streptolysin O antibodies are given in Todd units/ml, and anti-DNAse B antibodies in reciprocal titre units.

(See legends to Figs. 2.1-2.3 for description of statistical parameters depicted, and see Table 2.1 and Appendix A for comparative and univariate statistics).

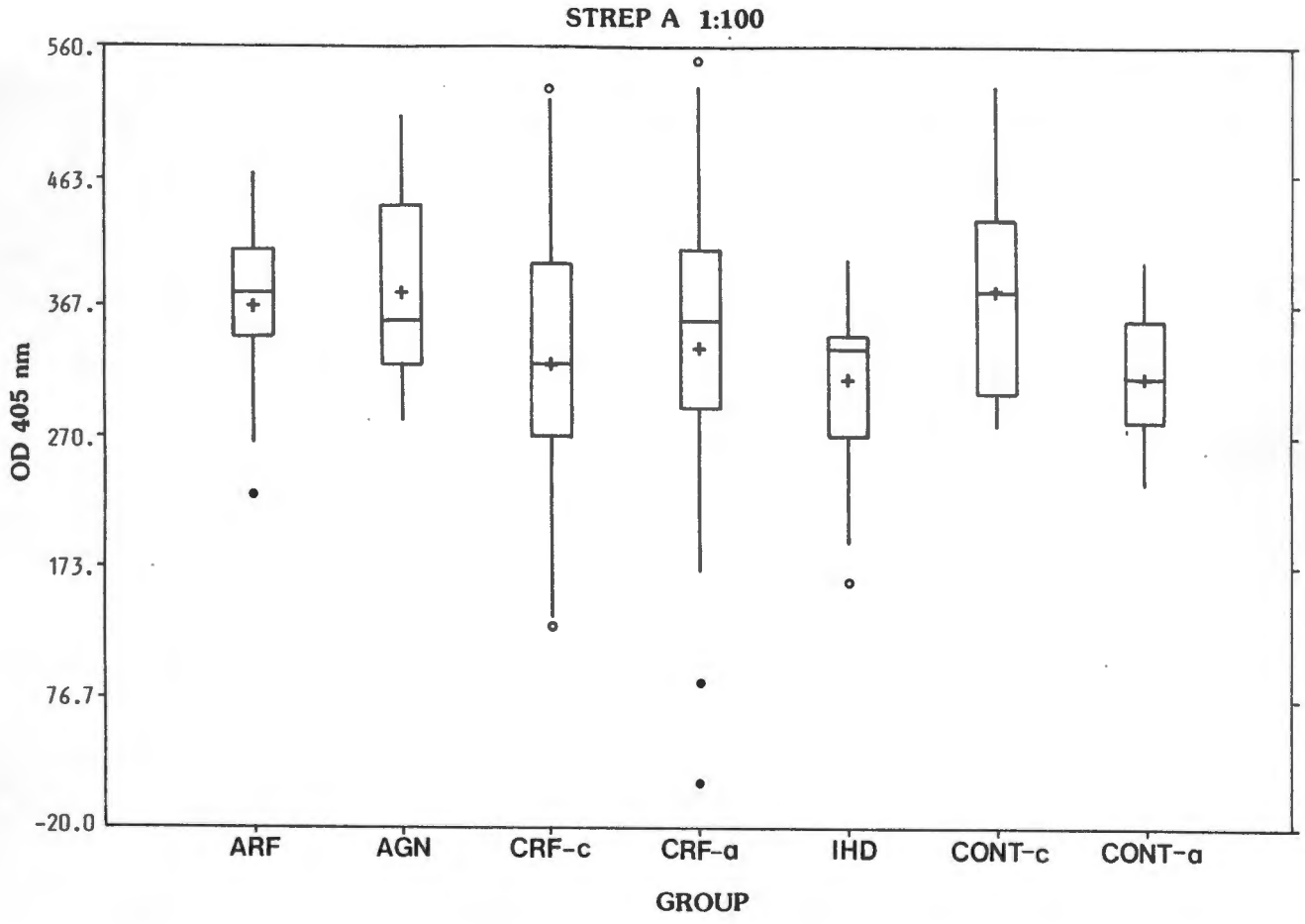


Fig. 2.7 Group A streptococcus ELISA

The reactivities of the 20 sera in each of the seven groups of subjects (section 2.2.1.2) were tested in ELISA against whole group A streptococci isolated from the throat swab of a patient with acute rheumatic fever (section 7.A.3) at dilutions of 1:100, 1:1000 and 1:10000. The figure depicts the results for the 1:100 serum dilution.
(See legends to Figs. 2.1-2.3 for description of statistical parameters depicted, and see Table 2.1 and Appendix A for comparative and univariate statistics).

Marginally elevated levels of antibodies to group A streptococci were found in only the adult CRF group at a serum dilution of 1:1000, and in none of the other rheumatic fever groups ($p = 0.0458$). Statistical correlation analyses, showed some association in the child CRF group, between antibodies to GAS (1:1000 dilution) and heart-reactive antibody (HRA: 1:100 dilution) ($r = 0.824$).

The relatively high reactivity of the child normal sera can be seen in Fig 2.7, and was probably due to the high incidence of streptococcal infections in this group of rural children (eg impetigo - also an *S.pyogenes* infection). Statistical comparisons against this group thus masked the comparatively high titres of anti-GAS antibodies in ARF and AGN sera.

It was subsequently shown that antisera to streptococcal groups B, C, D and G (Wellcome Diagnostics) cross-reacted with group A streptococci in this ELISA. This non-specificity may explain why all groups of sera displayed reactivity in this ELISA.

To compare reactivity of rheumatic fever and control groups of subjects against different streptococcal antigens, antibodies to purified M proteins were measured.

2.2.3.3 M Protein ELISAs

IgG antibodies to various streptococcal M proteins (M1, M3, M5, M6, M12, M18, M19 and M24) were measured by Dr. James Dale according to methods described by Dale et al. (1980, 1982). The method uses alkaline phosphatase conjugates which are more sensitive than the horseradish peroxidase conjugates used in the other ELISAs in this study.

It was shown that compared to normal adults, CRF adult patients have raised antibodies to all the tested M proteins. IHD patients had raised titres to M12, M18 and M19. Among the child groups, the control group had relatively high titres of antibody to all M proteins, except M5 and M6 (see Appendix A and Fig 2.8). As in the other streptococcal antibody assays (above), this high level of anti-streptococcal antibodies in the child control group had the effect of obscuring the relatively high levels of anti-M protein antibodies in the other child groups. In statistical comparisons between the child groups of sera and the normal adult group (which had low levels of antibodies to streptococcal antigens) the ARF and AGN groups showed significantly raised antibodies to all the tested M proteins (except M24).

Compared to AGN sera, the child rheumatic fever sera were shown to have significantly higher levels of antibodies to proteins M5 and M6. These were also the two M proteins to which the child control group did not have elevated titres of antibodies. Possibly, M5 and M6 are important

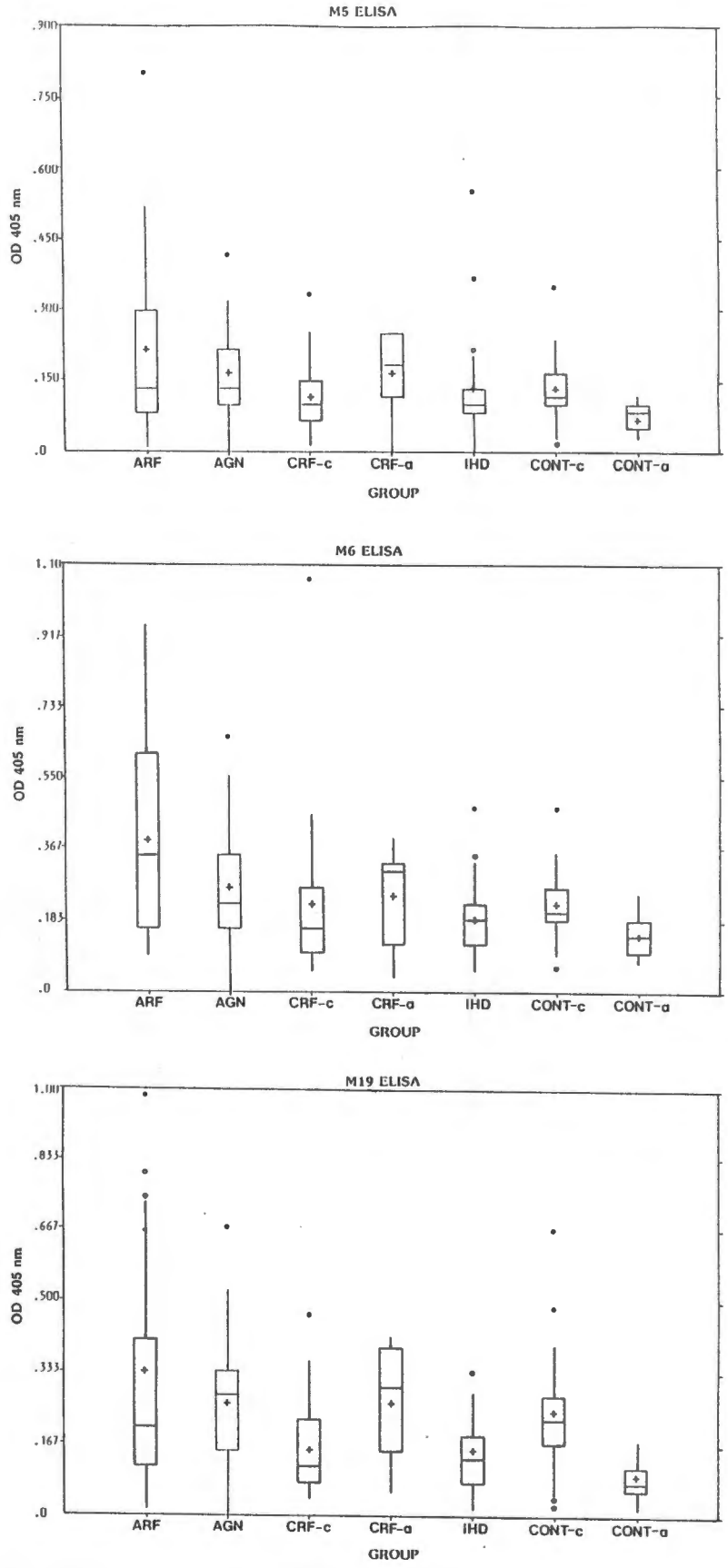


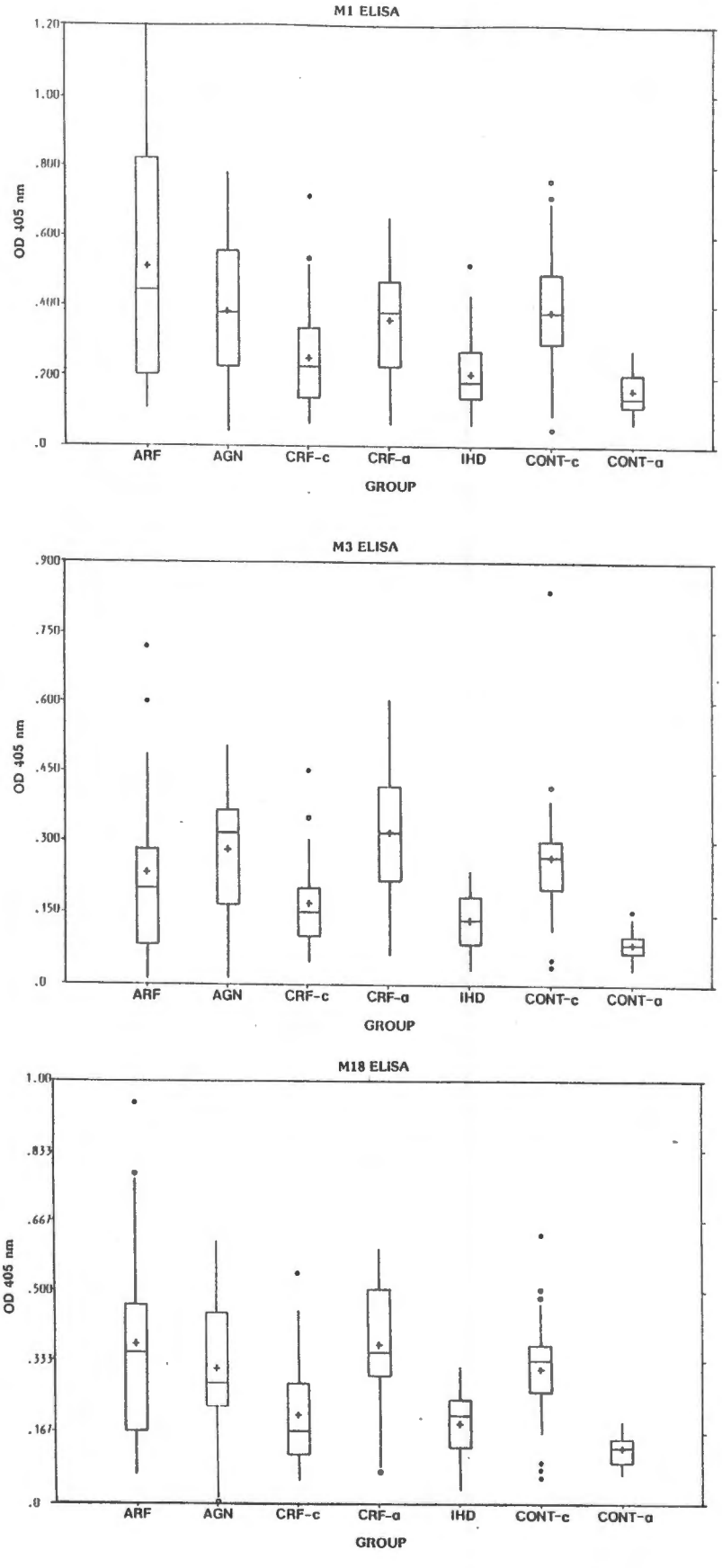
Fig. 2.8 (a & b) Group A streptococcal M protein ELISAs

The reactivities of the 20 sera in each of the seven groups of subjects (section 2.2.1.2) were tested in ELISAs against each of the purified group A streptococcal M proteins (section 2.2.3.3) at a dilution of 1:500.

(See legends to Figs. 2.1-2.3 for description of statistical parameters depicted, and see Table 2.1 and Appendix A for comparative and univariate statistics).

(a) ELISA results of antibodies to M proteins M5, M6 and M19 which have been associated with rheumatic fever.

(continued overleaf)



(legend continued from previous page)

(b) ELISA results of antibodies to streptococcal M proteins M1, M3 amd M18 (see previous page for legend).

rheumatogenic serotypes in rheumatic fever patients in Cape Town. Type M5, and to a lesser extent M6, are predominant serotypes among Chilean ARF patients; and M5 is the major serotype in England and the USA (see Introduction).

2.2.4 NUCLEAR AUTOANTIGENS

2.2.4.1 Antinuclear Antibodies (ANA)

Antibodies to nuclear antigens were determined by indirect immunofluorescence against HEp-2 cells, a human epithelial cell line with a large easy-to-read nucleus that binds a broader range of antibodies than tissue section substrates (Kozin et al., 1980).

The AFT HEp System (200 Test) supplied by Behring Diagnostics was used, together with an affinity purified goat anti-human IgG (H+L) FITC conjugate (Zymed). As a positive control, twelve SLE sera were tested. Sera from all groups were tested at a dilution of 1:40. There was no background fluorescence in the no-serum controls. Immunofluorescence staining patterns were scored as depicted in Table 2.2

TABLE 2.2

HEp-2 Antinuclear Autoantibody System: positive immunofluorescence patterns (Adapted from Behring Diagnostics, AFT HEp System)

Code No.	Pattern	Possible Autoantibody Specificity	Disease Association
1.	Homogeneous	Histone nDNA Single-stranded DNA Deoxyribonucleoprotein	Drug induced lupus High levels in SLE Also seen in other connective tissue diseases (in lower titres)
2.	Peripheral/rim	nDNA	SLE
3.	Speckled - fine	Smith antigen (Sm) Ribonucleoprotein (RNP)] Scl-70 SS-B	SLE Mixed Connective Tissue Disease (MCTD) Scleroderma Sjögren's Syndrome Polymyositis
4.	Speckled - discrete	Centromere	Scleroderma - CREST Syndrome
5.	Nucleolar	4 - 6s RNA	Progressive systemic sclerosis Sjögren's Syndrome
6.	Cytoplasmic - speckled	Mitochondria	Primary biliary cirrhosis Chronic active hepatitis
7.	Fine fibrous	Smooth muscle	

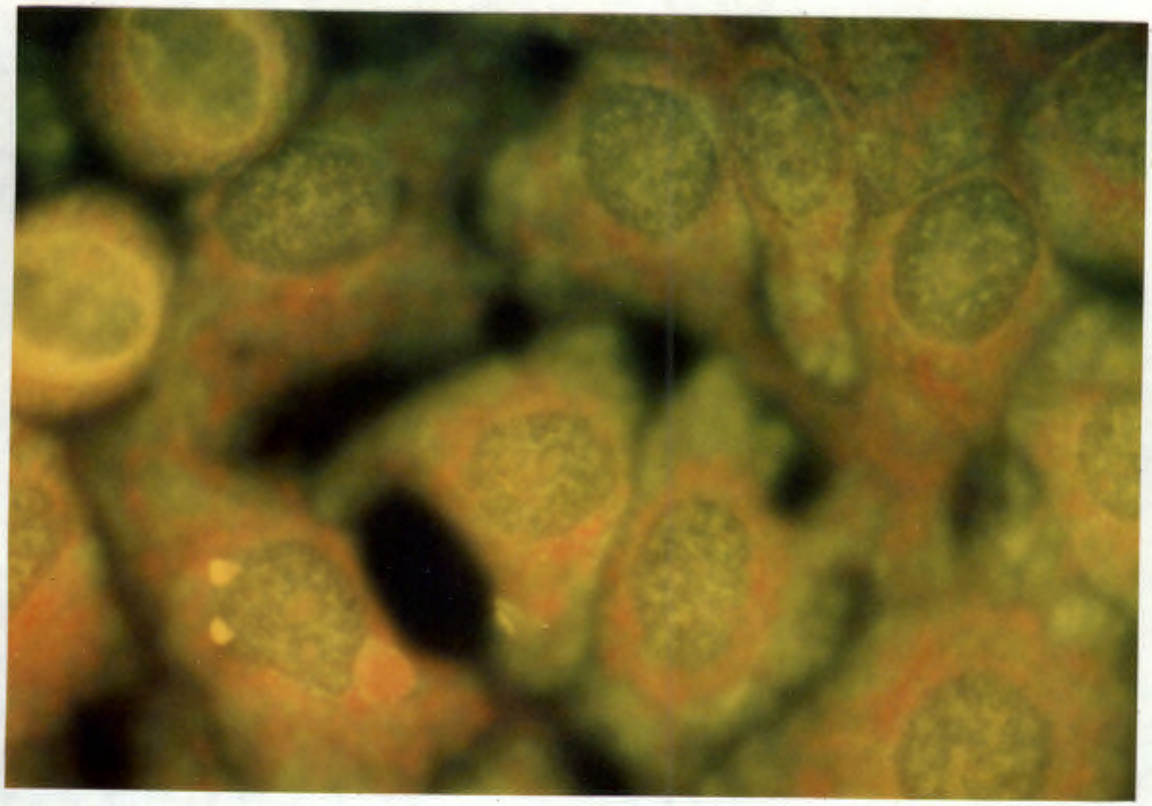
Positives that were ambiguous or displayed more than one staining pattern were titrated, and the predominant pattern at the highest dilution was scored. Although this selected scoring was somewhat biased, only five of the rheumatic fever patients displayed more than one staining pattern. These five all showed predominantly fine speckling (pattern 3) and in addition showed fine fibrous staining (pattern 7) and/or the speckled cytoplasmic staining indicative of mitochondrial autoantibodies.

Table 2.3 shows the number and percentage of sera in each group, staining with a particular pattern of immunofluorescence. A high percentage of rheumatic fever sera show evidence of ANA (ARF, 77.8%; CRF-a, 84.6%; CRF-c, 57.9%) compared to normal children and adults (CONT-c, 12.5%; CONT-a, 0%). Most rheumatic fever sera displayed a fine speckled pattern of staining (pattèrn 3) similar to that shown in Fig 2.9b. One ARF serum showed distinct evidence of mitochondrial autoantibodies (pattern 6), and another displayed a nucleolar pattern of binding (pattern 5, usually associated with Sjögren's syndrome or progressive systemic sclerosis).

The fine speckled pattern seen with the rheumatic fever sera has generally been associated with diseases such as mixed connective tissue disease, SLE (Fig.2.9a), Sjögren's syndrome and scleroderma (Table 2.2). However, patients with rheumatoid arthritis as well as some normal individuals, are known to contain ANA at low serum dilutions ($<1:20$).

These results present evidence of autoantibodies to nuclear antigens in rheumatic fever sera. Although these patterns were seen at relatively high serum concentrations of 1:40, and (except for some of the fine speckled staining of pattern 3) were no longer observable at a 1:80 serum dilution, the high percentage of these sera containing ANA compared to normal adults and children would seem to indicate that these patterns are not due to artefactual binding but specific.

Other investigators reported autoantibodies to mitochondrial and smooth muscle antigens in rheumatic fever (Boonpucknavig et al., 1984). The wide range of ANA binding seen among the adult CRF patients (Table 2.3) is consistent in this study with their generally high reactivity to various other autoantigens. The significance of these autoantibodies in rheumatic fever sera is unclear.



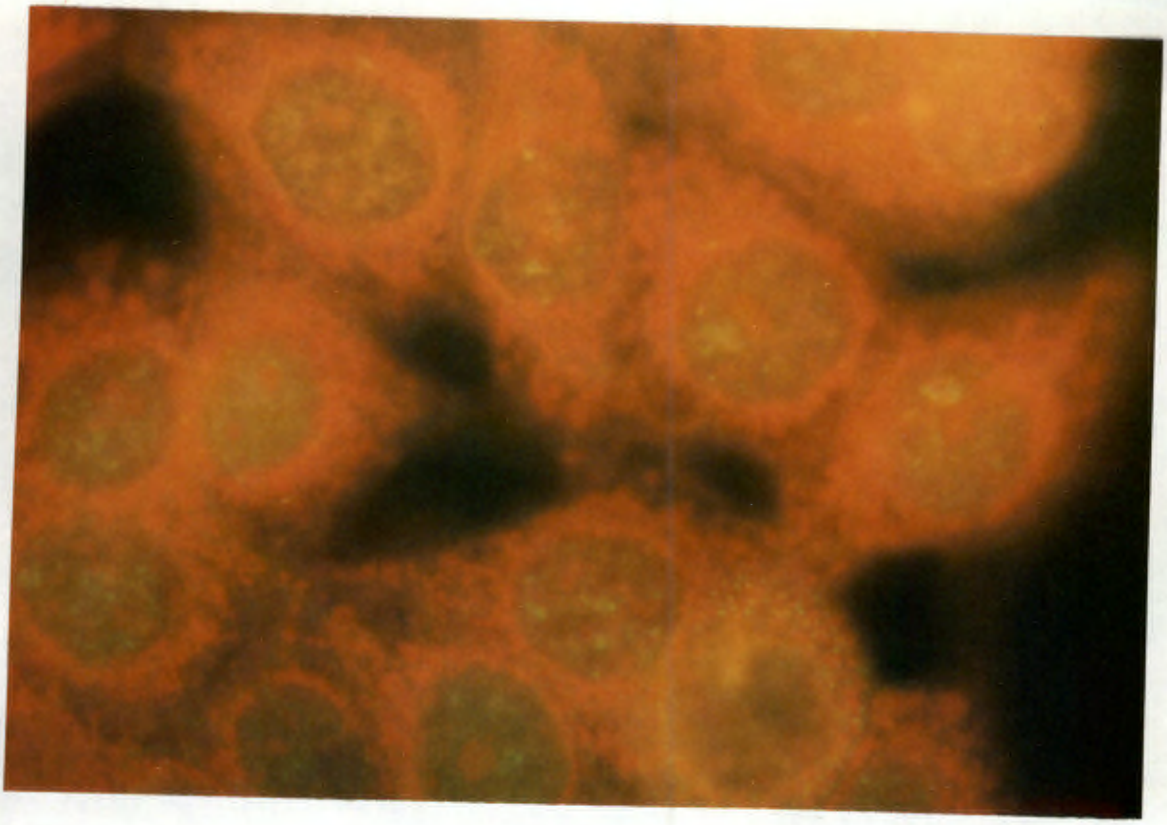
a

Fig. 2.9 Antinuclear antibodies

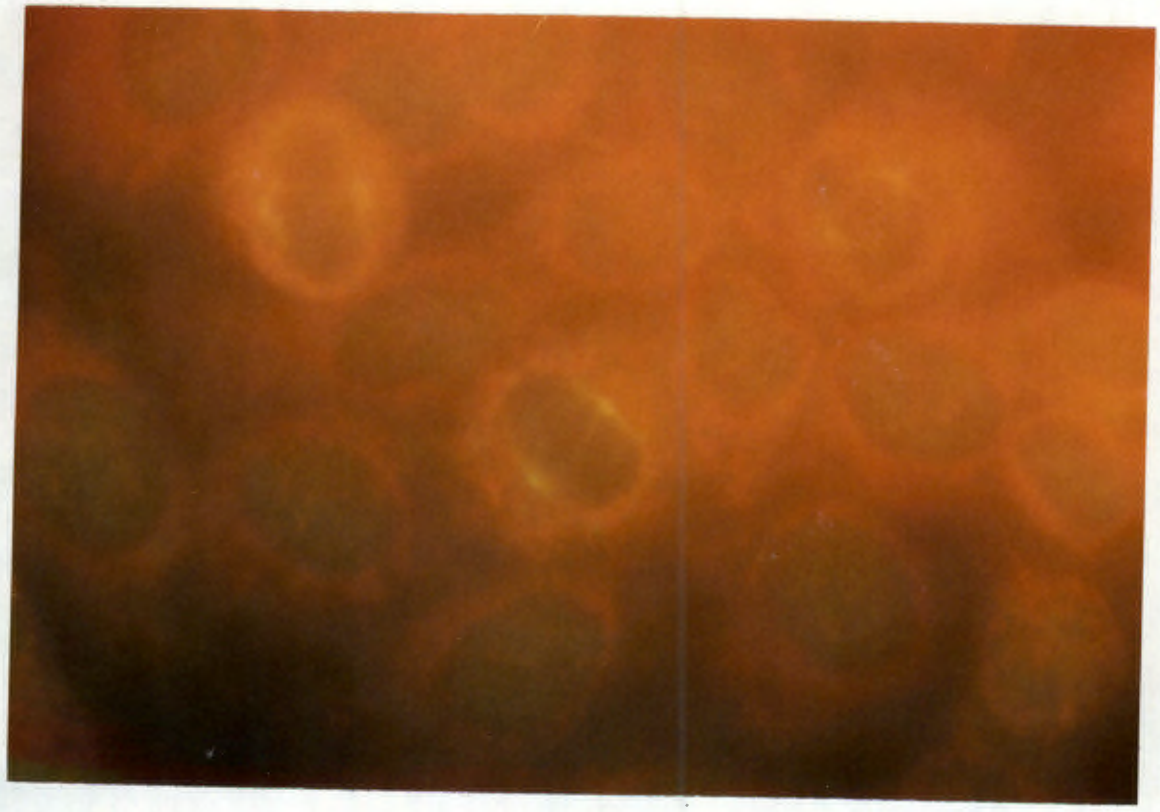
Antibodies to nuclear antigens were determined by indirect immunofluorescence against HEp-2 cells as the substrate, using the AFT HEp System supplied by Behring Diagnostics, together with an affinity purified goat anti-human IgG (H+L) FITC-conjugated second antibody. The slides were examined under a Nikon Optiphot microscope using a PlanApo 60X/1.4 oil lens. Positive slides were photographed using the attached camera (FX-35A, with automatic lightbox) and Kodak 400 ASA colour film (Kodacolor VR).
Twelve SLE sera were tested as positive controls. Sera were tested at a dilution 1:40 and ambiguous positives were titrated.
The immunofluorescence patterns against HEp-2 cells and the scoring codes are summarised in Table 2.2, and the results of this study are summarised in Table 2.3.

(a) Positive immunofluorescence staining of an SLE serum (dilution 1:80; a positive control) showing several patterns: homogeneous (1), peripheral, speckled fine (3), speckled discrete (4).

(continued overleaf)



b



c

(continued from previous page)

(b) Immunofluorescence staining pattern of an ARF serum (dilution 1:40) showing the fine speckled staining pattern (3) which was observed in 66.7% of ARF sera, 57.9% of CRF-c and 61.5% of CRF-a (Table 2.3). Cytoplasmic speckled staining (6) can also be seen (bottom of photograph).

(c) Fine speckled immunofluorescence staining (4) showing antibody binding to centromeres, observed with one adult CRF serum (Table 2.3).

TABLE 2.3

Antinuclear Antibodies (ANA) Using HEp-2 Cell Substrate: Positive Fluorescence Patterns in Different Groups of Patients

(Number of patients and percentage in each group presented)

Staining pattern	GROUP							
	ARF n = 18	AGN n = 12	CRF-C n = 19	CRF-A n = 13	IHD n = 19	CONT-C n = 16	CONT-A n = 8	SLE-Cont n = 12
1	-	1 (8.3%)	-	-	1 (5.3%)	-	-	2 (16.7%)
2	-	-	-	-	-	-	-	2 (16.7%)
3	12 (66.7%)	4 (33.3%)	11 (57.9%)	8 (61.5%)	3 (15.8%)	2 (12.5%)	-	6 (50%)
4	-	1 (8.3%)	-	1 (7.7%)	-	-	-	2 (16.7%)
5	1 (5.6%)	-	-	1 (7.7%)	-	-	-	-
6	1 (5.6%)	-	-	-	-	-	-	-
7	-	-	-	1 (7.7%)	1 (5.3%)	-	-	-
Total % with ANA	14 (77.8%)	6 (50%)	11 (57.9%)	11 (84.6%)	5 (26.3%)	2 (12.5%)	0 (0%)	12 (100%)

Code of Staining Pattern (see Table 2.2 for description of code)

1. Homogeneous
2. Peripheral or rim
3. Speckled - fine
4. Speckled - discrete
5. Nucleolar
6. Cytoplasmic - speckled
7. Smooth muscle - fine fibrous staining

2.2.4.2 Extractable Nuclear Antigens (ENA)

Antibodies to the extractable nuclear antigens, Sm (Smith antigen) and RNP (ribonucleoprotein) were measured in the rheumatic fever patients (ARF, CRF-c; CRF-a) and in the child and adult control groups, by counter-immunoelectrophoresis. Antibodies to these antigens are known to occur in certain systemic rheumatic diseases such as SLE, mixed connective tissue disease, Sjögrens' syndrome and scleroderma.

Of all the sera tested, only two patients (both ARF) showed evidence of antibodies to these nuclear antigens - one to SM and one to RNP. Neither patient had any history or clinical evidence of SLE. Both showed a fine speckled immunofluorescence pattern in the HEp-2 ANA assay (indicative of autoantibodies to Sm or RNP) but neither had anti-DNA antibodies. These patients both had raised ASO and DNAse titres, and the RNP-positive patient stained heart tissue intensely (4+) in the biotin-streptavidin-peroxidase assay (section 7.A.18).

Since rheumatic fever may also be viewed as a connective tissue disease, antibodies to RNP are plausible. Antibodies to the Sm antigens are surprising since this antigen is regarded as a diagnostic marker for SLE. Both Sm and RNP antigens are comprised of several interactive proteins associated with RNA, and have been implicated in mRNA splicing (Tan, 1989b). The presence of these autoantibodies in ARF sera has not previously been reported, but as only two patients were positive their pathological significance is doubtful.

2.2.4.3 Anti-DNA Antibodies

Anti-DNA antibodies are not known to occur in rheumatic fever. The finding in this study of antinuclear antibodies (ANA) as well as antibodies to Sm and RNP antigens, prompted this investigation of anti-DNA antibodies in rheumatic sera. Antibodies to native double-stranded DNA were measured in all groups of sera by the standard *Crithidia luciliae* assay.

Only three sera in the entire study group contained antibodies to ds-DNA: two from the IHD group and one AGN child.

2.2.4.4 Immunocytochemistry:Biotin-Streptavidin-Peroxidase and Immunofluorescence

All sera were tested for antibodies binding human heart sections, using a biotin-streptavidin-peroxidase system described in Materials and Methods (section 7.A.18).

Sera were tested at an optimal dilution of 1:5 and scored, on a scale from 1+ to 4+, according to their intensity of staining (see Table 2.4; Fig.2.10a,b,c,d). It was found that trypsinization of the sections (0.1% trypsin at 37°C for 12 minutes) to expose cross-linked antigens considerably enhanced the staining, as did pre-treatment with 0.3% hydrogen peroxide to inhibit endogenous peroxidases.

A commercially available rabbit anti-actin antiserum (Biomakor) was used as a positive method control. It reacted with an intense speckled pattern, showing some evidence of striational binding (Fig 2.10.e). Since antisera to denatured antigens often react more strongly in immunocytochemical assays (probably because fixation procedures denature tissue antigens), a rabbit antiserum raised

to the detergent extract of heart (section 7.A.1) was also used. It reacted with a similar speckled pattern of binding, but without evidence of striational binding (Fig 2.10.f). To control for interassay variability, two strongly-reactive (4+ staining) ARF sera were included in each assay.

TABLE 2.4

Staining of Human Heart Sections by Biotin-Streptavidin-Peroxidase

Intensity of staining	GROUP						
	ARF n = 20	AGN n = 19	CRF-C n = 20	CRF-A n = 16	IHD n = 21	CONT-C n = 33	CONT-A n = 10
4+	6 (30%)		5 (25%)				
3+	3 (15%)	3 (15%)	3 (15%)	2 (12.5%)			
2+	7 (35%)	3 (15%)	5 (25%)	2 (12.5%)	1 (5%)	4 (12%)	1 (10%)
1+	1 (5%)	2 (10%)	3 (15%)	6 (37.5%)	1 (5%)	5 (15%)	4 (40%)
Total % with staining	17 (85%)	8 (42%)	16 (80%)	10 (62.5%)	2 (10%)	9 (27%)	5 (50%)

Intensities scored as follows:

4+ Intense staining throughout the section and/or presence of striations
3+ Strong staining in parts of the section
2+ Diffuse/patchy staining
1+ Faint but evident staining

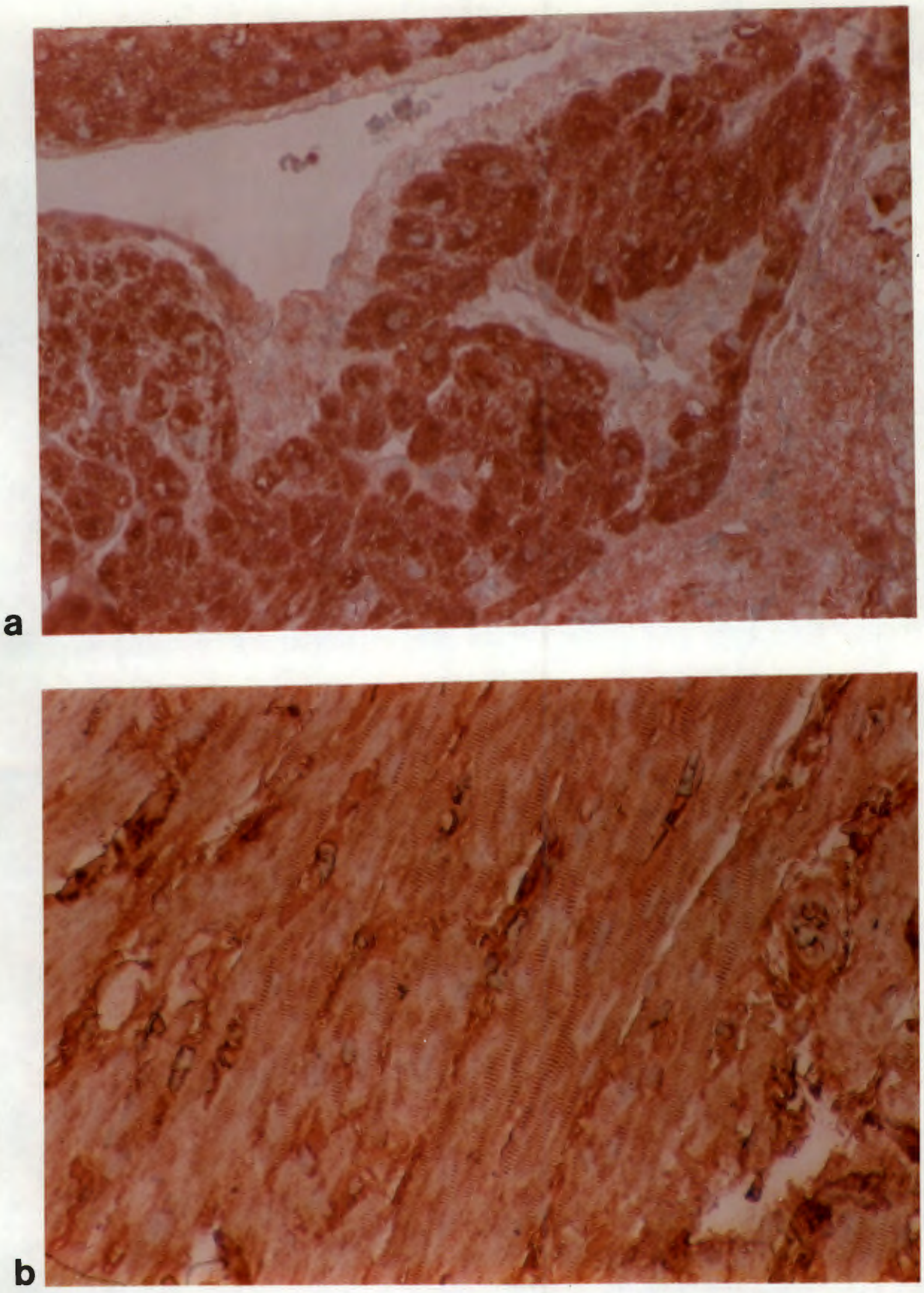
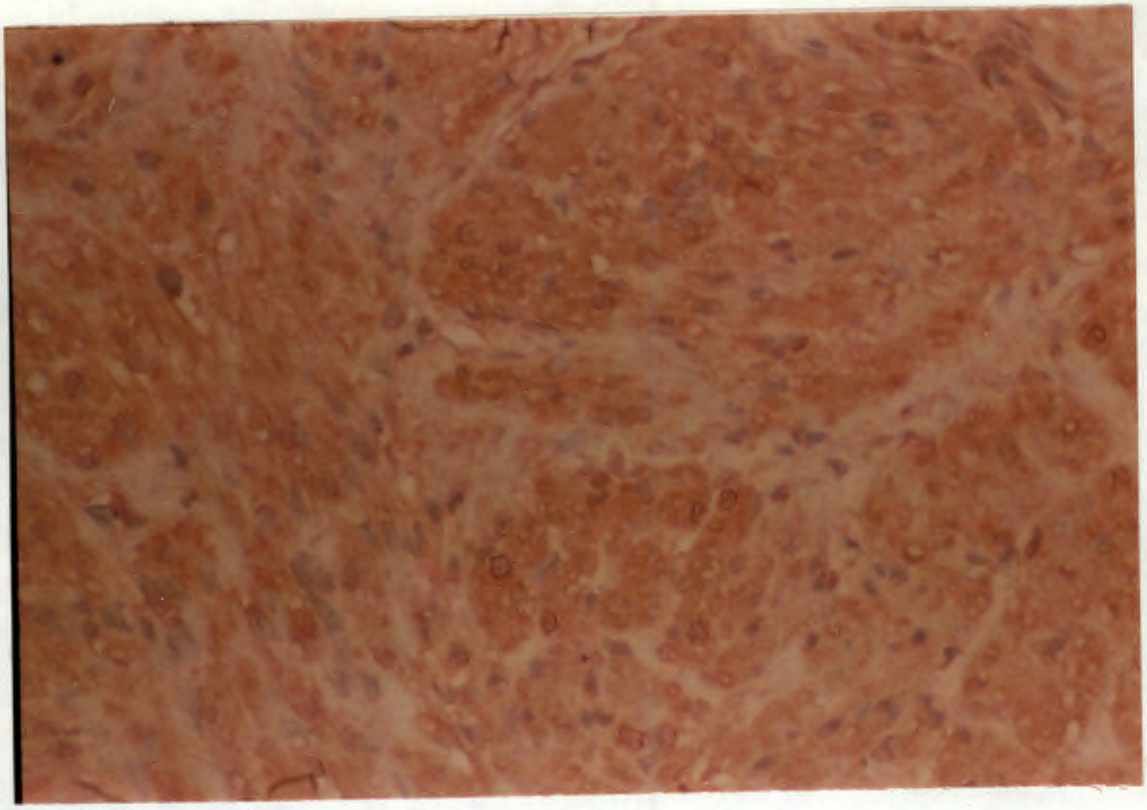


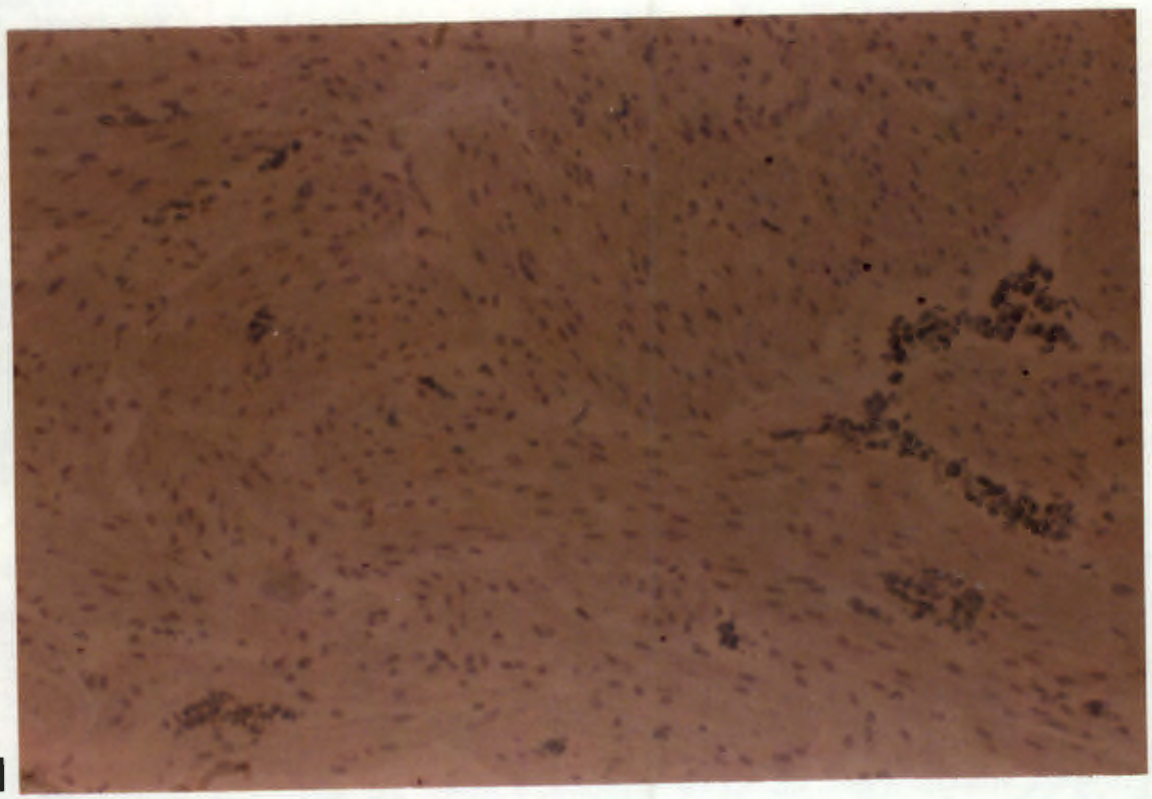
Fig.2.10 Biotin-streptavidin-peroxidase immunocytochemical staining of human heart paraffin embedded sections

Sera were tested at a dilution of 1:5 for antibodies binding to heart using a biotin-streptavidin-peroxidase assay (section 7.A.18). Intensity of the reaction was scored visually as one to four pluses. (Results are summarised in Table 2.4).

(a) 4+ intensity of staining by an ARF serum of human heart tissue in cross-section showing staining of muscle bundle fibres and no staining of connective tissue. **(b)** 4+ staining by ARF serum of longitudinal section showing striational binding pattern. *(continued overleaf)*



c

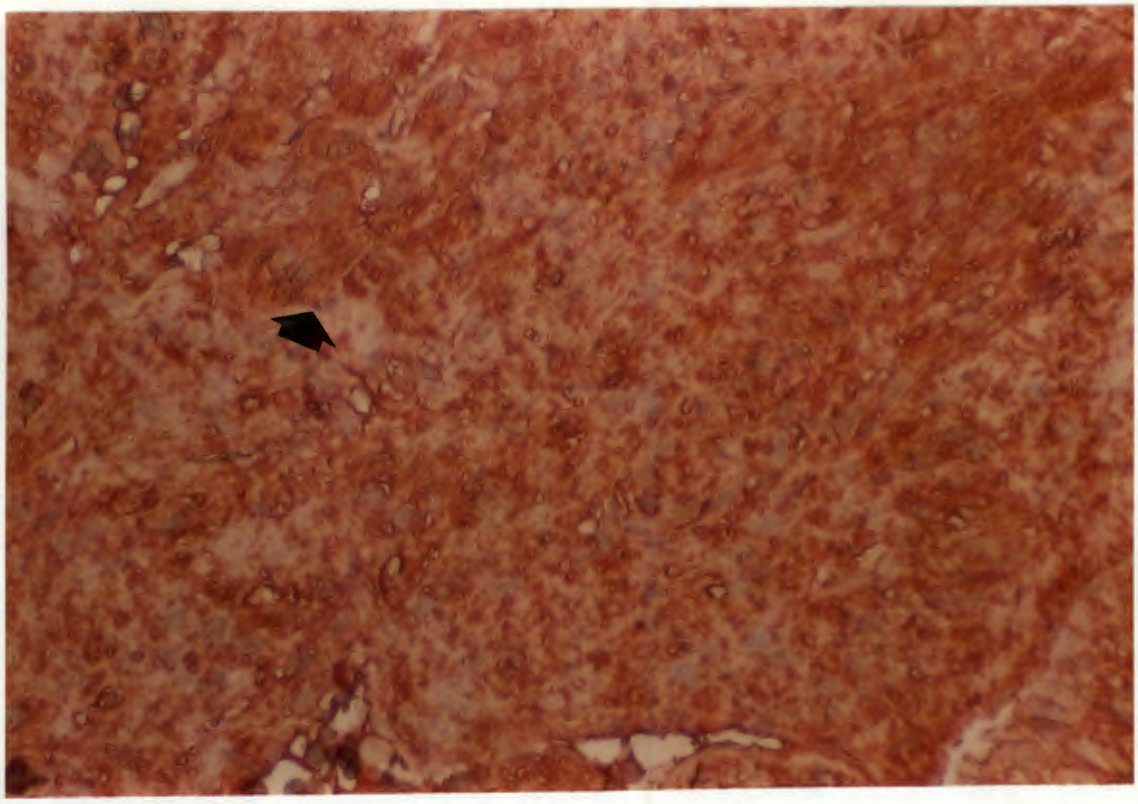


d

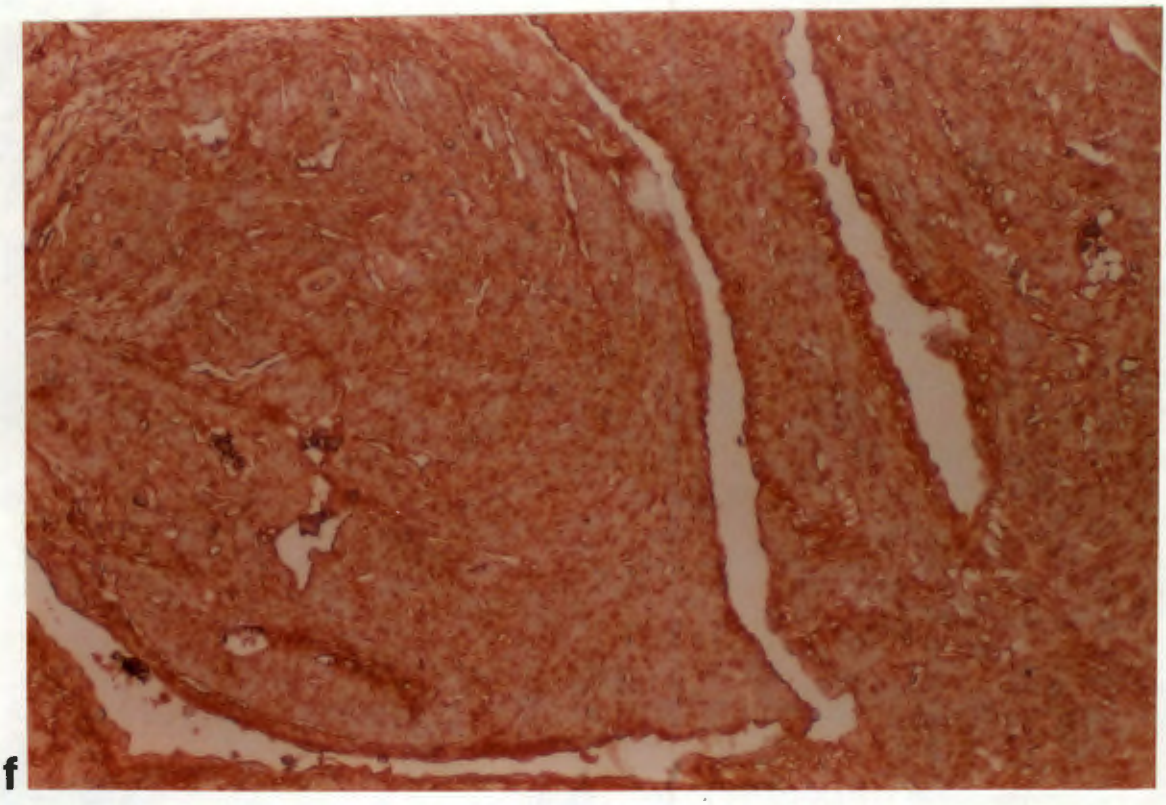
(continued from previous page)

(c) Example of 1-2+ staining of heart section by a non-rheumatic fever serum showing diffuse colour reaction in comparison with **(a)**.

(d) The no-serum control reaction showing no nonspecific binding of the biotinylated second antibody or streptavidin-peroxidase conjugate to the tissue.



e



f

(continued from previous page)

(e) Reaction of rabbit anti-actin antibody (Biomakor) with human heart Section showing intense speckled patterns with patches of striational staining.

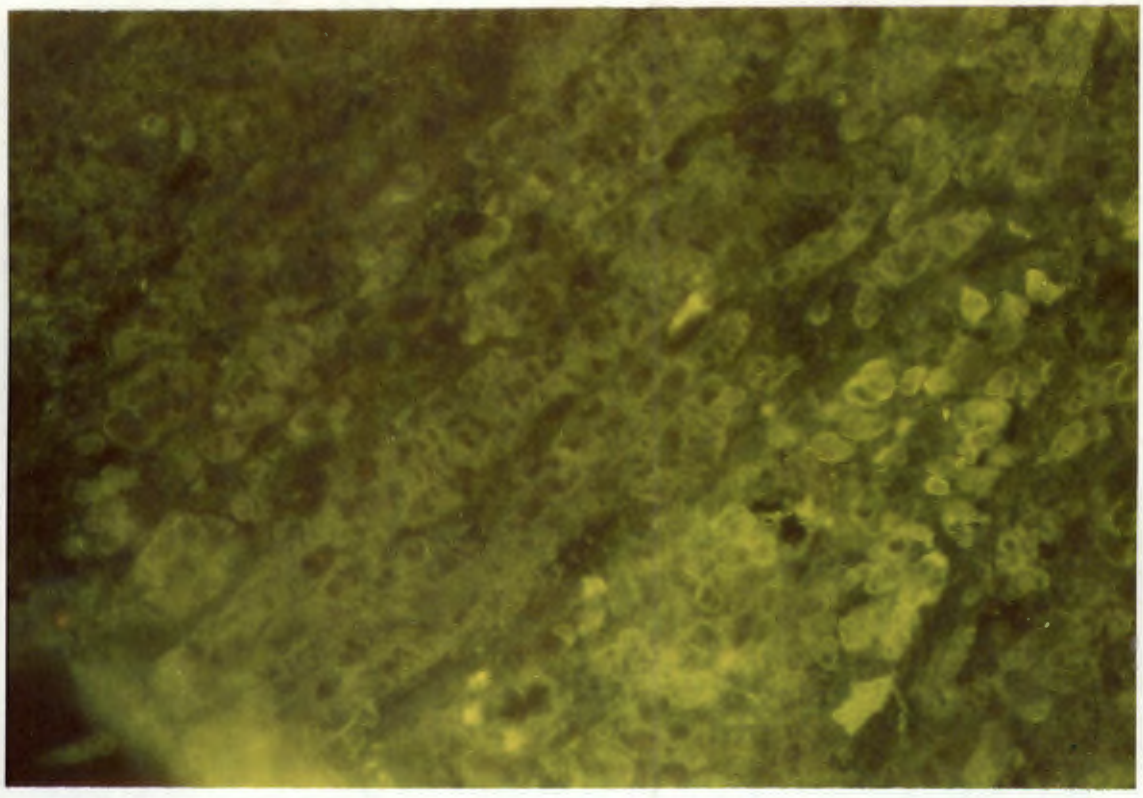
(f) Reaction of rabbit anti-crude heart extract antibody (section 7.A.1) showing a speckled staining pattern. (No evidence of striations)

Table 2.4 shows that antibodies to heart were present in all groups of sera, but the incidence and intensity of staining differed markedly. 85% of ARF and 80% of child CRF patients showed evidence of heart-reactive antibody (HRA) compared to 27% of normal children. Furthermore, whereas none of the controls showed staining more intense than 2+, 40-45% of the child rheumatic fever sera stained with an intensity of 3+ or 4+ (Fig.2.10a, b). The adult CRF sera also showed a higher incidence and intensity of staining compared to normal adults. Interestingly, the reactivity of the AGN patients was somewhat higher than might be expected (Table 2.4). Interpretation of these results is arguably somewhat subjective, and competition experiments might have permitted more sophisticated analyses.

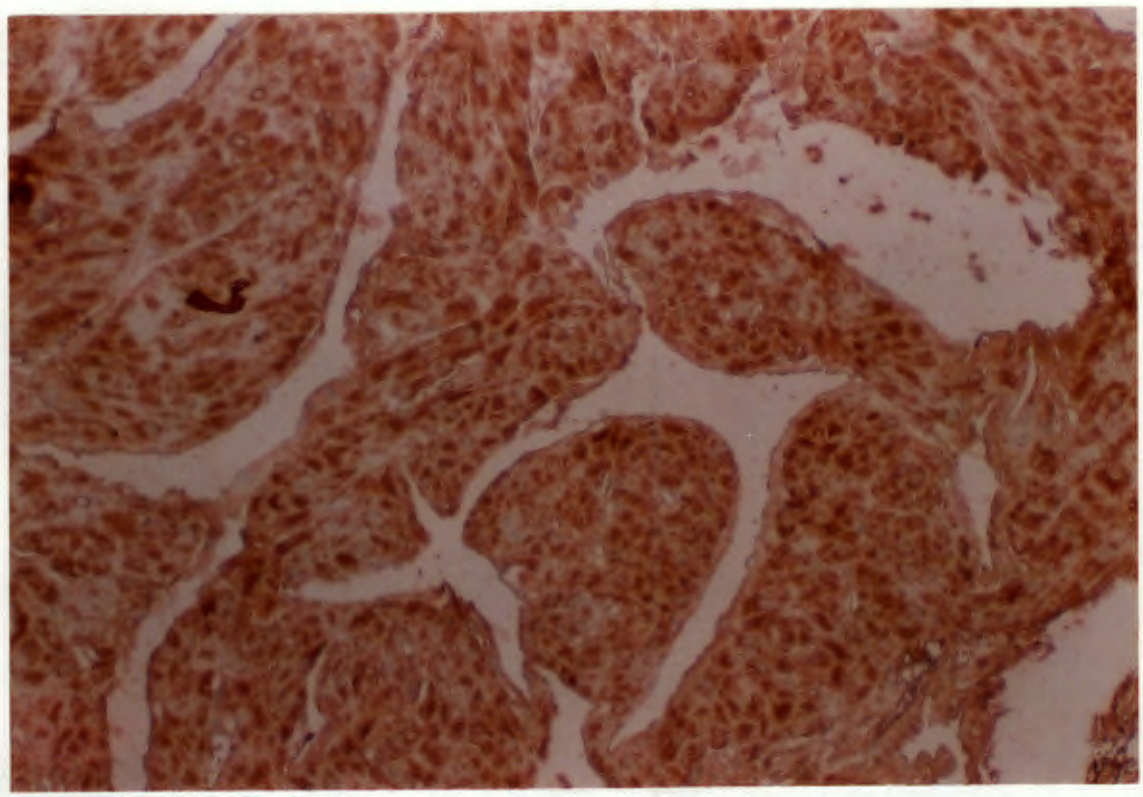
Examples of these binding patterns are shown in Fig 2.10(a,b,c,d). Intense staining of muscle fibres, both in longitudinal and in cross-section, can be clearly seen. The staining is not distinctly sarcolemmal but appears to permeate the cytoplasm of the cells. This could be an artefact of peroxidase staining of tissue sections, since the immunofluorescence staining depicted in Fig 2.11 shows a greater intensity of staining of the sarcolemmal membranes, which is in accord with previous reports of ARF serum reactivity against heart (Zabriskie 1985).

In preliminary investigations, indirect immunofluorescence was used, but the streptavidin-peroxidase method was preferred since there was no background binding, the method was sensitive and the staining was permanent (for future reference). Fig 2.11 compares the immunofluorescence and streptavidin-peroxidase staining of heart sections by a high titre ARF serum.

This study shows that the biotin-streptavidin-peroxidase assay for detection of heart-reactive antibody is sensitive and reproducible. The findings are in agreement with previous studies using indirect immunofluorescence, showing that up to 85% of ARF sera contained antibody binding to sections of normal heart. This assay also shows the presence of these antibodies in some normal individuals and children with acute glomerulonephritis. Although striational binding patterns were seen with several ARF sera, sarcolemmal binding was not distinctly evident using this assay.



a



b

Fig. 2.11 Comparison of immunofluorescence and biotin-streptavidin-peroxidase staining patterns of human heart tissue sections by an ARF serum.

(a) Frozen section of human heart incubated with an ARF serum diluted 1:10 (section 7.A.17), and stained with an Fab'$_2$ goat anti-human IgG (H+L) FITC-conjugated antibody (Zymed). Slides were observed under a Nikon Optiphot microscope using a PlanApo 60X oil lens, and photographed (FX-35A: Kodak 400 ASA). **(b)** Biotin-streptavidin-peroxidase staining (4+) of human heart tissue (section 7.A.18). Photographed using Nikon Optiphot microscope with FX-35A camera attachment (20 X magnification) and Kodak 400 ASA colour film.

2.3 CONCLUSIONS

In this study of sera from seven groups of rheumatic fever (RF) patients and control subjects:

1. All RF patients showed elevated concentrations of IgA. ARF and CRF-a patients also showed elevated levels of IgG.

2. All RF groups of sera, at a dilution of 1:100 in ELISA, contained raised levels of heart-reactive antibodies (HRA). CRF-a patients showed the highest mean titres at all dilutions.

3. CRF-a sera showed raised levels of anti-myosin antibodies (AMA) in ELISA at a 1:100 dilution, and these appeared to be correlated with raised heart-reactive antibodies.

4. ARF and AGN sera at dilutions of 1:100 and 1:1000 showed evidence of antibodies to actin in ELISA.

5. All RF groups of sera at a dilution of 1:1000 showed raised levels of antibodies to phosphorylase b (APA) compared to normal control groups; CRF-c and CRF-a sera also showed raised levels at a dilution of 1:100. In groups of sera with heart disease, these antibodies showed some correlation with heart-reactive antibodies.

6. Antibodies to cardiolipin do not appear to have a role in rheumatic fever.

7. Anti-streptolysin O (ASO) antibodies were significantly raised in ARF sera. Although ARF patients also had high titres of anti-DNAse B antibodies, the effect was obscured by statistical comparison with the control group of rural children who had exceptionally high anti-DNAse B titres.

8. An ELISA for detection of antibodies to whole group A streptococci, showed marginally elevated titres among CRF-a patients. However this assay also detected antibodies to other groups of streptococci.

9. CRF-a sera showed raised levels of antibodies to all eight streptococcal M proteins tested in ELISA. ARF and AGN sera also showed high titres to all M proteins (except M24) but the effect was obscured by the high titres to these proteins in the rural child control group. Antibodies to proteins M5 and M6 were raised in ARF patients compared to AGN and adult controls. These streptococcal serotypes may possibly be important among South African rheumatic fever patients.

10. There was evidence of antinuclear autoantibodies (ANA) in all groups of RF sera at dilutions of up to 1:80, as shown by fine speckled immunofluorescence staining against HEp-2 cell substrates. Individual RF sera also showed evidence of autoantibodies to mitochondria, centromeres and smooth muscle.

11. Among RF sera, one ARF patient contained antibodies to Sm antigen, and another to RNP antigen.

12. None of the RF patients showed any evidence of antibodies to double-stranded DNA.

13. 85% of ARF sera (and 80% of CRF-c) contained antibodies binding to sections of normal human heart, in a biotin-streptavidin-peroxidase assay. 40-45% of these showed intense staining. Several of these sera showed striational binding along the muscle fibre, but in cross-section a sarcolemmal pattern of binding was not distinctly evident.

14. Among the adult CRF patients, the raised levels of antibodies to several of the antigens investigated in this study may be indicative of polyclonal B cell activation. This does not necessarily preclude an antigen driven response.

15. RF sera showed binding to several streptococcal and cardiac antigens but antibodies to these antigens were also usually evident (although often at a lower frequency, or intensity of reaction) in normal and related-disease control sera. The pathological relevance of the anti-heart autoantibodies and their relationship to the anti-streptococcal antibodies in antigenic mimicry, remains to be elucidated.

3. IMMUNOBLOTTING: CARDIAC AND STREPTOCOCCAL ANTIGENS AGAINST RHEUMATIC FEVER AND CONTROL SERA

3.1. INTRODUCTION 64
3.1.1 Immunoblotting of cardiac antigens: Crude Extracts, Sarcolemmal membrane and Purified Proteins 64
3.1.1.1 Extracts of Heart Tissue: Procedures 64
3.1.1.1.1 Crude Extracts 64
3.1.1.1.2 Sarcolemmal Membrane Extracts 65
3.1.1.2 Immunoblotting of Heart Antigen Against Human Sera and Monoclonal Antibodies 65
3.1.1.2.1 Crude Extracts of Heart 65
3.1.1.2.2 Sarcolemmal Membrane Preparations 67
3.1.1.2.3 Purified Cardiac Antigens 67
3.1.1.2.3.1 Myosin 67
3.1.1.2.3.1.1 Rabbit Antiserum 67
3.1.1.2.3.1.2 Monoclonal Antibodies 68
3.1.1.2.3.2 Tropomyosin 69
3.1.2 Immunoblotting of Streptococcal Antigens 70
3.1.2.1 Crude Extracts and Purified M Proteins 70
3.1.3 Special Applications of Immunoblotting 70
3.1.3.1 Affinity Purification of Antibodies 70
3.1.3.2 Immunization 71
3.1.3.3 Antigens Recognized by T Lymphocytes 71
3.1.4 Cross-reactivity and Immunoblotting: Some Pitfalls 71

3.2 RESULTS 82
3.2.1 Human Heart Detergent Extracts 82
3.2.1.1 Non-rheumatic Heart Extract 82
3.2.1.1.1 Preliminary Investigations 82
3.2.1.1.2 Main Investigation 83
3.2.1.1.2.1 Identification and Descriptions of Antigen Bands 83
3.2.1.1.2.2 IgG Binding 86
3.2.1.1.2.3 IgA Binding 86
3.2.1.1.2.4 IgM Binding 86
3.2.1.2 ARF Heart Extract 94
3.2.2 Cross-reactivity: Human Heart and *S.pyogenes* Antigens 94
3.2.2.1 Eluted Antibody: Reactivity with Immunoblotted *S.pyogenes* Antigens 94
3.2.2.2 Eluted Antibody: Immunofluorescence Staining of Heart 95
3.2.3 Sarcolemmal Membranes 95
3.2.4 Nuclear Antigens 99
3.2.5 Reactivity of Anti-Streptococcal Sera with Heart Extract 102

3.2.6 Additional Experiments 106
3.2.6.1 Reactivity to Human Collagen Types II, III and VI 106
3.2.6.2 Ca^{2+} ATPase Binding 106

3.3 DISCUSSION 108
3.3.1 Human Heart Detergent Extracts 108
3.3.1.1 Isotype Binding Patterns of Groups of Sera 108
3.3.1.2 Hypothesis: Raised IgM Anti-heart Antibodies 109
3.3.1.3 Individual Protein Bands Recognised 110
3.3.1.4 Specificity and Pathological Relevance of Antigens 112
3.3.2 Cross-reactivity: Heart and *S.pyogenes* M Proteins 113
3.3.2.1 Reactivity of HRA with Immunoblotted Streptococcal Antigens 113
3.3.2.2 Immunofluorescence Staining of Eluted Antibody Against Heart 114
3.3.3 Sarcolemmal Membranes 114
3.3.4 Nuclear Antigens 115
3.3.5 Rabbit Anti-*S.Pyogenes* Serum Reactivity with Heart Antigens 116
3.3.6 Additional Experiments 116
3.3.6.1 Ca^{2+} ATPase Binding 116

3.4 CONCLUSIONS 118

3.1. INTRODUCTION

Immunoblotting has proven to be an important technique for the characterization of autoantigens and autoantibodies in numerous autoimmune diseases (Elkon et al., 1987). It has been used to identify autoantigens such as nuclear antigens (e.g. Habets et al., 1983), muscle proteins (Koga et al., 1987), thyroid antigens (Kotani et al., 1986) and bullous pemphigoid autoantigens (Labib et al., 1986). In addition to the efficient fractionation of autoantigens, the class, subclass, idiotype and specificity of autoantibodies can be determined.

The aim of this project was to analyse the reactivity of rheumatic fever (and control) sera to antigens from extracts of human heart tissue. Crude detergent extracts of human heart following separation on polyacrylamide gel slabs were electroblotted onto nitrocellulose, and the separated antigens were tested for reactivity with the seven groups of sera (described in Chapter 2). Various purified cardiac and streptococcal antigens were also studied using this method.

This section reviews previous reports of immunoblotting using patient sera, rabbit immune sera and monoclonal antibodies as probes to constituents of heart tissue and *S.pyogenes*.

3.1.1 IMMUNOBLOTTING OF CARDIAC ANTIGENS: CRUDE EXTRACTS, SARCOLEMMAL MEMBRANE AND PURIFIED PROTEINS

3.1.1.1 Extracts of Heart Tissue: Procedures

3.1.1.1.1 Crude Extracts

The method of extraction of heart tissue antigens may affect the detection of autoantigens. Various extracts of heart tissue antigen have been used. These include: saline extracts (Kusher and Kaplan, 1967; Zabriskie and Friedman, 1983); 3 M KCl sarcolemmal membrane extracts (Van de Rijn et al., 1977); dilute acid extracts (Zabriskie and Freimer, 1966; Espinosa and Kaplan, 1968), SDS (Dale and Beachey, 1983; Ayakawa et al., 1985; Doyle et al., 1986), Triton X-100 (Cunningham et al., 1983; 1984) and sodium deoxycholate methods (Shastry et al., 1988).

Recent investigators have favoured detergent extracts, although the zwitterionic detergent CHAPS (used in this study) has not previously been tried.

3.1.1.1.2 Sarcolemmal Membrane Extracts

A number of studies have localised the heart epitope(s) in rheumatic fever to the sarcolemmal membrane (Kaplan and Meyerserian, 1962; Kaplan, 1963; Zabriskie and Freimer, 1966; Zabriskie et al, 1970; Van de Rijn et al, 1977; Dale and Beachey, 1982a).

Immunoblotting of sarcolemmal membrane extracts has therefore been used in several studies to identify these antigens. In most studies, the membranes have been isolated by the method of Van de Rijn et al., (1977), and SDS extracts reacted by immunoblotting with monoclonal antibodies and rabbit hyperimmune sera (Dale and Beachey, 1982a; Cunningham et al., 1984; Dale and Beachey, 1985a; Sargent et al., 1987).

3.1.1.2 Immunoblotting of Heart Antigen Against Human Sera and Monoclonal Antibodies

Table 3.1 (pages 73-81) summarises reports in the literature of the molecular weight constituents present in human heart and streptococcal antigenic extracts, that have been found reactive with rabbit immune sera and monoclonal antibodies raised against such antigens. Except in the study by Zabriskie and Friedman (1983), none of these investigations by immunoblotting have used patient sera. This is remarkable because patient sera could be considered the most relevant to the pathogenesis of the disease.

3.1.1.2.1 Crude Extracts of Heart

Antibody-reactive constituents in immunoblotted heart and streptococcal antigenic mixtures can be grouped into three or four major mol.wt. regions. These are summarised below and in Table 3.1. The majority of immunoblotting experiments have been performed under reducing conditions of the antigen and it is possible that some of the minor constituents may be breakdown products of these antigens. Dale and Beachey (1982a) for instance, found differences in antibody reactivity against heart extracts separated under reducing and nonreducing conditions. Possibly, proteolytic cleavage might also produce spurious reactivities, although protease inhibitors are usually included in extraction procedures.

1. **<40 kD** molecular weight
   - (i) 30 kD constituent: in Triton X-100 extracts of human heart, reactive with rabbit hyperimmune serum to M5 and M25 protein (Cunningham et al., 1984).
   - (ii) 38 kD: in saline extracts of bovine heart, reactive with ARF sera (Zabriskie and Friedman, 1983).
   - (iii) 27 kD: in SDS extracts of sarcolemmal membranes, reactive with (pep M5) rabbit antiserum affinity purified to the cloned M5 protein (Poirier et al., 1985).

(iv) 32 kD: in SDS extracts of crude heart, reactive with antisera to *S.mutans*.

2. **40-45 kD** molecular weight

(i) 40 kD constituent: in Triton X-100 extracts of human heart reactive with rabbit hyperimmune serum to M5 protein. Also present in skeletal muscle but not in kidney. Also faintly reactive with normal rabbit serum (Cunningham and Russell, 1983).

(ii) 40 kD: in SDS crude extracts of human heart, reactive with antisera to M protein fragment SM5 (164-197)C (Sargent et al., 1987).

(iii) 40 kD: in Triton X-100 extracts of human heart and in SDS extracts of sarcolemmal membranes, reactive with murine mAbs (49.8.2 and 49.8.9) evoked by streptococcal M5 membranes (Cunningham et al., 1984).

(iv) 40 kD as one of multiple-bands: in SDS crude extracts of human heart, reactive with rabbit sera to pep M5. (Dale and Beachey, 1985a).

(v) 40 kD: in SDS crude extracts of human heart, reactive with antisera to *S.mutans* wall-membrane complexes (Ayakawa et al., 1985).

(vi) >40 kD: in Triton X-100 extracts of human heart, reactive with rabbit anti-M 25 antiserum (Cunningham et al., 1984).

(vii) 43 kD: in saline extracts of bovine heart reactive with ARF sera (Zabriskie and Friedman, 1983).

3. **67-70 kD** molecular weight

(i) > 67 kD: in Triton X-100 extracts of human heart, reactive with rabbit hyperimmune sera to M 5 protein (Cunningham and Russell, 1983).

(ii) 70 kD: in Triton X-100 extracts of human heart, reactive with rabbit antiserum protein M25 (Cunningham and Russell 1983).

(iii) 69 kD (and faint 68 kD): in SDS extracts of human heart atrial appendage, reactive with sera to *S.mutans* wall-membrane complexes (Ayakawa et al., 1985).

4. **200 kD** molecular weight

This constituent in SDS crude extracts of heart has been identified as myosin (Dale and Beachey, 1985a).

3.1.1.2.2 Sarcolemmal Membrane Preparations

Indirect immunofluorescence on frozen sections of heart showed that rabbit antisera evoked by group A streptococci cross-reacted with sarcolemmal membranes (Kaplan, 1963; Zabriskie and Freimer, 1966).

Dale and Beachey (1982a) showed that rabbit antisera to pep M5 reacted specifically with 1% SDS extracts of cardiac sarcolemmal membranes. The reaction could be inhibited by purified M5 and M19 proteins. The cross-reactive sarcolemmal antigen was a large polypeptide of 215 kD composed of four subunits (78 kD, 71 kD, 65 kD, 58 kD) cross-linked by disulphide bonds. This was the first evidence of specific cross-reactivity between streptococcal M proteins and a sarcolemmal membrane protein.

Using murine mAbs evoked by type M5 streptococcal membranes, Cunningham et al. (1984) detected a component of the same mol.wt. in similar SDS extracts of sarcolemmal membranes. Immunoblot inhibition experiments by Dale and Beachey (1985a) showed that this antigen was myosin. Other investigators have shown myosin associated with the internal membrane surface of the sarcolemma (Fallon and Nachmias, 1980).

This summary of the literature shows that antisera react with numerous constituents in heart extracts and it is therefore difficult to know which are of significance to antigenic mimicry in rheumatic fever.

3.1.1.2.3 Purified Cardiac Antigens

Since crude antigenic extracts may contain numerous irrelevant cross-reactive epitopes, specificity of cross-reactive antigens detected in such extracts should be verified using purified proteins (Lampson and Fisher, 1985). In rheumatic fever, two purified proteins that have been shown to be cross-reactive with streptococcal antigens are myosin and tropomyosin.

3.1.1.2.3.1 Myosin

3.1.1.2.3.1.1 Using Rabbit Antiserum

Krisher and Cunningham (1985) provided the first evidence that purified myosin was cross-reactive with streptococcal M protein. A murine monoclonal antibody (54.2.8) evoked by type 5 M protein, was found to react with myosin

(extracted from rabbit heart and skeletal muscle). The same mAb reacted with a high molecular weight protein in SDS crude sarcolemmal extracts of human heart. It was suggested that this component too was cardiac myosin. Examination of the structures of myosin and type 5 M protein by Manjula et al. (1985) showed homologies in the amino acid periodicity and the coiled-coil structure of the two molecules.

Further evidence was adduced by Dale and Beachey (1985a) who showed that only the 200 kD band from sarcolemmal extracts, displaying multiple bands in immunoblots, disappeared on pre-absorption of the sera with cardiac myosin. It was shown, more specifically, that this reactive component was the heavy chain of myosin. Reactivity of anti-pep M5 sera with this heavy chain could be partially inhibited by pep M6 protein, but not by pep M25, myosin light chains, actin or tropomyosin.

Using synthetic peptide derivatives of pep M5, Dale and Beachey (1986b) mapped the myosin-reactive M protein epitope to a stretch within 33 amino acids. The peptide SM (164-197) completely inhibited reactivity of anti-M5 sera with myosin heavy chain, whereas other peptides had no effect.

More recently, Cunningham et al. (1989) have mapped the myosin cross-reactive epitopes of the pep M5 molecule to an epitope within the fourteen residue carboxy terminus, which appears to involve the sequence GLN-LYS-SER-LYS-GLN.

3.1.1.2.3.1.2 Using Monoclonal Antibodies

The above results, obtained using rabbit antisera, were corroborated using monoclonal antibodies evoked by streptococcal antigens.

Cunningham et al. (1984) showed that murine mAbs evoked by type M5 streptococcal membranes reacted with a 200 kD band in SDS extracts of sarcolemma. Krisher and Cunningham (1985) demonstrated reactivity between one of these mAbs and myosin. Interestingly, this series of mAbs was shown to react more intensely with sarcolemmal extracts from rheumatic fever heart than from non-rheumatic heart.

Similar to rabbit immune sera to M5 protein, the murine mAbs were found to be reactive with only the heavy chain of myosin (Cunningham et al., 1985). mAb 36.2.8 reacted with skeletal light meromyosin, whereas mAb 58.2.8 reacted with skeletal and cardiac heavy meromyosin. Neither reacted with myosin light chain or was inhibited by myosin light chain and neither of the mAbs inhibited the ATPase activity of myosin.

Van de Rijn et al (1977) demonstrated cross-reactivity between heart and *S.mutans*, and Doyle et al. (1986) have shown that the cardiac antigen implicated may be myosin. Murine mAbs raised to *S.mutans* (mAb 22C4 and

mAb D159) reacted with a 200 kD constituent in extracts of heart, as well as with myosin and *S.pyogenes*. (The significance of this cross-reaction for rheumatic fever is not clear, although it has obvious implications for development of potential vaccines).

Human monoclonal antibodies, selected for their cross-reactivity between cardiac and group A streptococcal antigens, also reacted with myosin heavy chain (Cunningham et al., 1988).

These investigations present some compelling evidence that myosin (specifically the heavy chain) may be a relevant autoantigen implicated in antigenic mimicry in rheumatic fever.

3.1.1.2.3.2 Tropomyosin

Manjula and Fischetti (1980) revealed structural similarities between tropomyosin and M proteins of *S.pyogenes*, and these findings were substantiated immunologically by the demonstration that M5 and M6 shared epitopes with tropomyosin, and other α-helical coiled-coil proteins (Fenderson et al., 1989).

Murine mAbs evoked by purified M5 streptococcal membranes, and specific for either pep M5 or cloned M6 protein, reacted with tropomyosin. All were also reactive with myosin and some of them also reacted with actin, keratin or DNA.

The immunologic relationship between these M proteins and the mammalian muscle proteins (myosin and tropomyosin) could be of relevance to antigenic mimicry in rheumatic fever. Manjula and Fischetti (1980) suggested that the structural similarities between these proteins, might be responsible for the antiphagocytic property of the M protein.

It is known that the energy for phagocytosis is generated by a similar biochemical pathway to that of muscle contraction (Silverstein et al., 1977), and it is suggested that through their structural resemblance to tropomyosin, M proteins might interfere with the contractile machinery of leukocytes. It has been shown that in the absence of actin, the troponin-tropomyosin complex from muscle can confer Ca^{2+}-regulated Mg^{2+}-ATPase activity upon myosins isolated from guinea pig leukocytes and mouse fibroblasts (Stossel and Pollard, 1973; Adelstein et al., 1972). These findings suggest an interesting mechanism by which antigenic mimicry between *S.pyogenes* and muscle proteins may be implicated in the pathology of rheumatic fever - but they have as yet not been substantiated.

3.1.2 IMMUNOBLOTTING OF STREPTOCOCCAL ANTIGENS

3.1.2.1 Crude Extracts and Purified M Proteins

S.pyogenes antigens have also been analysed by immunoblotting, using similar monoclonal antibodies and rabbit antisera applied in the analyses of heart antigens. These studies indicate a broad region of reactivity in the mol.wt. range of 50-70 kD in SDS extracts of group A streptococci.

Murine mAbs evoked by streptococcal M5 membranes, reacted with a triplet at 58 kD and a group of bands between 62-67 kD (Cunningham and Swerlick, 1986). Two of these mAbs were shown to be reactive in immunoblotting with myosin (Krisher and Cunningham, 1985). Similarly, Jones and Fischetti (1987) showed that a mAb raised to M6 protein (*E.coli*-cloned), reacted with streptococcal constituents in the range 57-77 kD.

The reactive constituents in these streptococcal extracts have not been identified, but it is noteworthy that the mAbs which they bound were evoked by M proteins and showed cross-reactivity with myosin.

3.1.3 SPECIAL APPLICATIONS OF IMMUNOBLOTTING

Immunoblotting was also used in this study for the purpose of affinity purifying antibodies; for separating antigens to immunize rabbits; and for analyzing constituents in antigenic mixtures capable of stimulating lymphocytes (results not reported). These applications are briefly discussed here.

3.1.3.1 Affinity Purification of Antibodies

Sera can be affinity purified on immunoblots of antigenic extracts to give monospecific populations of antibodies (Olmsted, 1981; Smith and Fisher, 1984). The technique can be used as an expedient alternative to monoclonal antibodies or affinity chromatography. The nonspecific binding in whole serum, as well as the potential multispecific binding of monoclonal antibodies, may be averted.

The technique is also suited to the study of cross-reactivity between antigens: antibody can be eluted from one nitrocellulose band and reacted against another, to reflect whether the antigens share epitopes (Query and Keene, 1987). In a variation of the technique (see Chapter 5), antibody is eluted from purified plaques of a cDNA expression library, and reacted against immunoblotted antigens (Ozaki et al., 1986) or against tissue sections by immunofluorescence (Stanley et al., 1988)

3.1.3.2 Immunization

Immunoblotted antigens can be injected into animals to raise antibodies. The nitrocellulose band can be cut into small pieces or dissolved to a slurry in organic solvent, before immunization (Parekh et al., 1985). Alternatively, the antigen can be eluted from the nitrocellulose with dimethylsulphoxide or nonionic detergent (Knudsen, 1985; Anderson, 1985), or else implanted subcutaneously as an immunogen (Chiles et al., 1987). Antibodies raised by this method can be used to purify more of the original antigen.

3.1.3.3 Antigens Recognized by T Lymphocytes

Assays for the recognition of immunoblotted antigens by T cells have been developed by Young and Lamb (1986) and Lamb et al., (1988). Excised antigen bands are dissolved in dimethyl sulphoxide (DMSO) and precipitated into fine particles before being added to the wells in a lymphocyte proliferation assay (Abou-Zeid et al., 1987). Alternatively, they can be sterilized, cut into pieces of appropriate size, and then added to the lymphocyte cultures (Young and Lamb, 1986). By this technique, one can test the ability of antigens separated from a complex extract to induce a T cell response. (Results of these experiments are not reported in this study).

3.1.4 CROSS-REACTIVITY AND IMMUNOBLOTTING: SOME PITFALLS

The application of immunoblotting, a solid phase technique, to the study of cross-reactivity has some pitfalls which should be taken into account in interpreting results. These are discussed by Lampson and Fisher (1985), Van Regenmortel (1989) and Ghosh and Campbell (1986) and summarised below.

(1) Irrelevant, misleading cross-reactions may be detected in complex protein extracts, especially using monoclonal antibodies. Possibly, the dense concentration of antigen into a narrow band on the nitrocellulose, and the use an amplifying second antibody may contribute to this effect (Lampson and Fisher, 1985; Van Regenmortel, 1989).

(2) The relatively high concentration of irrelevant cross-reactions present in complex protein mixtures may obscure the effect of the antigen of interest. Antibody specificities should therefore be evaluated using not only the complex extract, but also purified proteins (Lampson and Fisher, 1985).

(3) Competition between different antibody subpopulations in serum, may obscure specificities detectable using monospecific or monoclonal antibodies. For instance, antigens detectable by human mAbs, might not be detected by the patient's serum.

(4) Denaturation of antigen by SDS during the electrophoresis, may both either destroy epitopes (especially conformational ones) or reveal new potentially cross-reactive epitopes (Lampson and Fisher, 1985). Binding of antigen to nitrocellulose paper may have similar effects (Ghosh and Campbell, 1986).

Bands on immunoblots will often vary in intensity in different immunoblots. It is important therefore to control for such effects by: (1) using antibody preparations of appropriate concentration and isotype; (2) using consistent antigen extracts that are protected against proteolysis; (3) using, where possible, purified proteins to confirm cross-reactions detected in crude extracts.

TABLE 3.1 *(see following 8 pages)*

IMMUNOBLOTTING: Summary of Published Reports on the Reactivity of Patient Sera, Rabbit Hyperimmune Sera and Monoclonal Antibodies Against Cardiac and Streptococcal Antigens.

The table summarises information reported in the literature on constituents of specific molecular weights (**mol.wt.band**) contained in various **antigenic extracts** of heart and streptococci which have been found by immunoblotting to be reactive with **ARF patient sera**, **monoclonal antibodies** (murine and human) or **rabbit hyperimmune** sera evoked by various streptococcal and cardiac antigens. **Control** antibody preparations used are shown. Various antigens cross-reactive with or inhibiting the reactions of the antibody reagents are listed (**other reactivities, inhibitions**). **References** describing the immunoblotting investigation are given (see also REFERENCES).

Abbreviations

K:	kilodaltons
Pos:	positive
mAb:	monoclonal antibody
α:	anti- (eg anti-M5 rabbit immune serum)
S/N:	supernatant
MW/mol.wt:	molecular weight
strep:	streptococcus
p.i.:	pre-immune
M :	streptococcal M protein
pep M5 (or 6, 19, 24 etc):	pepsin digests of the streptococcal M protein
SM5 (164-197)/(84-116)/(1-35):	these refer to synthetic peptides of streptococcal M5 protein comprising the given residue numbers

("36.2.2" and "T7", etc refer to designations of particular mAbs described in the quoted reference)

(see overleaf for Table 3.1)

MOL. WT. BAND	ANTIGENIC EXTRACT	ANTIBODY REACTIVITY					▪ OTHER REACTIVITIES ▪ INHIBITIONS ▪ REMARKS	REFERENCE
		A.R.F Sera	Control Human Sera	Monoclonal Antibody	Rabbit Hyperimmune Sera	Control rabbit/ mouse sera/ mAb S/N		
22K	Strep M5 membrane homogenate (Method: Van de Rijn et al 1976)	-	-	*α* Strep M5 membrane (54.2)	-	Negative	▪ sarcolemma (human) SDS extracted ▪ weak with kidney and skeletal muscle	Cunningham et al (1984)
30K	Strep M5 membrane homogenate (Method: Van de Rijn et al 1976)	-	-	-	-	Positive with p.i. mouse serum & mAb S/N	-	Cunningham et al (1984)
30K	4% Triton X-100 of human heart	-	-	-	*α* M5 *α* M25	Positive	-	Cunningham, & Russell (1983)
38K	Saline extract of bovine heart	Pos	-	-	-	-	-	Zabriskie & Friedman (1983)
40K	4% Triton X-100 of human heart	-	-	*α* strep M5 membrane	-	-	▪ human sarcolemma (Weak) ▪ whole strep A (strong)	Cunningham et al (1984)
40K	1% SDS extracts of human heart	-	-	-	*α* SM5 (164-197) C (affinity purified)	Negative	Complete inhibition by: ▪ SM5 (164-197) ▪ pep M5 No inhibition by: ▪ SM5 (1-35) or other peptides	Sargent et al (1987)
40K	4% Triton X-100 of human heart	-	-	-	*α* M5 *α* M25 (fainter)	Faint	Skeletal muscle (not kidney)	Cunningham & Russell (1983)

Table 3.1 (Immunoblotting)

MOL. WT. BAND	ANTIGENIC EXTRACT	ANTIBODY REACTIVITY					▪ OTHER REACTIVITIES ▪ INHIBITIONS ▪ REMARKS	REFERENCE
		A.R.F Sera	Control Human Sera	Monoclonal Antibody	Rabbit Hyperimmune Sera	Control rabbit/ mouse sera/ mAb S/N		
>40K	4% Triton X-100 of human heart	-	-	-	*α* M25	Faint	Kidney and skeletal muscle	Cunningham & Russell (1983)
43K	Saline extracts of bovine heart	Pos	-	-	-	-		Zabriskie & Friedman (1983)
43K	Kidney glomerular membranes	-	-	*α* kidney glomerulus (PM II)	-	-	▪ Strep A ▪ Pep M6 ▪ Pep M12 (not: Pep M1, M3,M5,M19,M24)	Goroncy-Bermes et al (1987)
43K	Strep M5 membrane homogenate (Method: Van de Rijn 1976)	-	-	*α* strep M5 membrane 36.2.2 36.2.1	-	Positive with p.i. mouse serum	▪ Whole strep A ▪ Kidney & skeletal muscle.	Cunningham et al (1984)
50K 52.5K 55.6K 56K 64K	*E.coli* cloned M5	-	-	-	*α* Pep M5	Negative with *E.coli* lysate	-	Kehoe et al (1985)
52.9K 55.4K 57.9K 40K 32.6K 27K	*E. coli* cloned M5	-	-	*α* Pep M5 (11C 5.6)	*α* Pep M5	Negative with *E.coli* control lysate	-	Poirier et al (1985)
57 - 77K	Extracts of Group A and Group G strep	-	-	*α* strep A M6	-	-	-	Jones & Fischetti (1987)

Table 3.1 (Immunoblotting)

MOL. WT. BAND	ANTIGENIC EXTRACT	ANTIBODY REACTIVITY					▪ OTHER REACTIVITIES ▪ INHIBITIONS ▪ REMARKS	REFERENCE
		A.R.F Sera	Control Human Sera	Monoclonal Antibody	Rabbit Hyperimmune Sera	Control rabbit/ mouse sera/ mAb S/N		
58K	5% SDS extract of ▪ whole Group A strep ▪ M type 5 strep membranes	-	-	*α* strep M5 membrane 36.2.2 54.2.8 49.8.9	-	-	▪ 36.2.2 myosin (M5,M6) ▪ 54.2.8 myosin (M5,M6) ▪ 49.8.9 Pep M5; heart extracts; <u>not</u> myosin	Cunningham & Swerlick (1986)
60 - 70K	Extracts of skin	-	-	*α* strep M5 membrane (54.2.8)	-	-	▪ Myosin ▪ M5 ▪ M6 ▪ Strep A whole	Swerlick & Cunningham (1986)
62 - 67K	5% SDS extract of ▪ whole Group A strep, ▪ M type 5 strep membranes	-	-	*α* Strep M5 membrane 36.3.3 54.2.8	-	-	Inhibited by myosin	Cunningham & Swerlick (1986)
62 - 67K	SDS extracts of whole *S.Mutans* and *S.Rattus*	-	-	*α* strep M5 membrane 54.2.8 36.2.2	-	-	▪ M5; M6 of *S.pyogenes* ▪ cardiac & skeletal muscle myosin	Ayakawa et al (1988)
>67K	Triton X-100 of human heart	-	-	-	*α* M5	Faint	▪ Kidney and ▪ Skeletal muscle tissue extracts	Cunningham & Russell (1983)
69K also 54K, 32K, 40K, 50K 68K	1% SDS extracts of human heart (atrial appendage) (not sarcolemma)	-	-	-	*α S. Mutans* BHT: membrane wall complexes	Positive with rabbit normal sera	-	Ayakawa et al (1985)

Table 3.1 (Immunoblotting)

MOL. WT. BAND	ANTIGENIC EXTRACT	ANTIBODY REACTIVITY					▪ OTHER REACTIVITIES ▪ INHIBITIONS ▪ REMARKS	REFERENCE
		A.R.F Sera	Control Human Sera	Monoclonal Antibody	Rabbit Hyperimmune Sera	Control rabbit/ mouse sera/ mAb S/N		
70K	4% Triton extracts X-100 of human heart	-	-	-	α M25	Faint	Kidney and skeletal muscle tissue extracts	Cunningham & Russell (1983)
70K	Human & rabbit cardiac tropo-myosin	-	-	α M5 membrane 36.2 54.2 27.4 112.2 654.1	-	Negative	Inhibited by: DNA 54.2, 654.1 Actin 36.2, 112.2 Keratin 27.4, 36.2 Myosin 27.4, 36.2, 54.2, 654.1	Fenderson et al (1989)
94K	Whole strep M5 homogenate	-	-	α strep M5 membrane (54.2)	-	Negative	▪ α sarcolemma (human SDS extracted) ▪ Weak in kidney and skeletal muscle ▪ Whole strep A	Cunningham et al (1984)
>94K	Triton X-100 of human heart	-	-	-	α M5	Negative	-	Cunningham & Russell (1983)
100-200K	Triton X-100 of human heart	-	-	α strep M5 membrane ("54 series")	-	Negative	▪ Weak/unreactive in kidney & skeletal muscle ▪ Whole Strep A ▪ Strep A membranes	Cunningham et al (1984)

Table 3.1 (Immunoblotting)

MOL. WT. BAND	ANTIGENIC EXTRACT	ANTIBODY REACTIVITY					▪ OTHER REACTIVITIES ▪ INHIBITIONS ▪ REMARKS	REFERENCE
		A.R.F Sera	Control Human Sera	Monoclonal Antibody	Rabbit Hyperimmune Sera	Control rabbit/ mouse sera/ mAb S/N		
200K	Rabbit skeletal muscle myosin	-	-	Human mAb selected against *S.Pyogenes* M5 & heart extract (Triton X-100) ▪ **PB1** (Patient Cellulitis) ▪ **T2B** (Patient Pharyngitis) ▪ **T1;T2;T6;T7.1; T7.3** (normal individuals)	-	Negative	In ELISA inhibited by: Actin: T6;T7.1 Keratin: T6 Keratin: PB1;T1;T2 DNA: PB1;T1 Not collagen or IgG	Cunningham et al (1988)
200K	Human heart sarcolemma SDS extract (Method: Van de Rijn et al 1977) ▪ Rheumatic ▪ Non-rheumatic heart	-	Positive	*α* strep M5 membrane ("54 series")	-	Negative	(Remark: reaction more intense in rheumatic sarcolemma than non-rheumatic sarcolemma	Cunningham et al (1984)
Myosin	Rabbit cardiac myosin (heavy chain)	Pos. by ELISA	Some reactivity	-	*α* Pep M5	Negative	Inhibited by: ▪ Pep M5 (totally), ▪ Pep M6 (partially), ▪ Pep M24 (none) Not Tropomyosin, actin or myosin light chains	Dale & Beachey 1985 (b)

Table 3.1 (Immunoblotting)

MOL. WT. BAND	ANTIGENIC EXTRACT	ANTIBODY REACTIVITY					▪ OTHER REACTIVITIES ▪ INHIBITIONS ▪ REMARKS	REFERENCE
		A.R.F Sera	Control Human Sera	Monoclonal Antibody	Rabbit Hyperimmune Sera	Control rabbit/ mouse sera/ mAb S/N		
40 - 200K	1% SDS extracts of human heart sarcolemma membrane	-	-	-	*α* Pep M5	Negative	200K band absorbed out by cardiac myosin	Dale & Beachey 1985 (a)
230K Myosin	1% SDS extracts of human heart sarcolemma membrane	-	-	-	*α* Pep M5	Negative	Inhibited by: ▪ Pep M5 (totally) ▪ SM5 (84-116) (almost total) ▪ SM5 (1-35) none at all	Dale & Beachey (1986)
Multiple Bands	Purified sarcolemma membranes	-	-	-	*α* Pep M5 (affinity purified)	-	Inhibition of: ▪ high & low MW proteins byPep M6 ▪ low MW proteins by Pep M19 ▪ total inhibition by Pep M5	Dale & Beachey 1985 (a)
Myosin	▪ Rabbit skeletal muscle myosin, ▪ cardiac ventricular myosin ▪ SDS heart extracts (myosin)	-	-	*α* strep M5 membrane (54.2.8)	-	Negative	Inhibited by: ▪ Group A strep ▪ M types, 5; 6; 12	Krisher & Cunningham (1985)
215K	SDS sarcolemma (human heart) ▪ unreduced: 215K, ▪ reduced: 78K; 71K;65K;58K	-	-	-	*α* Pep M5	-	Inhibited by: ▪ sarcolemmal membrane ▪ M5 strep ▪ M19 strep ▪ Pep M5 ▪ Pep M19 (not other serotypes)	Dale & Beachey (1982)

Table 3.1 (Immunoblotting)

MOL. WT. BAND	ANTIGENIC EXTRACT	ANTIBODY REACTIVITY					▪ OTHER REACTIVITIES ▪ INHIBITIONS ▪ REMARKS	REFERENCE
		A.R.F Sera	Control Human Sera	Monoclonal Antibody	Rabbit Hyperimmune Sera	Control rabbit/ mouse sera/ mAb S/N		
200K 80K 165K	1% SDS human heart extract	-	-	*α S. Mutans* P-4 ▪ **mAb D159** (200K; 85K) ▪ **mAb 22C4** (200K; 165K)	-	Negative	▪ Myosin ▪ *S. Pyogenes*	Doyle et al (1986)
42K 56K 82K 85K	Extract *S.Mutans* BHT cell membrane	-	-	*α S. Mutans* P-4 ▪ **mAb 22C4** (42K; 56K; 85K) ▪ **mAb D159** (82K)	-	Negative	▪ Myosin ▪ *S. Pyogenes*	Doyle et al (1986)
42K 46K 62K 82K	S.*Mutans* glass bead homogenates or mutanolysin digest	-	-	-	*α* heart tissue homogenate antiserum	Negative	(suggested correspondence to 85K, 56K, 42K of *S.Mutans* - Doyle et al 1986)	Ayakawa et al (1985)
200K 59K 55K 53K 21K	Human heart sarcolemmal membranes (SDS extracts)	-	-	-	Pep M5 affinity purified *α* M5 (*E.coli* cloned)	-	Inhibited completely by Pep M5	Poirier et al (1985)

Table 3.1 (Immunoblotting)

MOL. WT. BAND	ANTIGENIC EXTRACT	ANTIBODY REACTIVITY					▪ OTHER REACTIVITIES ▪ INHIBITIONS ▪ REMARKS	REFERENCE
		A.R.F Sera	Control Human Sera	Monoclonal Antibody	Rabbit Hyperimmune Sera	Control rabbit/ mouse sera/ mAb S/N		
Myosin	Cardiac and skeletal muscle myosin	-	-	*a* strep M5 membrane ▪ **32.2.2:** skeletal light mero-myosin ▪ **58.2.8:** heavy mero-myosin: ▪ Both with skeletal and cardiac heavy chain myosin	-	Negative	▪ Group A strep ▪ Pep M5 ▪ Not myosin light chain ▪ Not Na^+/K^+ ATPase	Cunningham et al (1985)
cloned MW 60K	*E.coli* cloned M6 protein	-	-	Human mAb from tonsillar lymphocytes selected against: *S.Pyogenes* M5 and Triton X-100 heart extract: **T6;T7;T7.3**	-	Negative	-	Cunningham et al (1988)

Table 3.1 (Immunoblotting)

3.2 RESULTS

Immunoblotting has been extensively used to investigate cross-reactivity in rheumatic fever, but remarkably few of these studies have used patient serum to detect the antigens. Monoclonal antibodies and rabbit immune sera have been preferred because of their known specificity and the high titre of potentially relevant antibodies they contain. Patient serum could, however, be considered more relevant to the disease.

The objective of this study was to compare the immunoblotting binding patterns of rheumatic fever and control sera to various cardiac and *S.pyogenes* antigens, in order to determine whether unique reactivities existed in the disease.

The same seven groups of sera analysed for reactivity to these antigens by ELISA and immunocytochemistry (Chapter 2), were used to analyze reactivity of autoantibody isotypes against separated cardiac polypeptides by immunoblotting. In addition, immunoblotting was used to analyse cross-reactivity between cardiac and group A streptococcal antigens, using patient (and rabbit immune) sera.

3.2.1 Human Heart Detergent Extracts

3.2.1.1 Non-rheumatic Heart Extract

Crude extracts of postmortem human heart without any evidence of rheumatic (or other) heart disease, were prepared using the detergent CHAPS (3-[(3-cholamidopropyl)-diemthylammonio]-1-propane-sulfonate), as described in Materials and Methods (section 7.A.11).

3.2.1.1.1 Preliminary Investigations

Preliminary investigations in this study examined the reactivity patterns of rheumatic fever and control sera against immunoblotted extracts of human heart, prepared using the following extractants: 4% Triton X-100, 4% SDS, 3M KCl, 5 mM octyl glucoside and 5 mM CHAPS detergent.

Compared to the other methods, heart tissue extracted with CHAPS demonstrated the most consistent differences in patterns of reactivity between rheumatic fever and control sera. This method of extraction was therefore standardly used in this study. In addition to its efficient solubilization of proteins, this zwitterionic detergent is efficient at breaking up artefactual protein aggregates, is only mildly denaturing and its electrical neutrality leaves

intact the natural charge properties of the protein. Furthermore, CHAPS is compatible with charge fractionation techniques such as SDS PAGE.

Protease inhibitors (thymol and PMSF) were added to extracts, which were aliquoted and stored at -80°C, and not refrozen after being thawed for initial use. No change in the binding patterns of the extracts was seen even if they were left at room temperature overnight before use (or after several months of storage at -80°C), suggesting that no major proteolysis occurred in the extract.

Preliminary control studies also involved a comparison of serum reactivities against extracts from five different human hearts (all obtained at postmortem within 8 hours after death). Although (compared to each other) sera reacted with different patterns, any particular serum reacted with the same pattern on all five heart extracts. Differences in reactivity patterns were thus not attributable to allotypic differences among individual heart donors. None of the five donors had postmortem evidence of rheumatic heart disease. Extracts were prepared so as to include tissue from different areas of the heart.

The staining pattern of individual sera was distinctive and reproducible with respect to both mol.wt. and intensity of staining. Preliminary studies also showed that the use of different blocking reagents (2% BSA; 5% BLOTTO; 1% gelatin; 3% FCS - all containing, in addition, 1% goat serum and 0.05% Tween 20) affected only the intensity but not the pattern of binding. Since the same distinctive pattern was obtained with repeated testing of particular sera against different hearts, the observed pattern was peculiar to the serum tested.

The initial use of a combined IgG, IgA, IgM peroxidase conjugate (Cappel) failed to reveal clearly discernible differences in binding between the groups of sera, and therefore separate class-specific Fab'_2 fragment peroxidase conjugates were used (Cappel).

3.2.1.1.2 Main Investigation

Twenty sera in each of the seven groups of subjects, were compared for IgG, IgM and IgA autoantibody isotype reactivity with antigens in the heart extract.

3.2.1.1.2.1 Identification and Descriptions of Antigen Bands

Eleven major bands of reactivity were distinguishable. Fig. 3.1 shows an Amido Black protein stain of these immunoblotted antigens from extracts of both normal heart and postmortem acute rheumatic fever heart (section 7.A.11). The mol.wt. (as well as the designated number) of each of the eleven bands stained by sera, are depicted. The mol.wt. of the stained antigens ranged from 116 kD to 28 kD. (Proteins smaller than 20 kD may have been missed by passing through the 0.45 μm pores of the nitrocellulose during transfer).

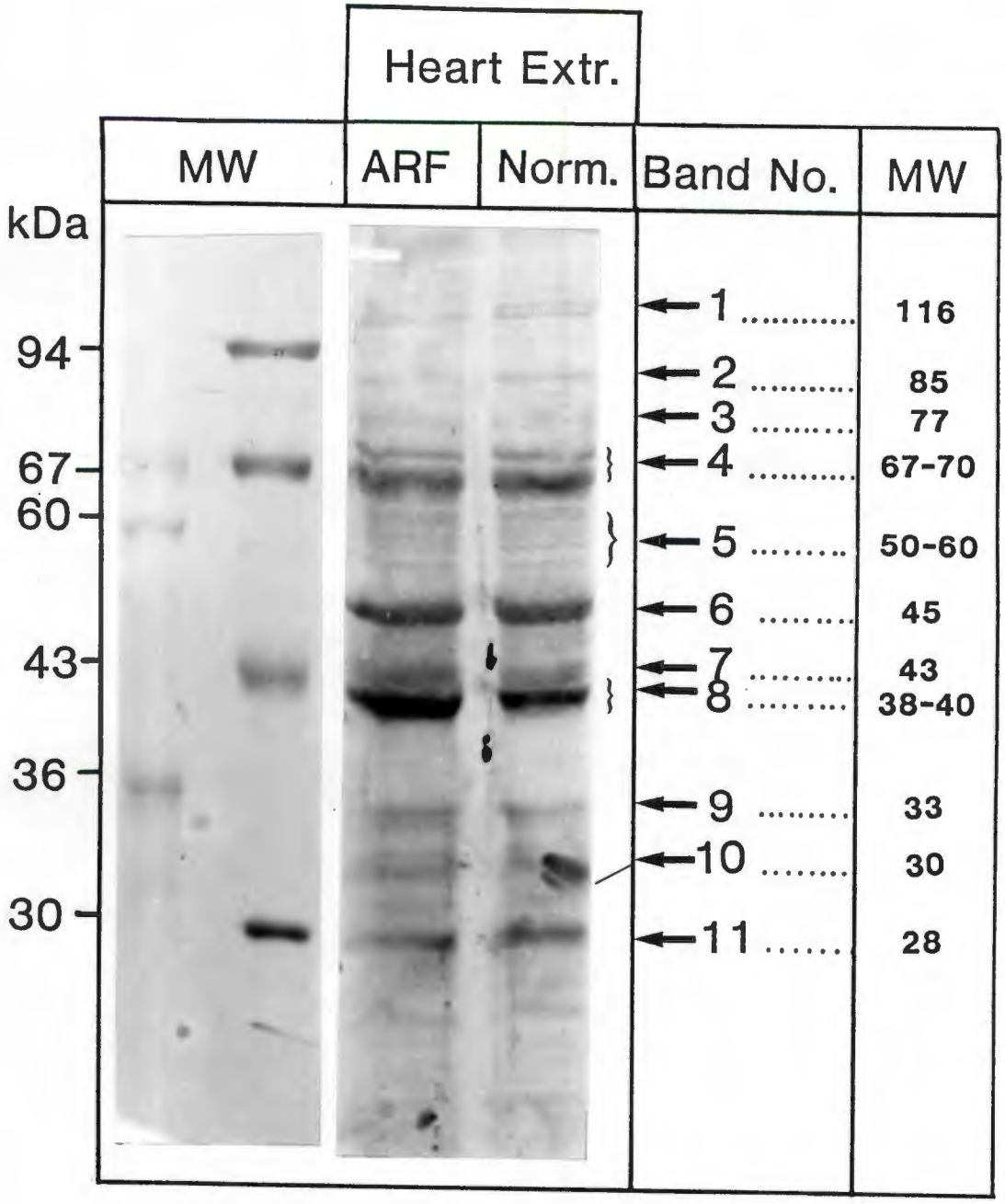


Fig. 3.1 Amido Black protein stains of immunoblotted crude extracts of normal and rheumatic fever heart showing the eleven bands reactive with rheumatic fever sera.

Extracts of postmortem normal and acute rheumatic fever heart were prepared using CHAPS detergent as described in section 7.A.11. The extracts were separated by 10% SDS PAGE, transferred to nitrocellulose (section 7.A.9) and stained with Amido Black (0.1% w/v in 2% acetic acid) followed by destaining in hot 2% acetic acid. Low and high molecular weight markers (Pharmacia) were likewise blotted and stained. The mol.wts. of the eleven major bands reactive with rheumatic fever sera were determined and designated Bands No.1-11.

The intensity of staining of each serum was scored as one to three pluses (+, ++, +++) by visual comparison. An average of two (and in many cases three) determinations was made for each serum, and intensities were scored by two independent observers.

Results, presented in Table 3.2, are presented as the percentage of sera in each group staining the eleven protein bands in the extract, at a particular intensity score.

The eleven protein bands were described as follows:

Band No.	Mol. Wt. and General Description of Staining:
No.1	116 kD: A faint doublet
No.2	85 kD A singlet except with ARF sera which appeared to stain it as a broad doublet.
No.3	77 kD: A faint and often diffuse band
No.4	67-70 kD: A prominent band at 67 kD, with a narrower sharp band above it at 70 kD.
No.5	50-58 kD: A broad area of reactivity comprised of about 3-5 narrow bands.
No.6	45 kD: A broad band staining strongly but nonspecifically with IgG (the peroxidase conjugate in the no-serum control also stained it). IgM binding was specific although less intense.
No.7	43 kD: A narrow sharp band showing strong IgG binding but faint with IgM.
No.8	38-40 kD: A prominent broad band reactive with all three antibody isotypes.
No.9	33 kD: Stained as a singlet by most sera, but by ARF sera usually as a broad doublet. IgM reactivity with this band appeared unique to ARF sera.
No.10	30 kD: A band stained by some sera as a broad doublet (especially by ARF sera); recognised by both IgG and IgM antibodies (and less intensely by IgA) in most sera except AGN and IHD.

No.11 28 kD: A faint diffuse band recognized by IgG and IgM class antibodies mainly in ARF sera, but also present (less intensely) in normal sera.

The antibody classes IgG, IgM and IgA thus recognised generally the same protein bands, but there were differences in the frequency, intensity and pattern of staining among the different groups of sera.

3.2.1.1.2.2 IgG Binding

IgG binding to the heart antigens was stronger than IgM and IgA in all groups of sera, both in terms of the total number of bands recognised and in the intensity of staining (Table 3.2a). Within groups of sera, there was considerable heterogeneity in the staining of individual bands. This heterogeneity was greatest among the ARF and adult CRF sera which also recognised some additional constituents, other than the eleven major ones (Fig. 3.2).

However, comparing the serum groups as a whole there were no remarkable differences in IgG recognition of these heart antigens.

3.2.1.1.2.3 IgA Binding

IgA binding to the antigens was generally weak (Table 3.2c). The intensity and number of antigens recognised was greatest among ARF and adult CRF sera, whereas the response of the AGN sera was remarkably lower than any of the other groups of sera. The response of the normal adult sera also showed an unexpectedly low response compared to the other control groups.

3.2.1.1.2.4 IgM Binding

IgM binding showed the most pronounced differences in staining between the groups. ARF sera stained all eleven bands at a higher frequency and intensity than any of the other groups (Table 3.2b). Some bands were not bound at all by IgM in certain groups of sera, notably by IHD and both adult and child CRF sera (Fig. 3.2).

In the mol.wt. range above 70 kD and below 40 kD, ARF sera showed evidence of unique IgM binding (Fig. 3.2). IgM recognition of band No.9 (33 kD) appeared unique to ARF sera, which stained it as a broad doublet. Excepting for one adult CRF serum (Fig.3.2c) and three normal adult sera (Fig.3.2e), which stained this band as a singlet, none of the other sera showed IgM binding to this antigen. Table 3.1c shows that the IgG response of ARF sera to this antigen was also more intense than that of the other groups.

IgM binding to bands No.4 (67-70 kD), No.8 (38-40 kD) and No.10 (30 kD), were also markedly more intense among ARF sera than in the other groups

(Table 3.2b). Although the intensity of staining of band No.1 (116 kD) was not great, the frequency of IgM recognition of this band (85%) among ARF sera was distinctly higher than that of other groups (10%) (Table 3.2b). Similarly, 100% of ARF sera showed IgM binding to band No.2 (85 kD), compared to between 5-and 30% in other groups.

Bands No.9, No.10 and No.11 were not recognised by IgM antibodies in AGN and IHD sera (Table 3.2b).

TABLE 3.2 (A, B, C) *(see following 3 pages)*

Immunoblotting of human heart extract against patient sera

Human heart CHAPS detergent extract (section 7.A.11) was separated by 10% SDS PAGE and transferred to nitrocellulose (section 7.A.9). Twenty sera in each of the seven groups of subjects (section 2.2.1.2) were compared for IgG, IgM and IgA autoantibody isotype reactivity (Table 3.2 A, B & C respectively) with antigens in the immunoblotted extract. Eleven major bands of reactivity were discernible (see Fig 3.1) and were designated bands No.1-11. The intensity of staining was scored as one to three pluses (+, + +, + + +) by visual comparison (average after two or three determinations). The percentage of patients in the various serum groups staining each band at a particular intensity was scored separately for each antibody isotype. (The number of patients showing no antibody binding to any of the bands was also recorded).

Notes:

* n = number of patients tested in each group

† The percentage of patients in each group giving a positive reaction at intensities scored as one to three pluses (+ + +, + +, or +). Scores are the results of visual comparisons made by three independent observers of two to three separate determinations.

‡ The number of patients in each group that reacted with none of the protein bands mentioned in the table.

▪ The designated number and apparent molecular weight of the eleven major protein bands detected by sera in the various groups.

• The seven patient groups tested.

TABLE 3.2A : IgG BINDING TO HEART ANTIGENS

Percentage of patients in each group whose sera stained specified protein bands at different intensities

Protein■ Bands	Staining Intensity	ARF● n = 20*	AGN n = 20	CRF-C n = 20	CRF-A n = 20	IHD n = 20	CONT-C n = 20	CONT-A n = 10
No. 1	+ + +	-	-	-	-	-	-	-
116K	+ +	†20	-	-	-	-	-	-
	+	80	40	60	100	100	40	80
No. 2	+ + +	-	-	-	-	-	-	10
85K	+ +	20	-	-	-	20	10	10
	+	70	20	60	60	80	90	55
No. 3	+ + +	-	-	10	-	-	-	-
77K	+ +	-	-	10	-	-	-	-
	+	70	5	50	60	100	100	80
No. 4	+ + +	40	-	-	-	-	-	20
67 -	+ +	25	-	60	60	20	30	30
70K	+	30	40	30	30	80	70	30
No. 5	+ + +	-	-	-	-	-	-	-
50 -	+ +	10	-	-	10	-	-	-
58K	+	70	45	60	80	85	90	80
No. 6	+ + +	40	-	-	10	-	-	-
45K	+ +	50	30	100	80	100	100	75
	+	10	30	-	10	-	-	10
No. 7	+ + +	-	-	-	-	-	-	-
43K	+ +	20	-	30	55	-	-	-
	+	75	-	70	40	100	90	70
No. 8	+ + +	-	-	-	-	-	-	-
38 -	+ +	85	20	40	60	-	-	-
40K	+	10	40	60	40	100	90	70
No. 9	+ + +	-	-	-	-	-	-	-
33K	+ +	90	-	-	10	-	-	-
	+	10	10	50	55	20	20	70
No. 10	+ + +	-	-	-	-	-	-	-
30K	+ +	50	-	50	70	-	-	-
	+	50	10	50	30	-	35	80
No. 11	+ + +	-	-	-	-	-	-	-
28K	+ +	-	-	-	-	-	-	-
	+	100	-	50	80	-	60	65
Patients with no bands‡		-	-	-	-	2	1	-

TABLE 3.2B : IgM BINDING TO HEART ANTIGENS

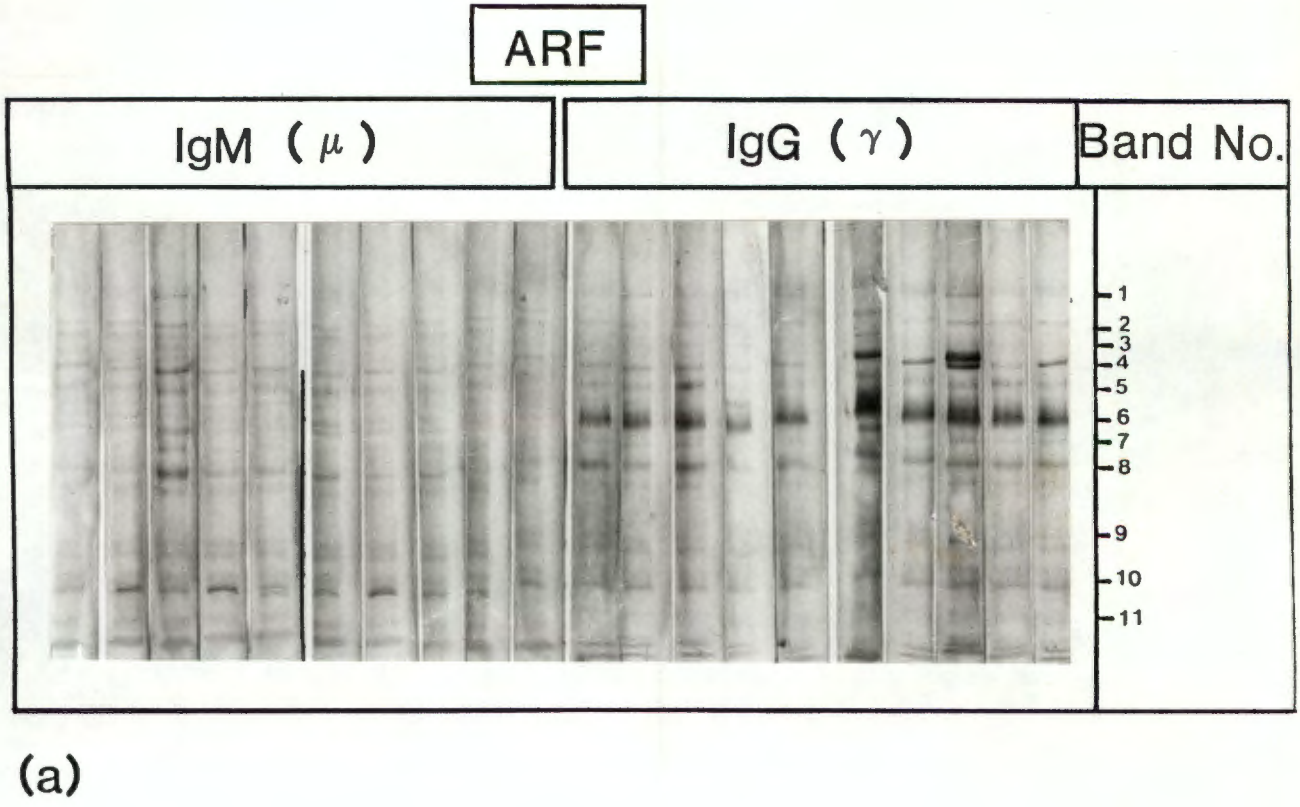
Percentage of patients in each group whose sera stained specified protein bands at different intensities

Protein* Bands	Staining Intensity	ARF• n = 20*	AGN n = 20	CRF-C n = 20	CRF-A n = 20	IHD n = 20	CONT-C n = 20	CONT-A n = 10
No. 1	+ + +	-	-	-	-	-	-	-
116K	+ +	10	-	-	-	-	-	-
	+	75	10	-	-	-	10	10
No. 2	+ + +	-	-	-	-	-	-	-
85K	+ +	20	10	-	-	-	-	-
	+	80	20	-	5	-	-	20
No. 3	+ + +	-	-	-	-	-	-	-
77K	+ +	-	-	-	-	-	-	-
	+	90	25	-	10	5	50	80
No. 4	+ + +	10	-	-	-	-	-	-
67 -	+ +	75	-	-	-	-	-	-
70K	+	10	90	20	25	-	75	80
No. 5	+ + +	-	-	-	-	-	-	-
50 -	+ +	20	-	-	-	-	-	-
58K	+	80	70	10	10	10	-	70
No. 6	+ + +	-	-	-	-	-	-	-
45K	+ +	-	-	-	5	-	-	-
	+	80	70	10	10	-	-	30
No. 7	+ + +	-	-	-	-	-	-	-
43K	+ +	25	10	-	-	-	-	-
	+	70	80	-	10	5	50	60
No. 8	+ + +	10	-	-	-	-	-	-
38 -	+ +	60	-	-	-	-	20	20
40K	+	30	75	45	50	-	80	70
No. 9	+ + +	-	-	-	-	-	-	-
33K	+ +	20	-	-	-	-	-	-
	+	80	-	-	10	-	-	10
No. 10	+ + +	50	-	-	-	-	-	-
30K	+ +	50	-	10	20	-	20	5
	+	-	-	70	60	-	80	35
No. 11	+ + +	-	-	-	-	-	-	-
28K	+ +	95	-	-	-	-	-	-
	+	5	-	-	-	-	50	70
Patients with no bands‡		-	-	-	7	7	3	1

TABLE 3.2C : IgA BINDING TO HEART ANTIGENS

Percentage of patients in each group whose sera stained specified protein bands at different intensities

Protein▪ Bands	Staining Intensity	ARF● n = 20*	AGN n = 20	CRF-C n = 20	CRF-A n = 20	IHD n = 20	CONT-C n = 20	CONT-A n = 10
No. 1 **116K**	+ + +	-	-	-	-	-	-	-
	+ +	-	-	-	-	-	-	-
	+	45	-	20	25	30	10	-
No. 2 **85K**	+ + +	-	-	-	-	-	-	-
	+ +	-	-	-	-	15	-	-
	+	40	-	30	35	25	5	-
No. 3 **77K**	+ + +	-	-	-	-	-	-	5
	+ +	-	-	-	-	10	-	-
	+	55	-	45	35	25	10	-
No. 4 **67 - 70K**	+ + +	-	-	-	-	-	-	5
	+ +	-	-	5	-	-	-	-
	+	50	-	45	40	40	15	15
No. 5 **50 - 58K**	+ + +	-	-	-	-	-	-	-
	+ +	5	20	10	25	25	-	10
	+	80	15	40	20	15	60	40
No. 6 **45K**	+ + +	-	-	-	-	-	-	-
	+ +	5	-	-	-	-	-	-
	+	50	30	50	45	40	35	45
No. 7 **43K**	+ + +	-	-	-	5	-	-	-
	+ +	5	10	-	20	-	10	5
	+	80	40	50	25	50	40	40
No. 8 **38 - 40K**	+ + +	-	-	-	-	-	-	-
	+ +	15	20	-	20	-	10	-
	+	60	20	15	30	15	75	5
No. 9 **33K**	+ + +	-	-	-	-	-	-	-
	+ +	-	-	-	-	-	-	-
	+	45	25	-	35	-	25	-
No. 10 **30K**	+ + +	-	-	-	-	-	-	-
	+ +	5	-	-	5	-	-	-
	+	30	-	-	40	-	30	-
No. 11 **28K**	+ + +	-	-	-	-	-	-	-
	+ +	-	-	-	-	-	-	-
	+	-	-	-	-	-	-	-
Patients with no bands‡		1	-	1	-	3	-	-



(a)

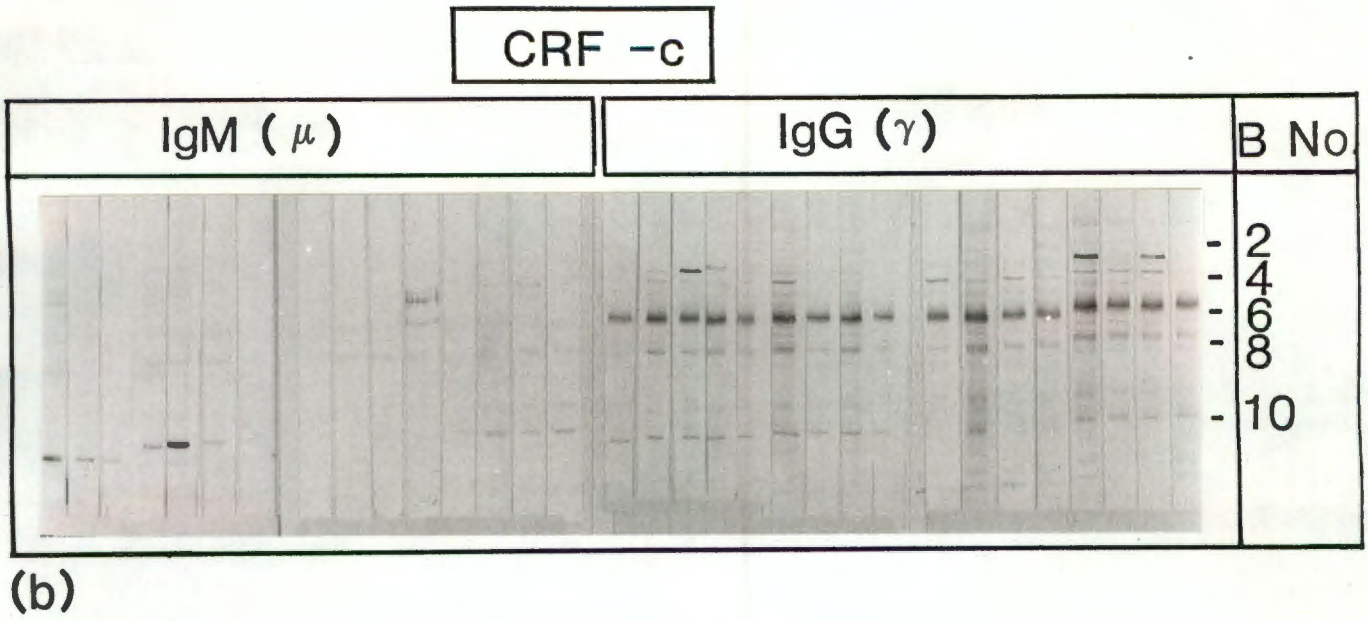
Fig. 3.2 Immunoblotting of human heart extracts against sera from the seven groups of patients and controls.

Extracts of human heart (section 7.A.11) were separated by 10% SDS polyacrylamide gel electrophoresis (section 7.A.10), and immunoblotted against sera (dilution 1:100) from the seven groups of patients and control subjects, ARF, CRF-c, CRF-a, IHD, AGN, CONT-c and CONT-a (see section 2.2.1). Each serum was assayed for binding of IgG (γ), IgM (μ) and IgA (α) antibody using peroxidase-labelled class specific second antibodies (section 7.A.9). The intensities and frequencies of these binding reactions for each of the groups of sera are presented in Table 3.2

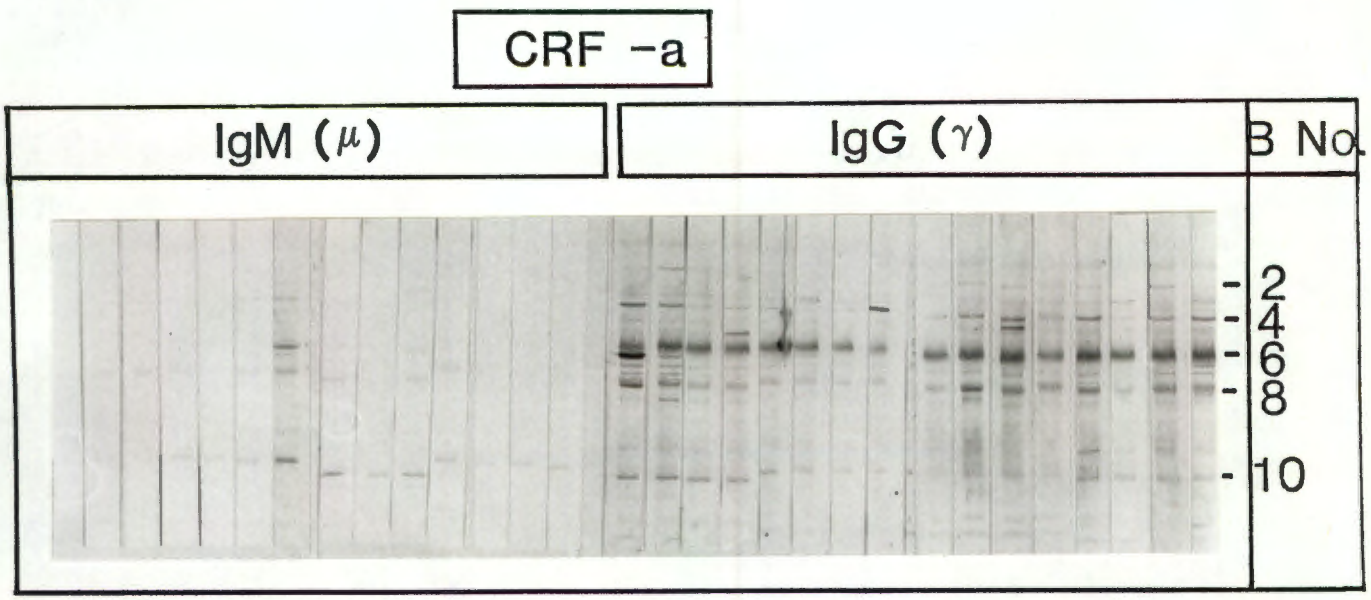
This figure illustrates representative immunoblots of the IgG (γ) and IgM (μ) reactivities of rheumatic fever (ARF, CRF-c, CRF-a) and control sera (CONT-c, CONT-a) against the heart extract. The numbers of the eleven major reactive bands in the extract (see Fig.3.1) are indicated. The minus first antibody controls are shown in Fig. 3.2**(e)**. (The photographs represent a reduction down to 40% of the original size of the blots, hence some reduction in resolution compared to the original blots.)

(a) Representative immunoblots of sera from acute rheumatic fever patients showing IgM (μ) and IgG (γ) antibody binding.

(Continued overleaf)



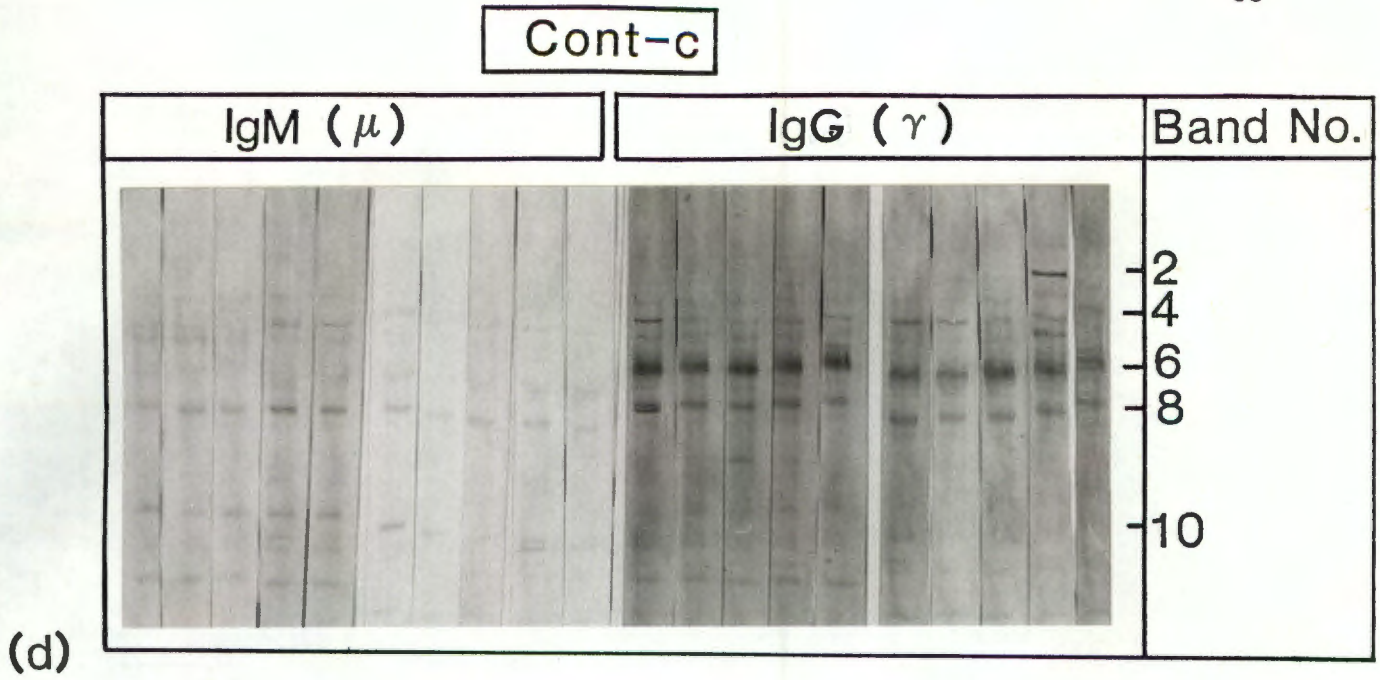
(b)

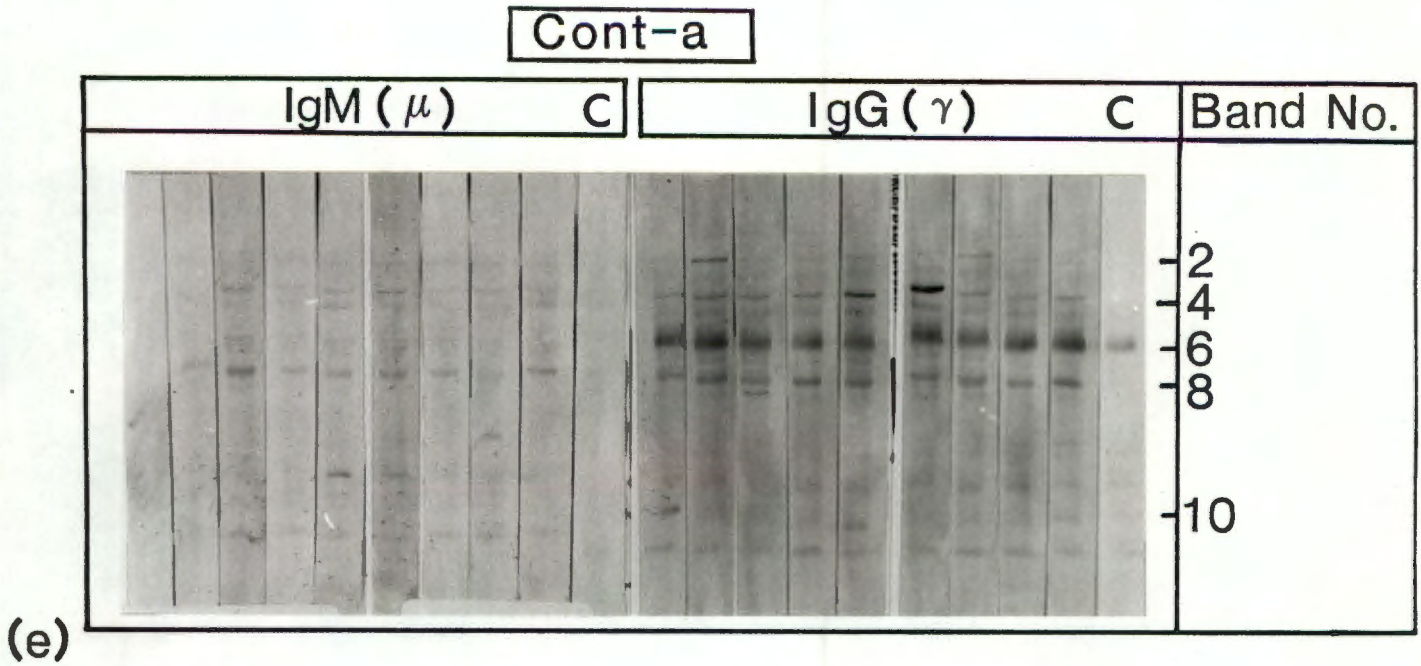


(c)

(continued from previous page)

Fig. 3.2 (b & c). Immunoblots of sera from chronic rheumatic heart disease child and adult patients (CRF-c and CRF-a) against human heart extract, showing IgM (μ) and IgG (τ) antibody binding. (See legend beneath Fig.3.2 (a)).





(continued from previous page)

Fig. 3.2 (d & e). Immunoblots of normal child and adult sera (CONT-c and CONT-a) against human heart extract showing IgM (μ) and IgG (γ) antibody binding. The minus first antibody controls are indicated by "C". (See legend beneath fig. 3.2 (a)).

3.2.1.2 ARF Heart Extract

Postmortem heart tissue was obtained from a patient who had died in the acute phase of rheumatic fever. Extracts of this heart were separated alongside extracts from normal heart on 10% SDS PAGE gels.

The protein banding pattern of the two extracts appeared identical. No additional or missing bands could be detected between them even when stained with the protein stain *AuroDye forte* (Janssen Life Sciences). Amido Black stains of the two electroblotted heart extracts are compared in Fig.3.1.

3.2.2 Cross-reactivity: Human Heart and *S.pyogenes* Antigens

To test for cross-reactivity between cardiac and *S.pyogenes* antigens, antibodies were eluted from heart antigen bands and reacted against immunoblotted streptococcal antigens.

Antibody was eluted from two heart antigen bands:

1. Band No 9 (33 kD): IgM recognition of this antigen was almost unique to ARF sera, and IgG reactivity was also more intense with ARF sera (Table 3.2).

2. Band No.8 (38-40 kD): Cardiac antigen(s) of this mol.wt. have been reported by several investigators to be cross-reactive with streptococcal M proteins (see Introduction and Table 3.1). In this study, both ARF and normal child sera reacted with this band. It was important to ascertain whether different subpopulations of antibodies in the two sera might be involved in binding this antigen.

Nitrocellulose bands No.8 and No.9, excised from single well immunoblots, were incubated separately both with a pool of five ARF sera and a pool of five normal sera (1:50 dilution; 2 hour incubation at room temperature). After washing, specific antibody was eluted in three steps (low pH; detergent; 3 M KSCN) and immediately neutralised to pH 7.4 (section 7.A.13). Residual nitrocellulose strips were developed with peroxidase-conjugated antibody to determine the efficacy of the elution, and the protein concentration of the eluate was determined to confirm this.

3.2.2.1 Eluted Antibody: Reactivity with Immunoblotted *S.pyogenes* Antigens

Eluted antibody was reacted against the following immunoblotted *S.pyogenes* antigens: type M5 protein, pep M5, pep M24, purified cell membranes, purified cell walls. (The M proteins were a gift from Dr. James Dale, University of Tennessee, Memphis, USA; the membrane and wall preparations were a gift

from Dr. John Zabriskie, Rockefeller University, New York). Positive binding was detected using an affinity purified goat anti-human combined IgG, IgA, IgM peroxidase-conjugated antibody (Kierkegaard and Perry).

Fig.3.3 shows the reactivity with streptococcal antigens of ARF serum antibody eluted from heart antigen band No. 8 (38-40 kD). The eluted antibody reacted with: (1) a 40 kD band in pep M5 digests; (2) a 34 kD band in pep M24; (3) a 43 kD antigen in the purified membrane extract (faint). The reaction with the 40 kD constituent in pep M5 digests was the most intense. Normal serum antibody eluted from band No.8 was unreactive with any of these streptococcal antigens.

Neither ARF nor control antibody eluted from band No.9 (33 kD) reacted with any of the streptococcal antigens.

3.2.2.2 Eluted Antibody: Immunofluorescence Staining of Heart

Antibody eluants from bands No.8 and No.9, were examined by indirect immunofluorescence against sections of normal human heart (section 7.A.17). The eluted antibody was first concentrated to a volume of 200 μl using an Amicon stirred ultrafiltration cell with a YM30 filter. Positive binding of antibody was detected using an affinity purified goat anti-human IgG (H+L) FITC-conjugated antibody (Zymed). Nonspecific protein binding sites on the tissue were blocked with 20% goat serum in PBS.

ARF antibody eluted from band No.8 (38-40 kD) bound to membranous structures of human heart as detected by immunofluorescence (Fig.3.4a,b). The pattern was not typically sarcolemmal. Normal serum antibody eluted from this band also appeared to stain membranous structures along the fibre of the muscle, but the binding was more diffuse than that of the ARF antibody, and also stained more connective tissue (Fig.3.4c).

Antibody eluted from band No. 9 (33 kD) did not bind to heart specifically.

There was negligible nonspecific binding of the FITC-conjugated antibody to the tissue.

3.2.3 Sarcolemmal Membranes

There are no reports showing reactivity of ARF patient sera against immunoblotted cardiac sarcolemmal membranes, whereas such reactivity has been shown by several investigators, using monoclonal antibodies and rabbit hyperimmune sera evoked by streptococcal antigens (see Introduction and Table 3.1).

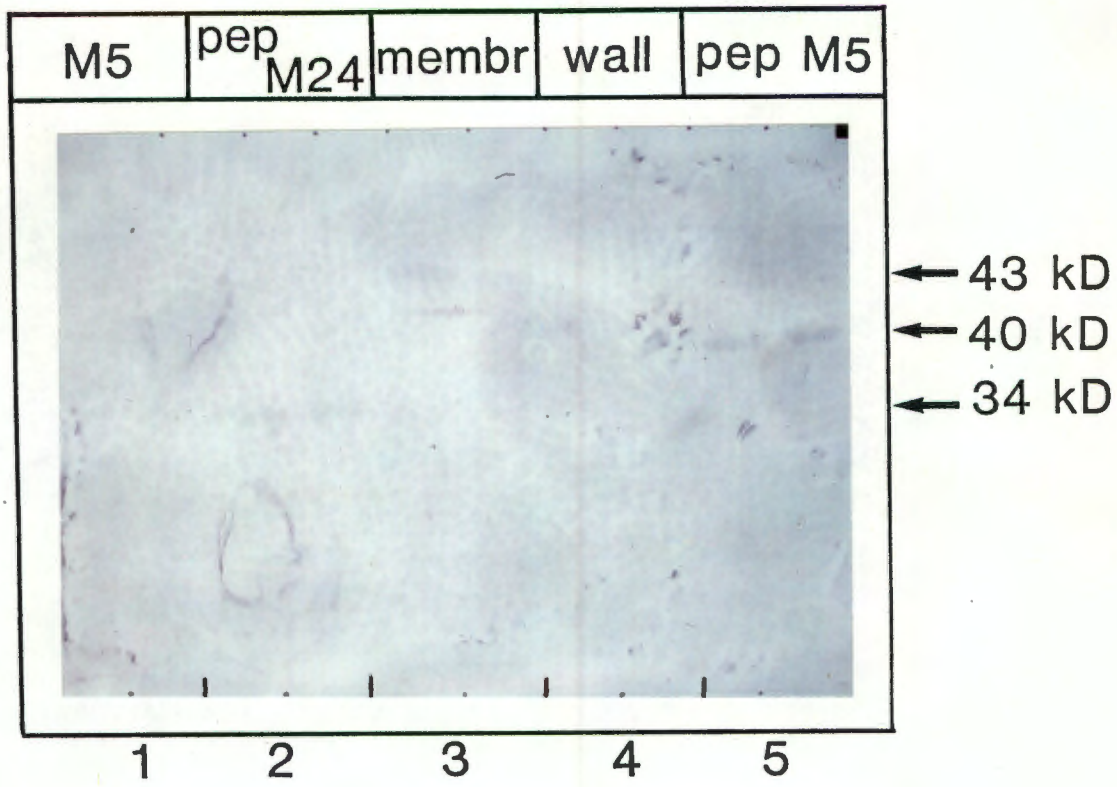
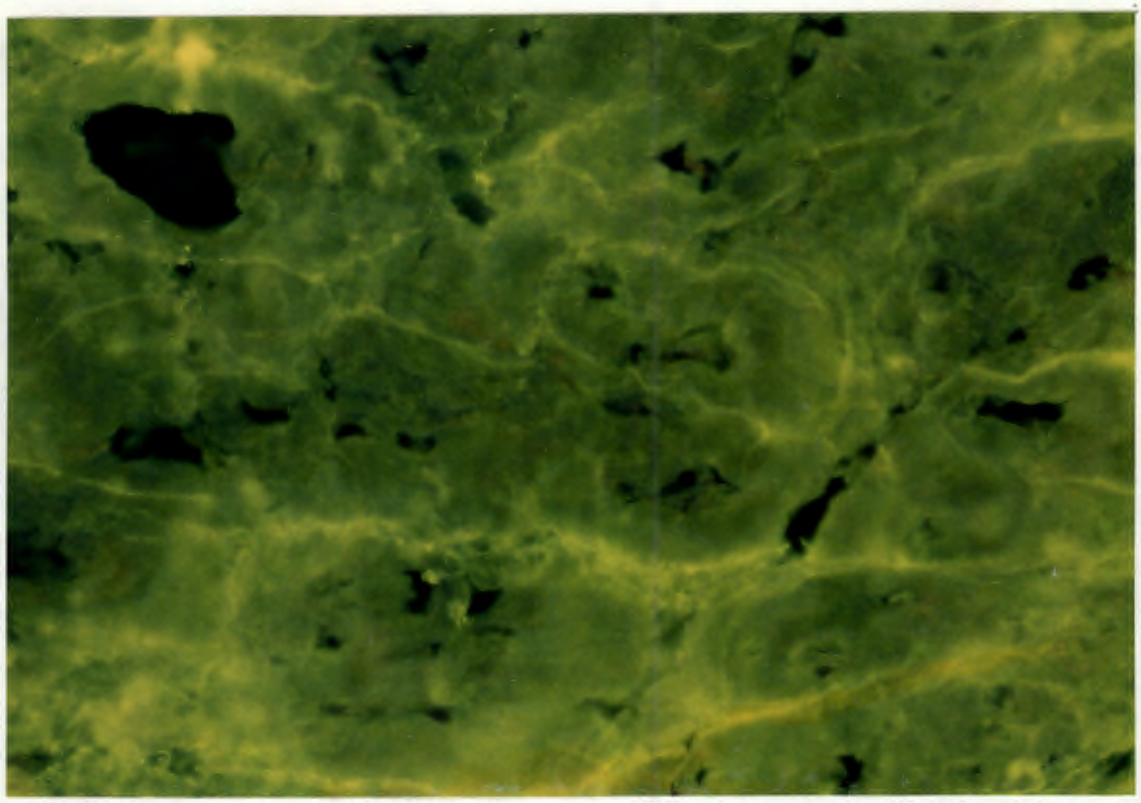


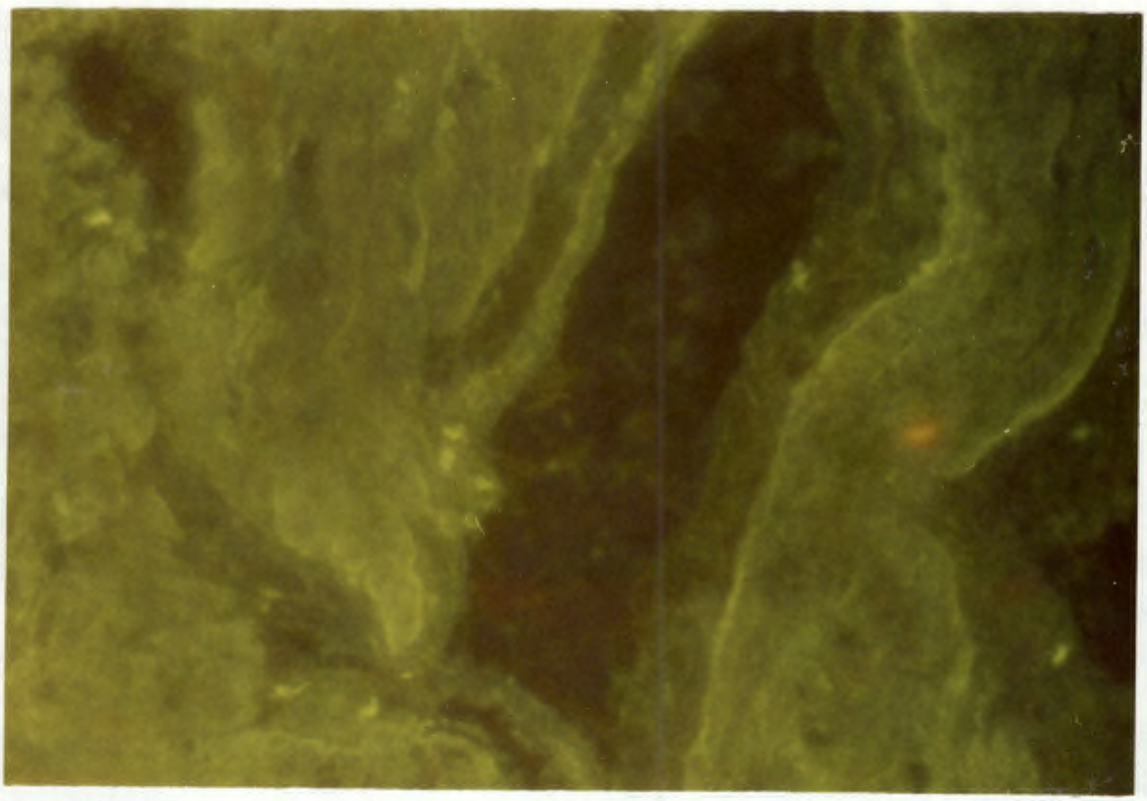
Fig. 3.3 Reactivity of ARF serum antibodies, eluted from heart extract band No.8, against immunoblotted group A streptococcal antigens.

A pool of five acute ARF sera was reacted against band No.8 (38-40 kD) (see Fig.3.1), the bound antibody was eluted according to the procedures described in section 7.A.13, and reacted against the immunoblotted group A streptococcal antigens M5, pep M5, pep M24, purified cell membranes and cell walls. Appropriate control elutions are described in section 3.2.2.

The eluted antibody is seen to react strongly with a 40 kD band in the pep M5 digest, and faintly with a 43 kD and 34 kD bands in the cell membrane extract and pep M24 digest, respectively.



a

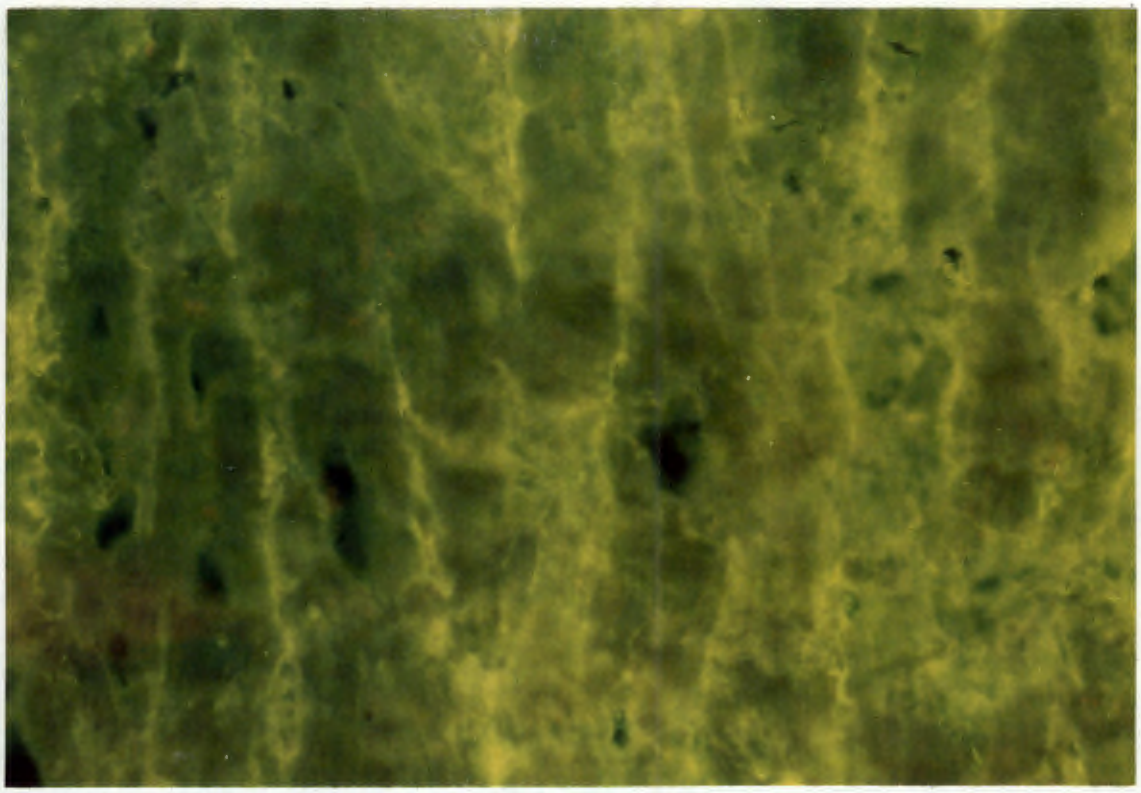


b

Fig. 3.4 Immunofluorescence staining of human heart tissue sections by ARF serum antibody eluted from immunoblotted heart extract band No.8.

(a & b) A monospecific population of ARF serum antibodies eluted the immunoblotted heart extract band No.8 (see legend to Fig.3.3) was concentrated by ultrafiltration and then tested by indirect immunoflourescence for binding to frozen sections of human heart, according to methods described in section 7.A.17. Binding to membranous structures can be seen in two different sections of heart.

(continued overleaf)



C

(continued from previous page)

Fig 3.4 (c) Immunoflourescent staining of heart tissue by normal serum antibodies eluted from heart extract band No.8.

Normal serum antibody eluted from heart extract band No.8 (see legend to Fig.3.3) was tested by indirect immunofluorescence for binding to sections of human heart, and showed a different pattern of binding compared to the eluted ARF antibody. Diffuse binding is seen along the fibre of the muscle and nonspecifically to connective tissue.

To demonstrate such reactivity in ARF sera, sarcolemmal membranes were purified from human heart (obtained freshly at transplant surgery) according to the method of Van de Rijn (1977) as described in Materials and Methods (section 7.A.12).

The purity of the membranes was assessed visually by electron microscopy. Fig.3.5 shows an electron micrograph of purified sarcolemmal membrane sheaths, indicating a fairly good degree of purity with minimal contamination by other cellular organelles or Z-band material. Purified membrane preparations were stored lyophilized, and reconstituted in sterile distilled water for use.

Membranes were separated by 10% SDS PAGE and reacted by immunoblotting against the twenty sera in each of the seven groups of subjects. Binding of IgG and IgM antibodies to the sarcolemmal membranes was detected using Fab'_2 class-specific peroxidase-conjugated antibodies (Cappel).

Rheumatic fever sera demonstrated binding of IgG to a 200 kD constituent in the membrane extracts. This band was absent in all the other groups of sera. Sixteen of the twenty ARF sera, twelve adult CRF and eleven child CRF sera displayed reactivity with this constituent. Fig.3.6 (a) compares the reactivity of normal and ARF sera against these immunoblotted membranes, indicating reactivity of ARF sera with the 200 kD constituent. In addition, IgG reactivity with a 40 kD constituent was evident in 80% of ARF sera (and 50-70% of CRF sera) compared with 30% of AGN sera, 25% of IHD and 30-40% of the child and adult normal sera.

Binding of IgM antibody was faint or absent in all groups of sera, and there was no binding to the 200 kD and 40 kD constituents (Fig.3.6 b).

3.2.4 Nuclear Antigens

Several autoimmune diseases are characterized by the production of autoantibodies to intracellular proteins and nucleic acids (Tan 1989a; 1989b). Autoantibodies to nuclear proteins are routinely detected by tissue immunofluorescence or cellular (e.g. HEp-2 cells) immunofluorescence assays, but immunoblotting allows more precise identification and characterization of these antigens.

In this study, nuclear extracts were prepared from both Girardi heart cells and from a primary culture of human heart cells (section 7.A.15). (These cells were not cardiomyocytes but comprised of endothelial-like and fibroblastic cells).

The nuclear protein extraction method of Dignam (1983) was used, with some modifications (section 7.A.14). The extracts were aliquoted, stored at -80°C, and not refrozen after initial thawing.

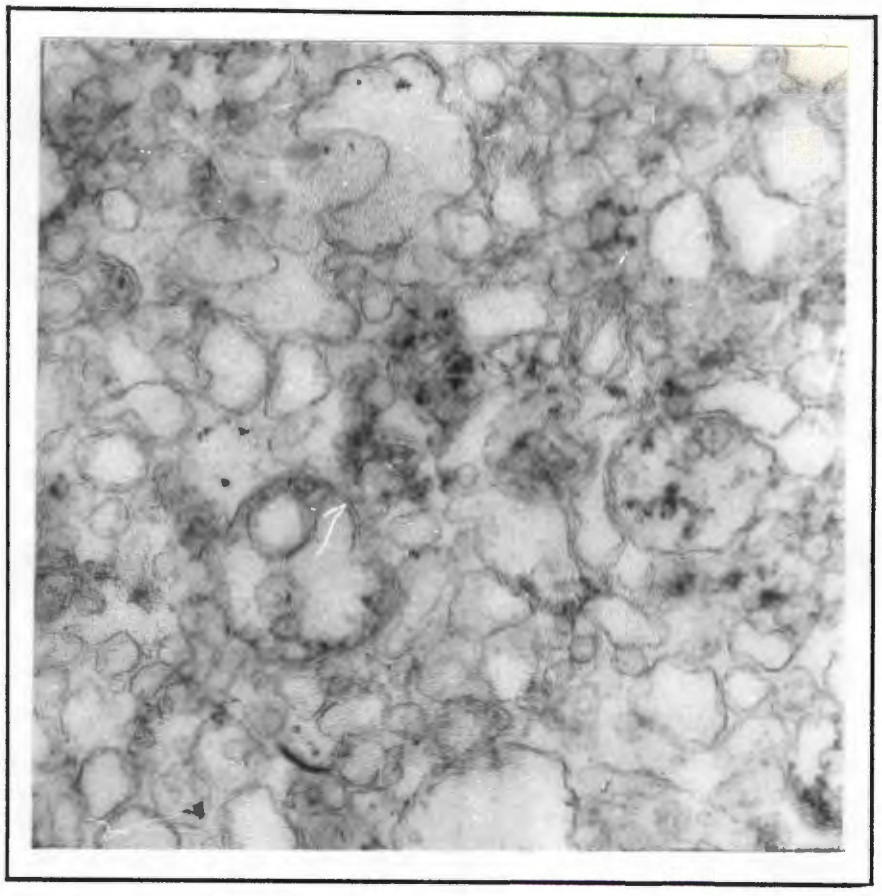


Fig. 3.5 Electron micrograph of purified sarcolemmal membranes.

Membranes were purified from human heart according to the method of Van de Rijn et al. (1977) (section 7.A.12). Numeorus membrane vesicles can be seen, with relatively little contamination by other cellular organelles. (Magnification 25000X)

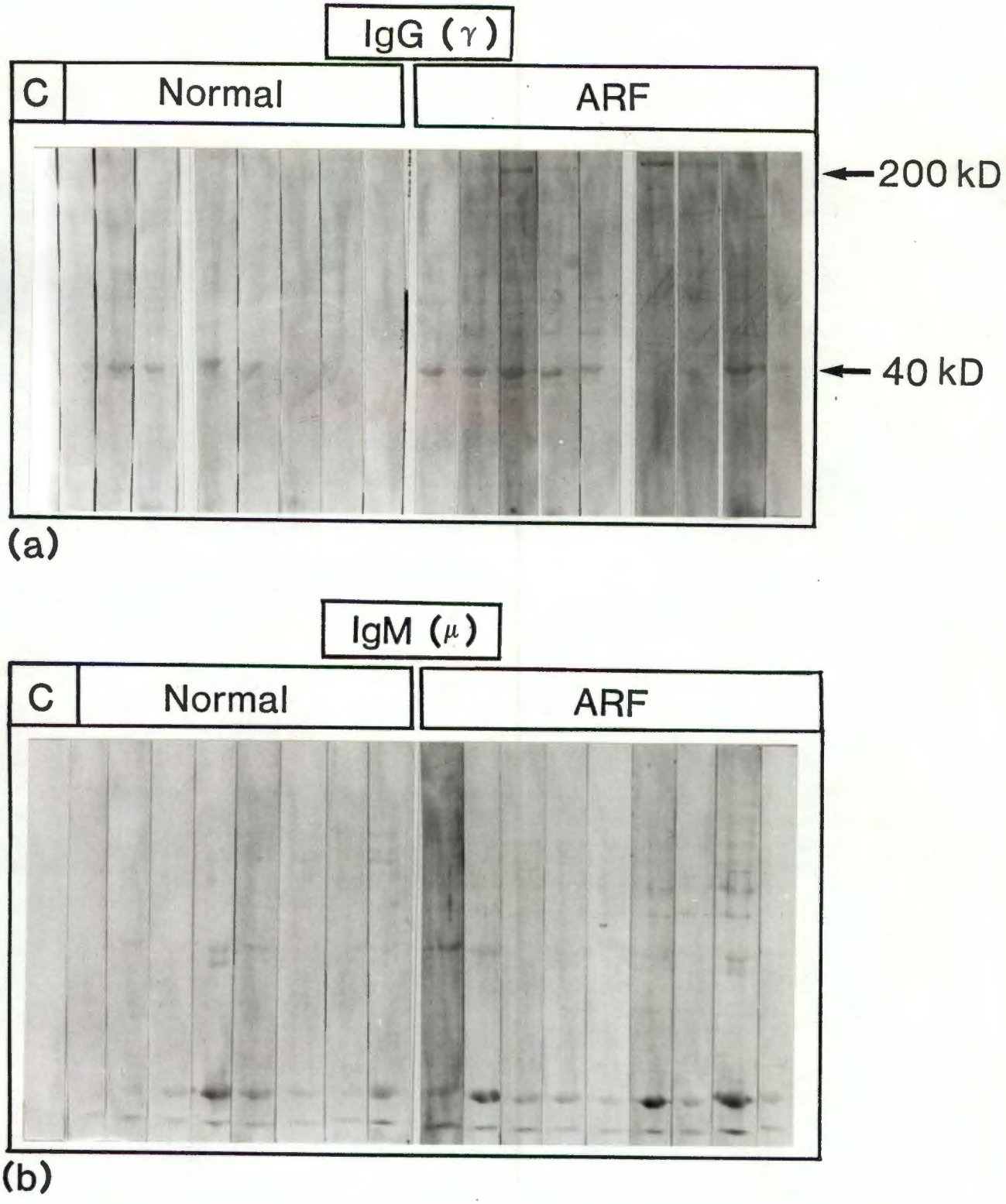


Fig. 3.6 Immunoblotting of purified cardiac sarcolemmal membranes against ARF and normal sera.

Purified sarcolemmal membranes (section 7.A.12 and Fig. 3.5) were separated by 10% SDS PAGE (section 7.A.10) and immunoblotted (section 7.A.9) against sera from the seven groups of patients and control subjects (section 2.2.1). Representative blots of ARF and normal sera reacted against the sarcolemmal membranes are shown. (The no-serum controls are shown by the lanes marked "C"). **(a)** ARF sera showed unique IgG (γ) reactivity with a 200 kD constituent in these extracts compared with sera from the other groups. A higher frequency of IgG (γ) reactivity against a 40 kD constituent was also seen among ARF sera. **(b)** IgM (μ) binding to the membrane extracts was negligible in all groups of sera.

Extracts from both cell lines were separated by 10% SDS PAGE, transferred to nitrocellulose and tested against twenty ARF sera, twenty adult control and twenty child control sera at dilutions of 1:100. Positive binding was detected using Fab'_2 goat anti-human IgG and IgM class-specific peroxidase-conjugated antibodies (Cappel). Protein stains of the electroblotted antigens, using the sensitive *AuroDye forte* stain (Janssen), showed no differences in the protein composition of the two extracts (results not shown).

Results showed considerable heterogeneity in the binding patterns of sera to nuclear antigens. IgM antibodies in ARF sera bound to more antigen bands in both cell extracts than did the normal sera, and the staining also appeared more intense. Furthermore, about 70% of the ARF sera had IgM antibodies to a narrow band at mol.wt. 140 kD in the primary cell extracts (Fig.3.7a). This constituent was also very faintly discernible in the Girardi cell extracts (Fig.3.7b). None of the adult or child normal sera showed binding to this antigen.

There were no discernible differences in IgG reactivity to the two nuclear extracts, between the ARF and normal sera. IgG did not bind the 140 kD antigen in any of the sera.

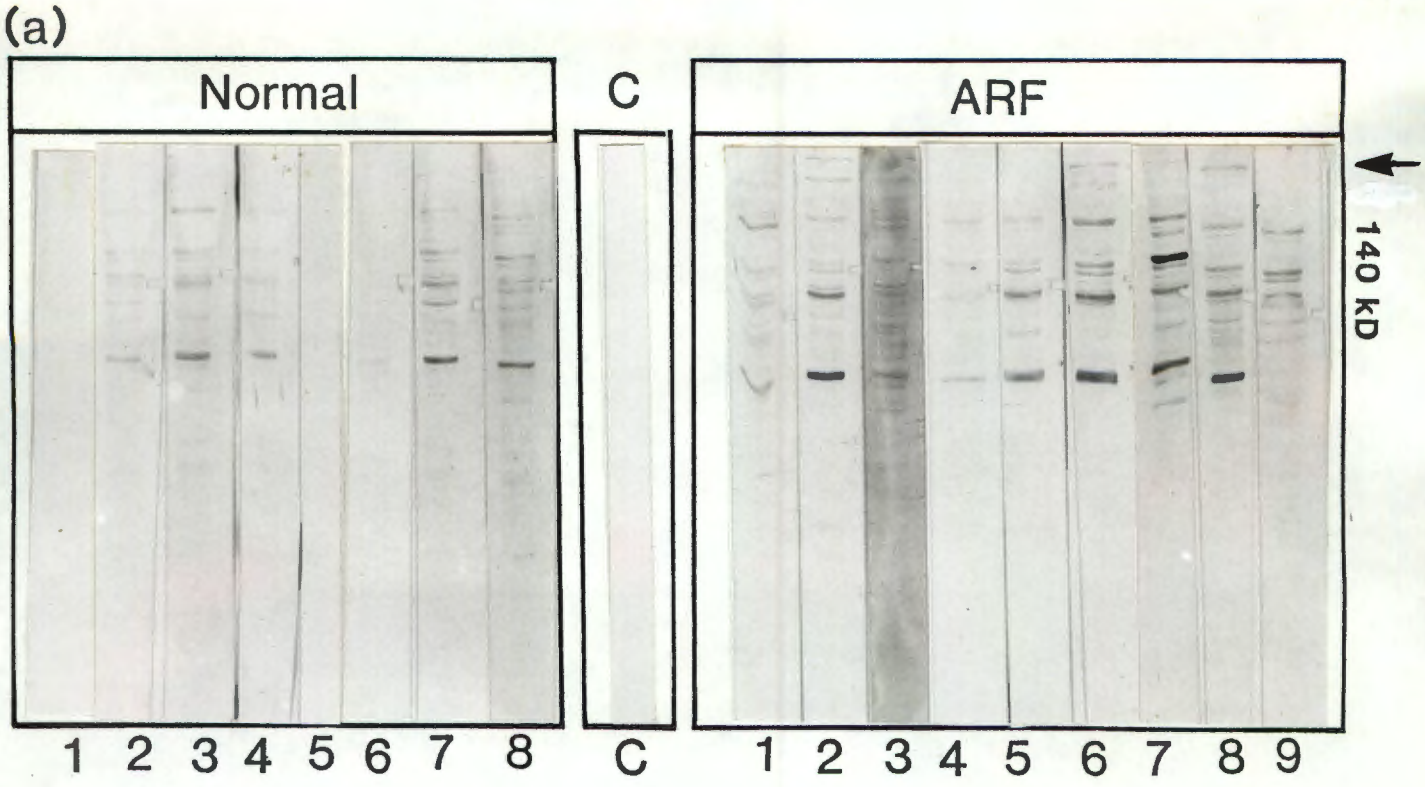
3.2.5 Reactivity of Anti-Streptococcal Sera with Heart Extract

Several investigators have used monoclonal antibodies and rabbit hyperimmune sera evoked by *S.pyogenes* antigens, to demonstrate cross-reactivity between these antigens and cardiac antigens by immunoblotting (see Introduction). Most have used SDS and Triton X-100 extracts of heart.

The purpose of this investigation was to examine the reactivity of rabbit anti-group A streptococcal antigen hyperimmune sera against various antigenic extracts of heart, and against purified cardiac myosin. The following cardiac antigen preparations were separated by 10% SDS PAGE and transferred to nitrocellulose for immunoblotting:

1. CHAPS detergent extract of human heart (section 7.A.11).
2. Human heart purified sarcolemmal membranes (section 7.A.12).
3. Heart cell nuclear extracts (section 7.A.14)
4. Myosin (rabbit heart) (Sigma)

The antigens were reacted against the following rabbit antisera: (1) antiserum to whole group A streptococci; (2) antiserum to the synthetic streptococcal peptides SM5 (164-197), (3) SM 24, (4) M19; (5) non-immune serum. Positive binding was detected using an affinity-purified goat anti-rabbit IgG (H+L) peroxidase-conjugated antibody (Cappel).



Girardi Cells

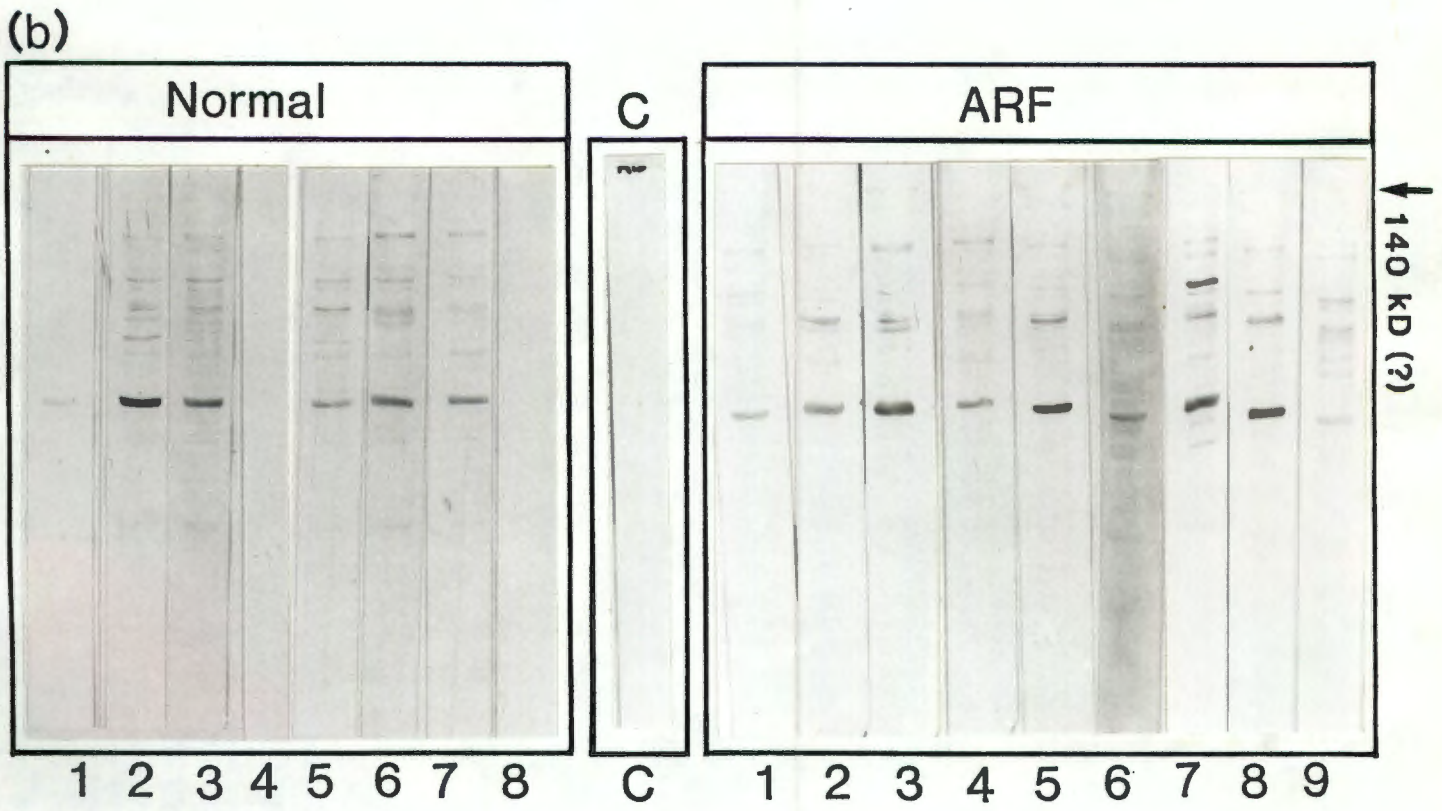


Fig. 3.7 Immunoblotting of nuclear protein extracts from Girardi and primary culture heart cells against ARF and normal sera.

Nuclear proteins extracted from a primary culture of human heart cells **(a)** and Girardi heart cells **(b)** (section 7.A.14) were immunoblotted against ARF sera, normal child and normal adult sera (section 3.2.4). 70% of the ARF sera showed evidence of IgM (μ) reactivity with a 140 kD constituent in extracts of the primary culture extracts (faintly visible in the Girardi cell extracts). (The no-serum controls are shown by "C").

Fig.3.8 shows the binding of these antisera to the various heart antigens.

(A) Anti-whole group A streptococcus antiserum: This antiserum was raised to *S.pyogenes* cultured from a throat-swab of an ARF patient with severe carditis (section 7.A.1).

Fig.3.8 (c) indicates faint reactivity of this immune serum with a 40 kD constituent present in both nuclear antigen extracts.

The nuclear extracts also displayed reactivity at 43 kD, at 36 kD and a broad region between 91 kD and 100 kD.

(B) Antiserum to SM5 (164-197): This antiserum to a 33-amino acid synthetic peptide of pep M5, was reactive with a series of polypeptides in both nuclear extracts: 140 kD, 49 kD, 46 kD, 43 kD, 40 kD, 34 kD, 33 kD and 29 kD. (The 46 kD and 43 kD bands appear nonspecific since they are also present in the non-immune serum).

The serum also reacted with a 53 kD constituent present in the crude extract of heart and in both nuclear extracts.

(C) Antiserum to M19: This antiserum reacted intensely with constituents of 55 kD and 50 kD in the nuclear extracts. Similar to the other rabbit immune sera, it reacted with polypeptides in the range 33-36 kD in both nuclear extracts.

This was the only serum that reacted with antigens in the sarcolemmal membrane preparations, showing intense staining with a range of antigens between 72-and 200 kD (The major bands discernible occurred at 170 kD, 130 kD, 81 kD and 72 kD).

(D) Antiserum to SM24: Antigens of mol.wt. 36 kD and 33 kD in the nuclear extracts reacted with this serum. In addition, it reacted strongly with a 40 kD constituent in the crude heart extract.

(E) Myosin: None of the sera reacted with rabbit cardiac myosin. However, protein staining of the gel and nitrocellulose indicated that the protein had not transferred well to the membrane.

(F) Rabbit Non-immune Serum: A pool of five non-immune rabbit sera was used to identify nonspecific (or natural autoantibody) binding to the heart antigens.

Faintly reactive bands were discernible in the nuclear antigen extracts at 70 kD, at 44-46 kD (a doublet) and at 30 kD. Reactivity with these bands by the immune sera may therefore be nonspecific.

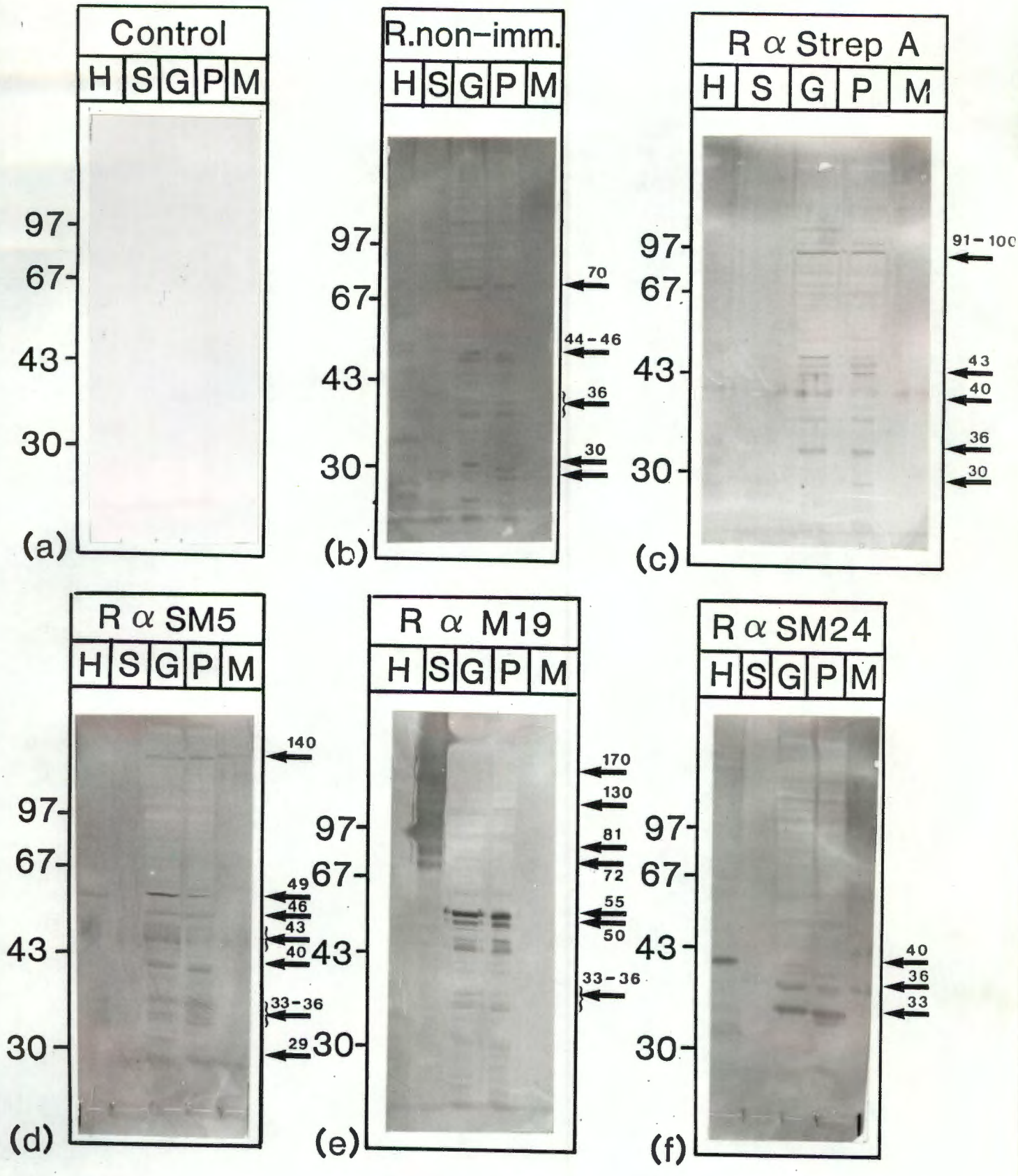


Fig. 3.8 Cross-reactivity of rabbit anti-group A streptococcal immune sera with various immunoblotted cardiac antigens.

Crude extracts of heart **(H)** (section 7.A.11), purified cardiac sarcolemmal membranes **(S)** (section 7.A.12), nuclear protein extracts of heart cells (**G**: Girardi cells, **P**: primary culture cells) (section 7.A.14) and cardiac myosin **(M)** were separated by 10 % SDS PAGE and immunoblotted against streptococcal immune sera raised to whole *S.pyogenes* and to the M proteins, SM5, M19 and SM24. Non-immune rabbit serum and no-serum controls are also shown. Antibody binding was detected using a peroxidase conjugated goat anti-rabbit IgG (H+L) second antibody. The reactivity patterns are described in sections 3.2.5 and 3.3.4.

3.2.6 Additional Experiments

3.2.6.1 Reactivity to Human Collagen Types II, III and VI

The reactivity of rheumatic fever sera and normal sera to highly-purified human collagen types II, III, and VI (Heyl, West Berlin) was also investigated by immunoblotting, but failed to indicate reactivity among any of the sera.

3.2.6.2 Ca^{2+}ATPase Binding

There has been some speculation on the possible implication of muscle and membrane ATPase enzymes in antigenic mimicry in rheumatic fever (see Introduction and Discussion). Sarcolemmal membranes contain a Na^+/K^+ATPase, and ATPase activity is associated with the globular headpiece and light chains of myosin. Ca^{2+}ATPase has a sequence similar to that of the α-subunit of Na^+/K^+ATPase and is mechanistically similar to it. It has a mol.wt. of 114 kD.

This simple investigation was undertaken to examine whether ARF sera contain antibodies to ATPase enzymes. Ca^{2+}ATPase enzyme isolated from rabbit skeletal muscle (a gift from Dr.David MacIntosh, Dept of Chemical Pathology, University of Cape Town) was separated by 10% SDS PAGE, electroblotted onto nitrocellulose and then reacted against ten ARF and ten normal sera. Antibody binding was assayed using both IgG and IgM class specific peroxidase-conjugated Fab'_2 goat anti-human antibodies (Zymed).

Extremely intense IgG binding was observed with one ARF serum (patient No. 53; Appendix B). Fig.3.9 shows the intense binding to the 114 kD ATPase protein with this serum. The reactions of three ARF and three normal sera are shown. Faint binding to the enzyme was discernible with the other sera. The band at 45 kD represents calsequestrin, an associated co-purifying anionic protein.

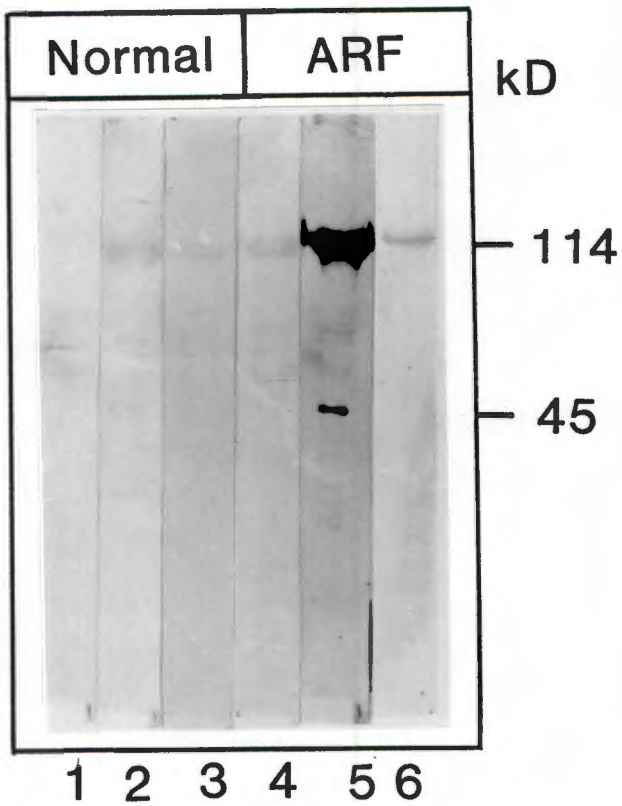


Fig. 3.9 Immunoblotting of muscle Ca^{2+}ATPase against ARF and normal sera.

Ca^{2+}ATPase isolated from rabbit skeletal muscle was separated by 10% SDS PAGE and immunoblotted against 10 ARF and 10 normal sera. One ARF serum showed intense reactivity with the 114 kD Ca^{2+}ATPase enzyme and the 45 kD co-purifying protein calsequestrin. The reactivity of this serum is shown in comparison with representative normal and ARF sera.

3.3 DISCUSSION

3.3.1 Human Heart Detergent Extracts

There have been few reports in the literature on the reactivity by immunoblotting of ARF sera against heart antigens. Possibly the polyspecificity and frequently low titre of the relevant antibody in patient serum, account for the preferential use of monoclonal antibodies and hyperimmune sera used in most studies (see Introduction).

3.3.1.1 Isotype Binding Patterns of Groups of Sera

Table 3.2 gives an indication of the polyspecificity of the patient sera to the immunoblotted heart antigens. Most of the eleven protein bands (except in the lower mol.wt range) were recognised by all seven groups of sera, even if only faintly and by just one class of antibody. All three immunoglobulin classes bound to the same heart antigens, but there were differences in the frequency and intensity of band staining among the different groups of sera.

ARF sera, compared to the other groups, showed a markedly higher frequency and intensity of IgM heart-reactive antibody. This finding is perhaps unexpected in view of reports (using immunofluorescence) that antibody bound to heart tissues is primarily IgG (Kaplan et al., 1961; Hess et al., 1964; Ehrenfeld et al., 1961). Increased IgG might be expected instead, unless deposition of IgG in heart tissue removes it from circulation.

During a conventional immune response there is often a shift from IgM, to IgG and IgA production as the disease progresses. Such a shift has, for instance, been found to occur in anti-histone antibody profiles among SLE patients (Fellows et al., 1988). In this study, such a shift may explain the change from IgM to IgG production discernible in the child and adult CRF groups (Figure 3.2 b & c). In both groups, there is very little binding of IgM to heart antigens.

Whether the raised IgM heart-reactive antibodies in ARF sera, and the shift to IgG in CRF sera, is of significance to the aetiology of rheumatic fever is not known. Natural autoantibodies are often IgM, whereas autoantibodies associated with disease tend to be IgG (Clough and Valenzuela, 1980). In animal models, it has been shown that effective T-cell function is essential for normal switchover from IgM to IgG production (Schimpl et al., 1973), and it has been suggested that abnormal T-cell function in SLE may affect this switchover resulting in high ratios of IgM to IgG anti-DNA antibodies (Clough and Valenzuela, 1980). It is thought that the poor regulation by IgG on IgM production, permits production of low affinity IgM antibody to DNA, and that this results in formation of long-lasting immune complexes leading to renal disease. The pathogenic effect is thus a consequence, not of the IgG molecule itself, but of its low concentration that impedes the switching off of IgM.

Although there are reports of abnormal B and T cell function in rheumatic fever, especially in response to extracellular streptococcal products (Gray et al., 1981), it is not known whether this affects the ratio of IgM to IgG heart-reactive antibodies. The raised IgM heart-reactive antibodies among ARF patients may be pathologically important. Diseases in which IgM autoantibodies clearly have pathological relevance include haemolytic anemia in cold agglutinin disease (Duggan and Schattner, 1986), a vasculitis caused by self-associating IgM cryoglobulins (Winfield, 1983) and peripheral neuropathy caused by anti-nerve autoantibodies (Ilyas et al., 1985).

An hypothesis for the pathological relevance of raised IgM antibodies to cardiac antigens (such as myosin and sarcolemmal membranes) in acute rheumatic fever, is presented below.

3.3.1.2 Hypothesis: Raised IgM Anti-heart Antibodies

It is known that IgM antibody can specifically enhance the humoral response to antigen, and it has been suggested that this may be achieved by a mechanism similar to normal immunization with antigen alone (Henry and Jerne, 1968; Heyman et al., 1985). IgM might concentrate antigen in the spleen thereby increasing its availability to cells involved in antibody production (Wason, 1973; Dennert, 1971).

The presentation to immunocompetent cells of suboptimal doses of heart antigen could potentiate the production of high-affinity IgG antibodies. Such suboptimal doses of heart antigen might be products of natural catabolism of heart cells being cleared by natural IgM autoantibodies (Grabar et al., 1983); or they may occur as a result of tissue injury; or they might be presented in immune complexes.

It has been suggested that, due to its poor solubility at physiological salt concentrations, myosin can persist in areas of cell necrosis, thus providing a stimulus for a chronic immune response (Alvarez et al., 1987). Conceivably, natural IgM autoantibodies, while "clearing" persistent small doses of myosin, could potentiate production of high affinity pathogenic IgG antibodies. With regard to the anti-sarcolemmal antibodies consistently found in ARF patients, it is interesting that myosin (previously considered to be an intracellular protein) has more recently been found to be associated with the internal membrane surface of the sarcolemma (Fallon and Nachmias, 1980).

Evidence for regulation of the humoral immune response by IgM natural autoantibodies, has been presented by Mahana et al. (1988). They were able to induce in mice, immunized with small doses of *polyspecific monoclonal natural IgM autoantibodies*, titres of specific anti-myosin IgG antibody (following challenge with myosin) that were significantly increased compared to

mice not neonatally pre-immunized with these autoantibodies. It was suggested that the altered anti-myosin response was the result of an early disturbance of an anti-idiotypic network in the newborn mice following injection of the monoclonal autoantibodies. Similarly, natural IgM autoantibodies have been shown to affect the intracellular metabolism of lymphocytes, leading to a modified immune response (Rosenblatt et al., 1985).

In rheumatic fever, one might speculate that individuals with particular anti-heart IgM autoantibodies (or raised levels of heart-reactive autoantibodies), might potentiate high affinity IgG anti-heart antibodies (through a mechanism such as described above). These may be the IgG anti-heart antibodies detected by immunofluorescence in rheumatic fever (Kaplan et al., 1961). A pathogenic mechanism such as described above might lead to the chronic destruction of heart valves and muscle seen in rheumatic heart disease.

3.3.1.3 Individual Protein Bands Recognised

The ensuing discussion describes the reactivity of the different groups of sera to some of these antigen bands, and compares them with antigens of similar mol.wt. reported by other investigators. (Such comparisons of mol.wt. are obviously superficial, and by no means imply identity of the antigens).

(a) Band No 1 (116 Kd):

The presence of IgM antibodies to this protein in 85% of ARF sera, compared to only 10% (or its complete absence) in other groups of sera, suggests that IgM recognition of this constituent may be distinctive of rheumatic fever.

There are no reports in the literature of heart autoantigens of this mol.wt. Cunningham et al. (1984) describe the reactivity of anti-streptococcal murine mABs (the "54 series") with three major proteins in the range 100 kD to 200 kD - but they do not state exact mol.wts.

(b) Band No.2 (85 kD):

Whereas IgG antibody to this protein was similar in most serum, IgM reactive antibody was noticeably greater in ARF sera compared to other groups (100% in ARF, compared to 30% in AGN, 5% IHD and 20% adult controls).

Heart antigens in this mol.wt. region have been shown to cross-react with *S.mutans* antigens. Doyle et al. (1986) demonstrated that murine mAbs evoked by *S.mutans* reacted with 82 kD and 85 kD human heart proteins. Similarly, Ayakawa et al. (1985) demonstrated that rabbits immunized with human heart homogenate, reacted with an 82 kD protein in homogenates of *S.mutans*. These findings indicate that antigens cross-reactive with human heart are present in streptococcal species other than *S.pyogenes*. Such cross-

reactions are of obvious importance to the development of vaccines against *S.pyogenes* (Van de Rijn et al., 1976).

(c) Band No 4 (67-70 kD):

Both rabbit antisera and murine mAbs evoked by streptococcal antigens, have been shown in various studies to react with heart antigens in this mol.wt. region.

Rabbit antisera to streptococcal M5 and M25 proteins react with proteins of mol.wt. 67-70 kD in Triton X-100 extracts of human heart (Cunningham and Russell, 1983), and a 71 kD protein in SDS extracts of sarcolemmal membranes has been shown to react with rabbit immune serum to streptococcal M5 protein (Dale and Beachey, 1982).

More interestingly, 70 kD corresponds to the mol.wt. of tropomyosin and 60 kD to keratin, which have been found in immunoblotting inhibition assays to react with murine mAbs evoked by streptococcal M5 protein (see Introduction and Table 3.1).

(d) Band No.7 (43kD):

This band may correspond to the 43 kD bovine heart protein reactive with ARF sera, described by Zabriskie and Friedman (1983).

Actin has a similar mol.wt (42 kD). Some evidence of anti-actin antibodies in rheumatic fever sera was found in this study (Chapter 2), and both murine and human monoclonal antibodies reactive with *S.pyogenes* antigens have been shown to cross-react with actin (Fenderson et al., 1989; Cunningham et al., 1988).

(e) Band No.8 (38-40 kD):

This study showed that all seven groups of sera contained autoantibodies reactive with a 40 kD cardiac antigen. Especially ARF sera showed strong IgM binding to this constituent.

There have been numerous reports, using both murine mAbs and rabbit antisera, of cross-reactivity between streptococcal M5 protein and a 40 kD heart antigen (Cunningham and Russell, 1983; Cunningham et al., 1984; Sargent et al., 1987). Furthermore, Zabriskie and Friedman (1983) showed that ARF sera reacted with a 38 kD protein in saline extracts of bovine heart.

Sargent et al. (1987) showed that rabbit immune sera raised to the streptococcal synthetic peptide SM5(164-197) reacted with a 40 kD constituent in crude extracts of heart, and the identical antiserum was shown

in this study to react with 40 kD antigens in nuclear extracts of heart cells (Fig.3.8).

(f) Band No 9 (33 kD):

Antibodies to this constituent of heart, occur at a higher frequency and intensity in ARF patients than in any of the other groups of sera (Tables 3.2). Both IgG and IgM bind this antigen in all ARF sera.

Autoantibody binding to a cardiac antigen of this mol.wt., has not been previously reported in rheumatic fever. An interestingly speculation is that this doublet may correspond to the two 32 kD subunits of tropomyosin.

(g) Bands No 10 and 11 (30 kD and 28 kD):

ARF sera also displayed the greatest intensity and frequency of binding (particularly IgM) to these two cardiac antigens than the other groups of sera.

Cunningham and Russell (1983) demonstrated reactivity of rabbit anti-M5 antisera to a 30 kD protein in Triton X-100 extracts of human heart.

3.3.1.4 Specificity and Pathological Relevance of Antigens

The incidence of autoantibodies in the normal adult sera in this study is perhaps higher than expected. Other investigators, using immunocytochemistry (Kaplan and Svec, 1964), have reported that only about 2% of normal adult sera contain heart-reactive antibodies. Immunoblotting is however a more sensitive technique, and this discrepancy illustrates the need for caution in interpreting and comparing the results of different solid-phase binding assays (Van Regenmortel, 1989; see Chapter 1). In particular, detection of cross-reactivity may be dependent on assay-specific variables, and irrelevant cross-reactions may be detected at a higher frequency in crude extracts of protein (see Introduction).

The rheumatic fever sera examined in this study do nonetheless show a noticeably different pattern of reactivity against cardiac antigens, compared with both the normal and related-disease control sera. These results showed some evidence of apparently unique recognition of particular constituents in the crude extracts by IgM antibodies in ARF sera. Immunoblot inhibition experiments using certain purified proteins (such as performed by Dale and Beachey, 1985a, who used myosin to identify a 200 kD band in crude heart extracts) could be used to confirm guesses about the identity of these constituents. Such experiments remain to be done.

The role of these immunoblotted antigens in the pathogenesis of the disease, might be further analysed using functional biological assays. In this project, an

extensive investigation was undertaken to examine the ability of immunoblotted antigen nitrocellulose bands to stimulate lymphocytes, using the methods of Abou-Zeid et al. (1987) and Young and Lamb (1986) (see Introduction). It was found that although the control antigens and mitogens used (ASO, BCG, PHA) stimulated the lymphocytes when spotted onto nitrocellulose prior to digestion with DMSO, neither these nor the heart and streptococcal antigens analysed in this study stimulated lymphocytes when separated by 10% SDS PAGE and immunoblotted (results not shown). Possibly stimulatory epitopes were denatured during immunoblotting. The pathological significance of these cardiac antigens is thus unknown.

3.3.2 Cross-reactivity: Heart and *S.pyogenes* M Proteins

3.3.2.1 Reactivity of HRA with Immunoblotted Streptococcal Antigens

The use of eluted heart-reactive antibody (HRA) to show antigenic mimicry between cardiac and *S.pyogenes* antigens, does not differentiate between "true crossreactivity" or "partial crossreactivity" (Berzofsky and Schechter, 1981; see Chapter 1) nor does it give any indication as to the biological significance of the reaction, but it might serve as clues to finer analyses such as epitope mapping.

The finding, in this study, that antibody eluted from the 40 kD heart band cross-reacted with streptococcal M5 protein, concurs with findings in other studies using rabbit antisera. Sargent et al. (1987) used rabbit antisera to show cross-reactivity between a 40 kD protein in human heart, and the *S.pyogenes* M protein fragments, pep M5 and SM5(164-197). Similar cross-reactions between a 40 kD cardiac antigen and streptococcal M5 and M25 proteins were shown by Cunningham and Russell (1983) and Cunningham et al. (1984) (see Introduction).

Some studies have shown that pep M5 and pep M6 have heart cross-reactive epitopes, but that pep M24 lacks such reactivity (Dale and Beachey, 1985a, 1985b; Kotb et al., 1989). In this study, the binding of eluted HRA to a 34 kD fragment in pep M24 contradicts such findings. Although the staining was faint, eluted normal serum antibody showed no reactivity with this, or any of the other, streptococcal antigens. This cross-reaction may be a function of antibody affinities and assay conditions, and should be confirmed either by inhibition experiments or in the context of a different assay.

The lack of reactivity of eluted HRA with the crude extract of protein M5 is puzzling. Possibly, it could be due to altered antigenicity following immobilization of the protein on nitrocellulose, or else the relevant epitopes may be masked under these specific assay conditions.

Although the almost unique IgM reactivity of ARF sera to the 33 kD heart protein made it seem promising as a cross-reactive autoantigen implicated in rheumatic fever, eluted antibody from the band failed to react with streptococcal antigens. Possibly, it may cross-react with other relevant M proteins, such as M6 and M19, which were not tested in this study.

In summary, the strong reaction between eluted ARF serum antibodies from the 40 kD heart antigen, with streptococcal pep M5 protein, suggests that they may share cross-reactive epitopes. This finding is consistent with similar cross-reactions of heart proteins of identical mol.wt. with streptococcal M5 protein, defined using rabbit antisera and murine mAbs (see Introduction).

3.3.2.2 Immunofluorescence Staining of Eluted Antibody Against Heart

The recognition of membranous structures of heart tissue by ARF antibody eluted from the 40 kD band indicates that the autoantigen may be a membrane protein, although sarcolemmal membrane staining was not evident (Fig.3.4 a & b). The differences in immunofluorescence staining of heart, between ARF and normal serum antibody (Fig 3.4c) eluted from the 40 kD band, may indicate that the antibodies recognise different epitopes on the antigen, or that different subpopulations of antibody in the two sera recognise the same epitope.

3.3.3 Sarcolemmal Membranes

The reactivity of rheumatic fever sera with constituents of 200 kD and 40 kD in human cardiac sarcolemmal membranes, is interesting since it concurs with similar findings using monoclonal antibodies and rabbit antisera (evoked by *S.pyogenes* antigens). This study presents the first evidence by immunoblotting of reactivity with these sarcolemmal proteins, using patient sera.

Rabbit antisera to streptococcal M5 protein and to M25 were reactive with a 40 kD protein in Triton X-100 extracts of human heart (Cunningham and Russell, 1983). More specifically, an antiserum to the peptide SM5 (164-197), also reacts with a 40 kD polypeptide in such heart extracts (Sargent et al., 1987). Dale and Beachey (1985) showed that antisera to pep M5, reacted with multiple bands between 40 kD and 200 kD in SDS extracts of sarcolemmal membranes. Murine mAbs evoked by streptococcal M5 membranes and cross-reactive with whole group A streptococci, reacted similarly at 40 kD (Cunningham et al., 1984). The finding of reactivity in 80% of ARF patient sera with a 40 kD constituent in these purified sarcolemmal membranes is therefore in agreement with previous findings using cross-reactive mABs and rabbit immune sera. Reactivity with this constituent in up to 30% of control sera, may indicate natural autoantibody reactivity to this antigen. Whether this binding represents the same subpopulation of antibodies found in ARF sera is not clear.

The 200 kD protein band appeared unique to rheumatic fever sera (80% of ARF and 55-60% of CRF sera tested). A protein band of this molecular weight has also been found in sarcolemmal and crude heart extracts using mAbs and rabbit antisera to streptococcal antigens (see Introduction). Cunningham et al. (1988) showed by immunoblotting that 88% of ARF and 91% of RHD sera were reactive with myosin (200 kD mol.wt.). A 200 kD protein detected in sarcolemmal as well as crude extract of heart was abolished by absorption of the sera with myosin (Dale and Beachey, 1982; Doyle et al., 1986; Dale and Beachey, 1985a). These studies all used mAbs and rabbit antisera. The present study has identified a 200 kD protein in purified sarcolemmal extracts using human rheumatic sera. This band may or may not correspond to myosin.

A shortcoming of this study was that inhibition assays were not done to verify the identity of the band. Moreover, the reason this constituent was not discernible in the total heart extracts (Fig.3.1) may be due to either differences in the methods of preparation of the two antigenic extracts, or else to quantitative differences between the amounts of this constituent present in the purified sarcolemmal extracts compared with the crude extracts.

Various studies have found cross-reactivity between myosin (mol.wt. about 200 kD) and the streptococcal M proteins, using immunoblot inhibition assays. Myosin is relevant to cross-reactivity with sarcolemmal membranes since it has been found associated with the internal membrane of the sarcolemma (Fallon and Nachmias, 1980). Inhibition assays to determine cross-reactivity between myosin and M proteins were not done in this study.

In summary, the 40 kD and 200 kD bands detected in ARF and CRF sera in this study, confirm findings that have been made using mAbs and rabbit antisera to streptococcal antigens and suggest that these may in fact be autoantigens relevant to rheumatic fever. Whether they are important to the pathogenesis of the disease is not known.

3.3.4 Nuclear Antigens

There are no reports in which immunoblotting has been used to measure reactivity in ARF sera to nuclear antigens. The finding of a 140 kD polypeptide reactive with 70% of ARF sera but absent in normal sera, may be of significance to rheumatic fever, although this is a preliminary finding that needs to be substantiated using larger numbers of sera and variations in assay conditions. The detection of autoantibodies to nuclear antigens has been found to depend on the sensitivity of the assay and the precise conditions used - such as the serum dilution and the substrate used (Stott, 1985; Habets, 1986). Nonspecific binding due to the polyspecificity of the autoantibodies can complicate the interpretation of results, and adequate controls are therefore important.

3.3.5 Rabbit Anti-*S.Pyogenes* Serum Reactivity with Heart Antigens

Several of the polypeptides that were found in this study to react with rabbit immune sera against *S.pyogenes* antigens, correspond in mol.wt. to polypeptides reactive with ARF sera. This does not necessarily mean that these epitopes and antibody populations are cross-reactive.

Reactivity of the rabbit antisera to the nuclear antigen extracts was strikingly distinctive. Since binding of these rabbit anti-*S.pyogenes* sera to cardiac nuclear antigens has not previously been investigated, the significance of these specificities cannot be assessed in terms of published reports. Nuclear antigens are often markers for autoimmune disease (see Chapter 1). It would be extremely valuable if such markers of diagnostic significance could be found for rheumatic fever. All of the rabbit and anti-*S.pyogenes* sera reacted with a 33 kD protein band in the nuclear extracts, but whether this protein might represent a significant autoantigen is unclear.

The strong reaction of the anti-SM24 rabbit immune serum to a 40 kD constituent in crude extracts of heart (Fig.3.8f), appears to contradict previous reports that types 5 and 6 M protein but not type M24, share epitopes with human heart tissue (Dale and Beachey, 1985a). The elution experiments performed in this study (section 3.2.2.1) in which ARF serum antibody eluted from a 40 kD heart tissue polypeptide reacted with a 34 kD constituent in pep M24 digests (Fig.3.3), substantiate this finding that type 24 M protein may share epitopes with human heart tissue.

3.3.6 Additional Experiments

3.3.6.1 Ca^{2+}ATPase Binding

The finding of extremely intense reactivity of an ARF serum with Ca^{2+}ATPase from sarcoplasmic reticulum is a preliminary finding that might be more thoroughly investigated. The possible implication of ATPase enzymes in antigenic mimicry in rheumatic fever has been raised by several investigators. Some of the arguments are the following.

The detection of cross-reactivity between streptococcal M5 protein and myosin (see Introduction) has led to tentative speculation that the ATPase enzyme associated with the light chains of the molecule might be implicated in this cross-reaction. Cunningham et al. (1986) showed that murine mAbs evoked by purified membranes from M type 5 *S.pyogenes*, cross-reacted with the heavy chains of the molecule (both heavy and light meromyosin fragments), but failed to bind to the light chains of the molecule or to inhibit the ATPase activity of the globular headpiece associated with these molecules. The authors also speculated that the binding to sarcolemmal membranes by these mAbs could implicate the Na^+/K^+ ATPase enzyme associated with these membranes. It

was thought that the enzyme might share epitopes with myosin and M5, but this was not verified.

Manjula and Fischetti (1980) suggested another way in which muscle ATPase enzymes might be implicated in antigenic mimicry between *S.pyogenes* and heart. The M protein has structural resemblance to (and is immunologically cross-reactive with) tropomyosin which, in the presence of actin, has been shown to confer Ca^{2+}-regulated Mg^{2+}-ATPase activity upon myosins in leukocytes (Stossel and Pollard, 1973; Adelstein et al., 1972). It was suggested that through its resemblance to this regulatory protein in muscle contraction, M protein might conceivably interfere with the contractile machinery of cells, and that this might be at the basis of antiphagocytic properties (see Introduction).

Other evidence for the possible implication of ATPase enzymes in antigenic mimicry has been presented by Lane and Hoeffler (1980) who showed cross-reactivity between SV40 virus T antigen and a 68 kD host cell protein with ATPase activity. It was suggested that mimicry of this ATPase-associated protein by the virus might change its function to suite the survival of the virus in the cell.

The evidence for the involvement ATPase enzymes in antigenic mimicry in rheumatic fever has not been unequivocally shown, but the hypothesis is attractive in that it indicates a mechanism by which cross-reactivity between the bacterial and mammalian epitopes might lead to a pathological interference in cellular function in the patient.

3.4 CONCLUSIONS

1. A previously unreported procedure, in rheumatic fever studies, of preparing crude extracts of human heart, using the zwitterionic detergent CHAPS, was used to compare the IgM, IgG and IgA binding of sera from seven groups of rheumatic fever, related-disease and normal subjects to immunoblotted cardiac antigens. It was shown that:

   (i) ARF sera stained the immunoblotted heart antigens at a greater frequency and intensity, than any of the other groups of sera. In particular, IgM binding by ARF sera was stronger than that of other groups of sera.

   (ii) There were eleven major constituents in the heart extract with which sera reacted, ranging in mol.wt. from 116 kD to 28 kD.

   (iii) A doublet at 33 kD appeared to be uniquely recognised by IgM antibodies in all ARF sera.

   (iv) Both child and adult CRF sera showed very little IgM binding to heart, whereas IgG binding was intense.

   (v) IgA binding in all groups of sera was weak.

   (vi) Within all groups, there was considerable heterogeneity in the reactions of individual sera, particularly with regard to IgG binding.

   (vii) All seven groups of sera contained antibodies that reacted with antigens in this heart extract by immunoblotting.

   (viii) Some of the antigens in the extract corresponded in mol.wt. to antigens in different types of heart extract reported by other investigators to be reactive with monoclonal antibodies and rabbit immune sera evoked by *S.pyogenes* antigens.

2. Antibody derived from ARF sera and eluted from the 38-40 kD heart antigen, showed cross-reactivity with immunoblotted *S.pyogenes* antigen pep M5, and faint reactivity with pep M24 and purified group A streptococcal membranes. The eluted antibody showed binding to membranous structures of heart tissue by indirect immunofluorescence.

3. IgG antibodies in ARF sera bound a 200 kD antigen in purified human heart sarcolemmal membranes, that was not recognised by normal sera.

A 40 kD antigen was recognised also by normal sera, but at a higher frequency by ARF sera.

4. IgM antibodies in ARF sera bound a 140 kD antigen in nuclear extracts of a primary culture of heart endothelial-like cells. The antigen was also faintly recognised in Girardi heart cells. The antigen was not recognised by any normal sera.

5. Rabbit antisera to various *S.pyogenes* antigens, showed distinctive patterns of binding to various extracts of cardiac antigens. In particular, intense binding was observed to heart cell nuclear antigens. The antiserum to protein M24 bound a 40 kD antigen in the crude heart extract, thus indicating that this *S.pyogenes* protein may, contrary to previous reports, share epitopes with cardiac antigens.

6. One ARF serum showed intense binding to an ATPase enzyme isolated from rabbit skeletal muscle. Cross-reactivity between *S.pyogenes* M proteins and enzymes involved in muscle contraction, may explain the antiphagocytic properties of the M protein. Such antigenic mimicry between bacterial and human proteins may provide clues to the pathogenesis of rheumatic fever.

7. Immunoblotting thus showed some binding reactions that appear unique to rheumatic fever sera and might provide clues to the pathogenesis of the disease.

4. HUMAN MONOCLONAL ANTIBODIES DERIVED FROM LYMPHOCYTES OF ACUTE RHEUMATIC FEVER PATIENTS

4.1 INTRODUCTION 121
4.1.1 Rheumatic Fever: Use Of Murine And Human Monoclonal Antibodies 121
4.1.1.1 Mouse Monoclonal Antibodies 121
4.1.1.1.1 Heart and S.pyogenes 121
4.1.1.1.2 Myosin and Tropomyosin 121
4.1.1.1.3 Multispecificity of Anti-streptococcal mAbs 122
4.1.1.1.4 Reactivity with DNA 123
4.1.1.1.5 Human Sarcolemma 123
4.1.1.1.6 Other Streptococcal Serotypes (and Myosin) 123
4.1.1.1.7 Shortcomings of Murine mAbs 124
4.1.1.2 Human Monoclonal Antibodies in Rheumatic Fever 124
4.1.2 Problems in Development of Human Monoclonal Antibodies 125
4.1.2.1 Range of Immunogens and Poor Source of Lymphocytes 125
4.1.2.2 Instability of Human Hybridomas 126
4.1.2.3 Immortalization of Human B Cells 126
4.1.2.4 Expansion of Human Monoclonal Antibody Cell Lines 127
4.2 RESULTS 128
4.2.1 Screening and Characterization of Monoclonal Antibodies 128
4.2.1.1 ARF Patients 128
4.2.1.2 Hybrid Screening and Selection 128
4.2.1.3 Stability of Hybridomas in Culture 129
4.2.1.4 Karyotyping of Selected Hybrids 129
4.2.1.5 Characteristics of Immunoglobulin Produced 129
4.2.1.6 Propagation of Hybridomas in Nude Mice 132
4.2.1.7 Stability of Antibody Production 132
4.2.1.8 CD5+ Cell Surface Marker 132
4.2.1.9 Anti-Streptococcal Antigen Reactivity 132
4.2.1.10 Selection of mAbs against Group A Streptococci 132
4.2.1.11 Cross-reactivity with other Streptococcal Groups (B,C,D,G) 133
4.2.1.12 Cross-Reactivity with Non-streptococcal Bacteria 134
4.2.2 Anti-Human Heart Antigen Reactivity 134
4.2.2.1 Binding to Heart Sections: Immunoperoxidase Sandwich Assay 134
4.2.2.2 Immunoblotting of Heart Extract and Sarcolemma 135
4.2.2.3 Organ Non-Specificity of mAb Binding 135
4.2.2.4 Nonspecific Binding to Mammalian Tissue Antigens 135
4.2.3 Cross-reactive Binding of Human mAbs to Heart and Streptococcal Ags 135
4.2.3.1 Immunoblotting 135
4.2.3.2 ELISA Inhibition Assay 139
4.2.4 Binding to Nuclear Antigens 139
4.3 DISCUSSION 141
4.4 CONCLUSIONS 146

4.1 INTRODUCTION

Human monoclonal antibodies offer a tool to the understanding of how the human B cell repertoire functions, and have been used to analyse reactivity to autoantigens in diseases like SLE, rheumatoid arthritis, Hashimoto's thyroiditis, juvenile diabetes and rheumatic fever (Shoenfield et al., 1982; Sasaki et al., 1984; Haskard and Archer, 1982; Eisenbarth et al., 1982; Satoh et al., 1983; Cunningham et al., 1988).

This discussion reviews the application of human and murine monpclonal antibodies (mAbs) to the analysis of antigenic mimicry in rheumatic fever, and describes some of the difficulties which have been encountered in developing human monoclonal antibodies.

4.1.1 RHEUMATIC FEVER: USE OF MURINE AND HUMAN MONOCLONAL ANTIBODIES

4.1.1.1 Mouse Monoclonal Antibodies

The specificities of the several of mAbs mentioned here have been discussed in the context of immunoblotting (Chapter 3) and are therefore mentioned only briefly again here.

4.1.1.1.1 Heart and *S.pyogenes*

Cunningham and Russell (1983) developed a panel of thirteen hybridomas from mice immunized with whole *S.pyogenes* (type M5) or their membranes. The secreted mAbs were screened by ELISA against Triton X-100 extracts of heart and *S.pyogenes*, and two groups of reactivity were identified: those that reacted with only *S.pyogenes*, and those that reacted with both *S.pyogenes* and heart extract. Monoclonals derived from some of these "polyclones" (e.g.No. 36, 49 and 54) were used in several subsequent studies by Cunningham and co-workers.

4.1.1.1.2 Myosin and Tropomyosin

Krisher and Cunningham (1985) showed that a murine mAb against *S.pyogenes* (derived from polyclone "54" in Cunningham and Russell, 1983), cross-reacted with skeletal and cardiac muscle myosin. This mAb reacted with the same high mol.wt. protein bands in heart extract as did a mAb raised to cardiac myosin, and immunoblot inhibition experiments showed that absorption with group A streptococci, abolished reactivity of this mAb to both these extract bands and to myosin. These results suggested a sharing of determinants between *S.pyogenes* and myosin. Structural resemblance between type M5 protein of

S.pyogenes and myosin was demonstrated by Fischetti and Manjula (1982) and Manjula et al. (1985), thus reinforcing the possibility of antigenic mimicry between these bacterial and mammalian proteins.

Subsequently, mAbs to *S.pyogenes* were used to localise the cross-reactive determinants on myosin to subfragments (either heavy or light meromyosin) of the myosin heavy chain (Cunningham et al., 1986). These experiments showed a difference in the reactivity patterns of the mAbs to skeletal and cardiac myosin: some reacted with both skeletal and cardiac myosin whilst others were specific for skeletal myosin.

More recently, murine mAbs evoked by M type 5 proteins have demonstrated cross-reactivity with tropomyosin (Fenderson et al., 1989). Five mAbs reactive with tropomyosin were reactive with either pep M5 or M6 (*E.coli*-cloned) protein. The tropomyosin antigens were shown by immunoblotting to be either the 70 kD dimer or the 35 kD monomer, or both. Cardiac myosin and tropomyosin were also shown to share epitopes.

4.1.1.1.3 Multispecificity of Anti-streptococcal mAbs

Murine mAbs derived from the hybridoma polyclones of Cunningham and Russell (1983) were shown to react with antigens from other tissues (Cunningham and Swerlick, 1986; Fenderson et al., 1989).

One mAb (54.2.8) reacted with myosin, DNA and synthetic nucleotide homopolymers, another (36.2.2) reacted with myosin and the α-helical coiled-coil proteins keratin and actin, and a third reacted with vimentin and with cardiolipin (weakly). None reacted with rheumatoid factors or with collagen (a protein with a highly repetitive structure implicated in some autoimmune disorders). These cross-reactive anti-group A streptococcal mAbs were grouped into those that reacted, (1) with myosin, keratin and actin, a family of α-helical coiled-coil proteins, or (2) with DNA and myosin.

Both groups of these heart-reactive mAbs, reacted with streptococcal M5 and M6 proteins. Protein structure analyses by Fischetti and Manjula (1982) and Manjula et al. (1985) provided a structural basis for these cross-reactions by identifying, in most M proteins, a periodicity of seven amino acid residues similar to that present in myosin, tropomyosin and the desmin-keratin family of α helical proteins.

However, whether these cross-reactions between mammalian antigens and *S.pyogenes* are pathologically relevant to rheumatic fever is disputable. Avrameas and co-workers demonstrated cross-reactivity among such conserved mammalian autoantigens (myosin, actin, DNA, thyroglobulin, neurofilaments, renin and tubulin) using a panel of murine natural monoclonal

antibodies (Dighiero et al., 1983, 1985). This finding brings into question the pathological relevance of some autoantigens implicated in antigenic mimicry.

4.1.1.1.4 Reactivity with DNA

Anti-DNA antibodies occur in autoimmune diseases like SLE but such antibodies have not been found in rheumatic fever patients sera. Cunningham et al. (1988) however showed that human serum antibodies affinity purified against myosin did react with DNA, and furthermore that several of their human mAbs reactive with myosin were inhibited by DNA. Other studies have shown (1) that a murine mAb against myosin reacted with DNA (Lafer et al., 1981), and (2) that DNA-reactive mAbs derived from SLE-prone mice, reacted with endogenous bacteria, including *S.faecalis* (Carroll et al., 1985). Cross-reactivity between myosin and DNA, and between bacterial antigens and DNA, may thus be specific but the significance of these cross-reactions in rheumatic fever is not evident.

4.1.1.1.5 Human Sarcolemma

Several studies have suggested that cross-reactive heart antigens in rheumatic fever are associated with the sarcolemmal membrane (Zabriskie and Freimer, 1966; Dale and Beachey, 1982a; Zabriskie and Friedman, 1983).

Murine mAbs evoked by *S.pyogenes* (type M5) membranes could be divided into two groups: strong reactors with streptococcal and sarcolemmal membranes, or weak reactors with these membranes (Cunningham et al., 1984). The major reactive determinants in the sarcolemmal extracts were high molecular weight proteins of about 200 kD. These polypeptides may have included myosin which has been detected in similar extracts (Dale and Beachey, 1985a), and has been associated with the internal surface of the sarcolemma (Fallon and Nachmias, 1980).

A comparison of mAb binding to extracts of normal and rheumatic sarcolemma, showed no differences in pattern but only in intensity of binding, indicating that the reactive epitopes were not unique to rheumatic heart. Furthermore, normal mouse sera also showed some binding to these antigens, suggesting natural autoantibody reactivity.

4.1.1.1.6 Other Streptococcal Serotypes (and Myosin)

Some evidence suggests that epitopes are shared between *S.pyogenes*, *S.mutans*, *S.rattus* and myosin.

mAbs binding a 62 kD component in membrane extracts of *S.pyogenes* were found to bind components of mol.wt. 62-67 kD in SDS extracts of *S.mutans* and *S.rattus* (Ayakawa et al., 1988). Furthermore, reactivity to *S.rattus* extracts could be abolished by absorption of the mAbs with myosin. These

results concur with those of Doyle et al. (1986) who found that mAbs to *S.mutans* reacted with *S.rattus* and myosin, and substantiate other reports of possible antigenic mimicry between streptococcal antigens and myosin as a relevant human autoantigen.

4.1.1.1.7 Shortcomings of Murine mAbs

The application of murine mAbs to the study of human disease has some disadvantages. First, immunization of animals may entail preparation of the antigen in a manner that can result in loss or change of antigenicity (Seibert, 1928; Wallis et al., 1989). Second, animal models show different patterns of infectivity, so that monoclonal antibodies derived from them could be unrepresentative and misleading. Third, even where the animal model closely resembles the human pattern of infection, the variation in the immunologic repertoires of the two species may result in different antibody responses.

Human mAbs are more faithful representatives of the human autoantibody repertoire and are therefore likely to be better tools for studying human disease.

4.1.1.2 Human Monoclonal Antibodies in Rheumatic Fever

Cunningham et al. (1988) developed ten human mAbs from normal subjects and from patients with streptococcal infections (pharyngitis and cellulitis with raised ASO titres), and compared their specificities with those of murine mAbs evoked by streptococcal antigens (Cunningham and Russell, 1983; Cunningham et al., 1984; 1986). Both tonsillar lymphocytes and PBL were used. These human mAbs were screened by ELISA against human heart antigen and group A streptococci.

The specificities of human mAbs and the murine mAbs were quite similar. Both types could be categorised into mAbs that reacted, (1) with myosin, keratin and/or actin, or (2) with myosin and DNA (Cunningham and Swerlick, 1986). Most of the human mAbs fell into the myosin-keratin-actin group.

Several of these mAbs were derived from normal subjects with no evidence of *S.pyogenes* infection (as judged by ASO titres), indicating that the lymphocytes capable of producing these cross-reactive autoantibodies are present in normal, uninfected individuals. This finding raises the perplexing question of the significance of autoantibodies in health and disease (Chapter 1). Apparently however lymphocytes had to be stimulated with streptococcal antigens prior to cell fusion, in order to obtain hybridomas secreting anti-myosin antibodies.

Human mAbs that reacted with DNA are puzzling in that no evidence for antinuclear antibodies could be found in sera of the ARF patients. Anti-myosin antibodies affinity-purified from ARF sera did however react with DNA and it

was suggested that reactivity of myosin antibodies with DNA could be due to its charge and configuration. The cross-reactivity these human mAbs showed between pep M5, M6, myosin and DNA may be similar to the cross-reactivity displayed by the murine mAbs between DNA, myosin and bacterial antigens (section 4.1.1.4).

More recently, Cunningham et al. (1989) used overlapping synthetic peptides covering the entire pep M5 protein sequence to map the myosin-reactive epitopes recognised by these human and murine mAbs (as well as by affinity purified myosin-specific ARF and CRF patient sera). The majority of human and mouse myosin cross-reactive antibodies recognised an epitope involving the sequence GLN-LYS-SER-LYS-GLN within the 14-residue carboxy terminus of pep M5. This exact sequence was not found in myosin itself, but it was argued that similar sequences or conformational epitopes might be recognised since previous work had clearly demonstrated cross-reactivity of these antibodies with the heavy chain of myosin (Cunningham et al., 1986).

The human mAbs used in the above studies were not derived from patients with rheumatic fever, but from two patients with high ASO titres (one with repeated streptococcal pharyngitis and the other with recurrent cellulitis), and from normal subjects. The innovation of the present study was to develop human mAbs from lymphocytes of ARF patients. A number of difficulties were experienced in trying to isolate such mAbs, pertaining in particular to the source of lymphocytes (PBL from a young patient), the instability of the hybridomas in culture, and the expansion of the antibody-secreting hybrids in culture and in mice. These difficulties appear to be general problems in human mAb technology, and are briefly reviewed below.

4.1.2 PROBLEMS IN DEVELOPMENT OF HUMAN MONOCLONAL ANTIBODIES

4.1.2.1 Range of Immunogens and Poor Source of Lymphocytes

The range of immunogens that can be injected into humans as well as the immunization schedules applicable are obviously restricted (compared to animal models), and the source of immune lymphocytes is usually limited to peripheral blood lymphocytes (PBL) which perform less well than those from spleen and tonsils (Olsson et al., 1983).

Various reasons have been postulated for the poor performance of PBL as hybridoma fusion partners: (1) insufficient memory or antigen specific B cells (Seigneurin et al, 1983; Olsson et al., 1983); (2) B cells not in an appropriate state of differentiation (Schwaber et al., 1984; Cote and Houghton, 1985); (3) existence of suppressor and cytotoxic T cells (Cote and Houghton, 1985); (4)

insufficient number and low mitotic activity of B cells (Burnett et al., 1985; Westerwoudt, 1985).

4.1.2.2 Instability of Human Hybridomas

Human hybridomas secreting antibody have often been found to be unstable in culture and to lose the structural genes coding for immunoglobulin (Schwaber et al., 1984) or to become overgrown by non-secreting lymphocytes (Zurawski et al., 1978). Epstein-Barr virus transformation of B cells can result in the overgrowth by non-secretors (probably due to polyclonal activation of cells), whereas production of human mAbs through fusion with immortal partner cells can result in chromosomal instability with preferential loss of the human chromosomes (Kozbor and Croce, 1985; Teng et al., 1985).

Possible reasons for loss or decrease in immunoglobulin production could be attributed to defects in synthesis (Sikora et al., 1983; Teng et al., 1985) or secretion (Kozbor and Croce, 1985; Gaffar et al., 1986b). Most studies have failed to investigate the possibility of immunoglobulin being present in the cytoplasm and have instead measured either total immunoglobulin or specific antibody in cell culture supernatants. Studies on the morphology of the human cell lines indicate that they often have many free polyribosomes and a poorly developed rough endoplasmic reticulum compared to mature plasma cells (Larrick et al., 1983; Kozbor et al., 1983). Such defects could impede efficient translation, assembly and secretion of immunoglobulin.

Other reasons given for defective immunoglobulin synthesis by human monoclonal cell lines include: defects in relevant regulatory genes (Raison et al., 1982; Schwaber et al., 1984); improper combination of H and L chains (James and Bell, 1987); shortage of important differentiation and growth factors (Winger et al., 1983) and inhibitory effects of microorganisms such as mycoplasma (Van Meel et al., 1985)

4.1.2.3 Immortalization of Human B Cells

Problems have been encountered in the choice of cell fusion partners for immortalizing human B cells.

Human myelomas grow poorly in culture, and therefore in human-human hybridomas, Epstein-Barr virus transformed lymphocytes have been used instead since they have the additional advantage that they secrete little immunoglobulin compared to myelomas (Kozbor et al., 1983).

A second strategy for immortalizing human lymphocytes has been to use heteromyelomas created by the fusion of lymphoblastoid lines with human or mouse myelomas. Such fusions (with mouse myelomas in particular) have produced a number of partner cells which form stable hybrid-hybrids with human lymphocytes (Teng et al., 1983; 1985).

Thirdly, human lymphocytes have been fused with mouse myelomas instead of with human myelomas or mouse-human heteromyelomas (Wunderlich et al., 1981; Thompson et al., 1986). The fusion rates are apparently higher with these mouse myelomas than with human partners (Cote et al., 1983, 1986). Such heterohybrids are known to preferentially lose their human chromosomes, but these cell lines can be stabilised by early cloning (Thompson et al., 1986).

4.1.2.4 Expansion of Human Monoclonal Antibody Cell Lines

While human/human hybridomas grow fairly well in stationary and suspension cultures, growth in immunodeficient (nude) mice is comparatively poor, despite some improvements such as co-inoculation with human fibroblasts (Olsson and Brams, 1985) or irradiating the mice prior to inoculation (Bogard et al., 1985).

Human/mouse heterohybrids take quite readily in nude mice probably due to the fact that they don't express the human HLA antigens which would lead to immune rejection (Raison et al., 1982).

In conclusion, although the development of stable antibody secreting human monoclonal antibody cell lines presents some serious technical difficulties, these antibodies have the advantage over murine mAbs in being more representative of the human immune repertoire and, in the study of autoimmune and infectious diseases, can offer a valuable inside view of antibody reactivity patterns in the disease. Furthermore, their homogeneity and potential specificity can make them more useful probes than whole serum for the identification of autoantigens.

4.2 RESULTS

Human mAbs derived from patients with acute rheumatic fever were fused with mouse myeloma cells to form heterohybridomas which were screened against both *S.pyogenes* and human heart antigens. Two mAbs displaying cross-reactivity between these antigens were cloned and characterised. The ensuing discussion describes the development of these mAbs, and the investigations performed to characterize them.

4.2.1 SCREENING AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES

Purified and enriched populations of peripheral blood lymphocytes (PBL) from two patients with acute rheumatic fever were fused with SP2/0-14 mouse myeloma cells to form mouse-human heterohybridomas, according to procedures described in Materials and Methods (section 7.A.21).

4.2.1.1 ARF Patients

ARF donor patients (designated M and VR) were children who fulfilled the Jones Criteria for diagnosis of rheumatic fever (Table 1.1), and had active carditis. Patient M, from whom both mAbs described in this study were derived, was 4 years old and had an ASO titre of 480 Todd units.

4.2.1.2 Hybrid Screening and Selection

Hybridomas were screened both against group A streptococci by ELISA (section 7.A.3), and against sections of human heart by a peroxidase-conjugated second antibody assay (section 7.A.23). Only hybrid clones demonstrating reactivity against both streptococci and heart tissue were selected for expansion and further characterization.

Table 4.1 shows that, fourteen days after fusion, only hybrids derived from lymphocytes of patient M, secreted antibody reactive with both *S.pyogenes* and human heart. Since the objective of the study was to isolate only such cross-reactive mAbs, only these clones were selected for subcloning and characterization. After subcloning by limiting dilution, two clones secreting cross-reactive antibody were identified. The two monoclonal antibodies were designated MA6 and MB4.

TABLE 4.1

Percentage of Seeded Hybridoma Clones Secreting Antibody Reactive with Group A Streptococcus and/or Human Heart Sections

HYBRIDOMA DERIVATION	STREP ELISA	IMMUNOCYTOCHEMISTRY (HEART)	STREP-ELISA & IMMUNO-CYTOCHEMISTRY (HEART)
Patient M	29	8	8
Patient VR	43	6	0

4.2.1.3 Stability of Hybridomas in Culture

Hybridoma MA6 survived twenty passages. (Culture supernatant was collected over a period of three months before the cell line was frozen in liquid nitrogen). Hybridoma MB4 was unstable and survived only five culture passages. (It was thus not fully characterised).

4.2.1.4 Karyotyping of Selected Hybrids

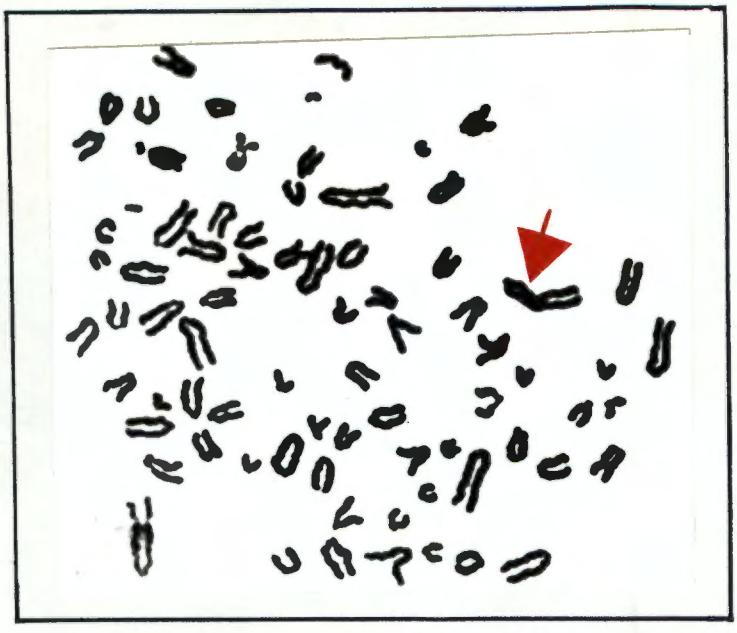
Karyotyping of the hybridomas was done as described in Materials and Methods (section 7.A.26). Hybridoma MA6 had an average of 85 chromosomes per cell compared with 70 chromosomes in the parent SP2/0-Ag14 mouse myeloma cell line.

Fig.4.1 shows karyotypes of: (a) an SP2/0-Ag14 murine plasmacytoma cell with acrocentric chromosomes and one large metacentric chromosome; (b) a normal human peripheral blood lymphocyte showing metacentric chromosomes; (c) an MA6 hybridoma cell showing both murine acrocentric chromosomes and a few additional human metacentric chromosomes.

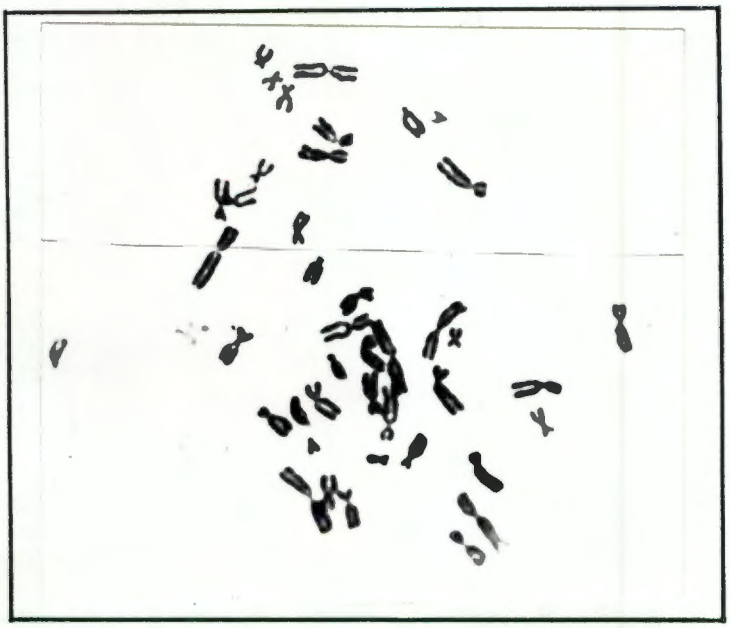
4.2.1.5 Characteristics of Immunoglobulin Produced

Both selected hybridoma clones produced IgM antibody as determined in ELISA using heavy-chain specific anti-human IgG, IgA or IgM conjugates. The immunoglobulin concentration of hybridoma MA6, measured by radial immunodiffusion four days after subcloning, was 28 μg/ml.

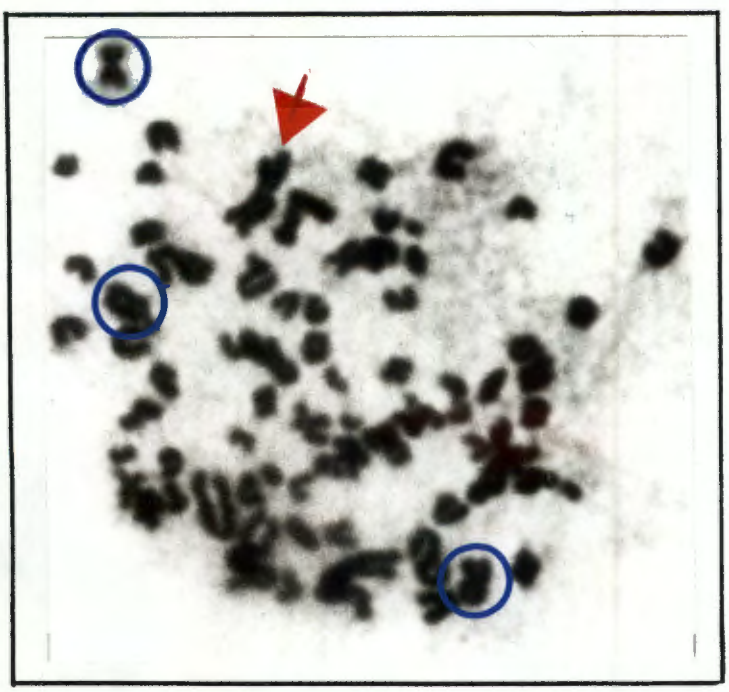
The secreted and cytoplasmic immunoglobulins of MA6 were determined by ^{14}C-leucine labelling and immunoprecipitation of hybridoma cell lysates and culture medium, according to the method of Choi (1977). The radiolabelled immunoprecipitates, separated by 10% SDS-PAGE and autoradiographed, showed the presence of both cytoplasmic and secreted immunoglobulin light and heavy chains (Fig.4.2).



(a)



(b)



(c)

Fig. 4.1 Karyotyping of murine myeloma SP2/0-Ag14 cells, human peripheral blood lymphocytes and mouse-human hybridoma cells.

Cells were exposed to hypotonic treatment in 0.075M KCl for 20 minutes, fixed and washed in acetic acid-methanol solution, spread on glass slides and then stained with Giemsa stain (section 7.A.26).

(a) SP2/0-Ag14 murine plasmacytoma cell showing acrocentric chromosomes and one large metacentric chromosome (arrow).

(b) Peripheral blood lymphocyte from acute rheumatic fever patient M (section 4.2.1.1) showing metacentric chromosomes.

(c) Mouse-human heterohybridoma cell showing numerous mouse acrocentric chromosomes, one large metacentric chromosome (arrow) and at least three human metacentric chromosomes (circles)

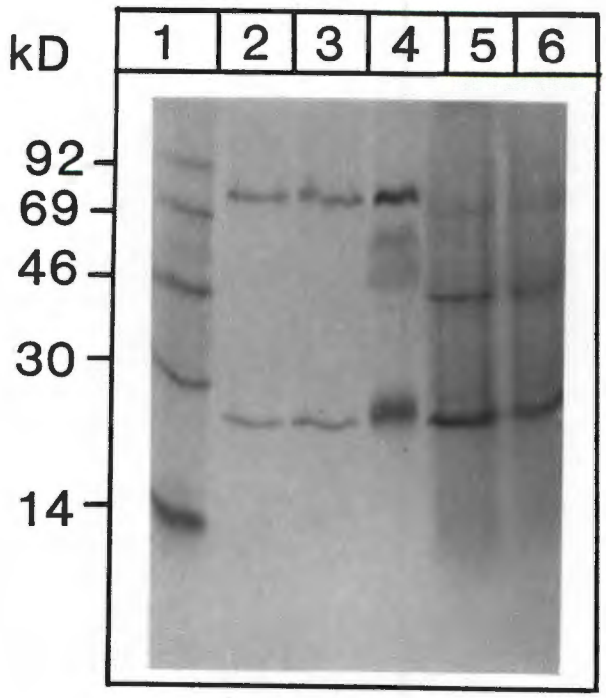


Fig. 4.2 Autoradiograph of immunoprecipitated cytoplasmic and secreted immunoglobulin of hybridoma MA6.

Hybridoma cell lysates and culture medium supernatants were immunoprecipitated following labelling with ^{14}C-leucine, as described in section 7.A.27. The radiolabelled immunoprecipitates were fractionated by 10% SDS PAGE and subjected to autoradiography for 6 days. Similar control immunoprecipitations were performed on culture media derived from normal human PBL which had been stimulated for 6 days in culture with pokeweed mitogen (PWM)

Lane 1:	mol.wt. markers
Lanes 2 & 3:	culture medium from hybridoma MA6
Lane 4:	culture medium from PWM stimulated normal human PBL
Lanes 5 & 6:	cell lysates of hybridoma MA6

Normal unfused PBL stimulated with pokeweed mitogen (lane 4) secreted proteins of mol.wt. 72 kD, 60 kD, 51 kD which may correspond to the mol.wts. of the heavy chains of IgM, IgG and IgA respectively. A fourth band at 25 kD corresponds to the mol.wt. of immunoglobulin light chains. The MA6 hybridoma cell lysate (lanes 5 and 6) contained proteins of mol.wt. 70 kD, 46 kD and 25 kD, whereas the culture supernatant of this hybridoma contained proteins of 72 kD and 25 kD (lanes 2 and 3).

4.2.1.6 Propagation of Hybridomas in Nude Mice

The mouse-human heterohybridomas failed to grow in pristane-primed BALB/c mice. They were propagated successfully in pristane-primed "nude" (athymic) mice, but several mice grew solid tumours and produced little or no ascites. In mice that developed ascites, the yield was generally between 1.5 and 2.5 ml per mouse.

4.2.1.7 Stability of Antibody Production

The stability of hybridoma antibody production was assessed by testing reactivity in the group A streptococcus ELISA after each passage of cells over a period of six months. A reduction of about 30% in ELISA antibody reactivity over this period was evident, indicating that the hybridomas were not completely stable in culture.

4.2.1.8 $CD5^+$ Cell Surface Marker

The MA6 heterohybridoma was tested for the $CD5^+$ cell marker using the monoclonal antibody T-1 (Dako: M705), and an FITC-labelled goat anti-mouse IgG antibody conjugate (Cappel). Results were negative.

4.2.1.9 Anti-Streptococcal Antigen Reactivity

4.2.1.10 Selection of mAbs against Group A Streptococci

Hybridomas MA6 and MB4 were selected for reactivity against *S.pyogenes* by ELISA (section 7.A.3). These hybridomas were also reactive with heart tissue antigens. Table 4.1 shows that the percentage of wells positive in the streptococcal ELISA was four to five times greater than positives detected by immunocytochemistry.

4.2.1.11 Cross-reactivity with other Streptococcal Groups (B,C,D,G)

The specificity of MA6 for *S.pyogenes* was assessed by comparing its reactivity against the streptococcal groups A, B, C, D and G, using the similar ELISA procedure with whole bacteria glutaraldehyde-fixed to PVC plates (section 7.A.3).

The reactivity of the monoclonal antibody was also compared with the IgG and IgM reactivity of serum from patient M.

Results presented in Table 4.2, show that MA6 reacted with all groups of streptococci, as did the donor patient's serum. Serum IgM absorbance (A_{405}) readings were similar in all streptococcal groups, but IgG reactivity to groups A, C and G was noticeably stronger. The readings are generally low because both the no-antibody control and the no-antigen controls were subtracted from each value.

TABLE 4.2

Reactivity of mAb MA6 and Serum "M" to Streptococcal Groups (A,B,C,D,G) in ELISA

ANTIBODY	ISOTYPE	DIL.	Streptococcal Groups				
			A	B	C	D	G
MA6*	IgM	1:50	.152	.174	.281	.129	.215
Serum M	IgM	1:100	.258	.241	.278	.265	.305
Serum M	IgG	1:100	.469	.179	.493	.141	.494

* Ammonium sulphate precipitated culture supernatant

Table 4.3 compares the reactivity to group A streptococci of MA6 and MB4 (1:2 dilutions) culture supernatants, with the reactivity of the PBL-donor patient serum (M) and a pool of five normal sera (5N). Both the no-antibody and the no-antigen controls were subtracted from each average value, giving low results.

TABLE 4.3

Reactivity of MA6 and MB4 and Patient Serum (M) to Various Antigens
Comparison of Reactivity of Human mAbs and Human Sera with Various Antigens in ELISA (OD 405nm)

ANTIBODY	ANTIBODY DILUTION	STREP A	MYOSIN	PHOSPHORYLASE B
mAb MA6	1:2	.315	0	.513
mAb MB4	1:2	.163	0	.192
HT medium (control)	1:2	.151	.024	.034
Serum M	1:100	.152	.045	.084
Pool 5N	1:100	.185	.032	.065
PBS-Tween (control)	-	.094	.013	.056

4.2.1.12 Cross-Reactivity with Non-streptococcal Bacterial Species: *E.coli*

Both MA6 and MB4 cross-reacted with *E.coli* antigens. Immunoblotting of culture supernatants against *E.coli* sonicates showed strong binding (to constituents of 35 kD and 38 kD; results not shown), and ELISA inhibition experiments (using *E.coli* sonicates in the *S.pyogenes* ELISA) showed similar cross-reactivity of MA6 with *E.coli*. Approximately 18 μg/ml of *E.coli* absorbant, compared to 13.5 μg/ml of *S.pyogenes* absorbant, was required to absorb 50% of MA6 binding to *S.pyogenes* (dilution 1:500).

4.2.2 ANTI-HUMAN HEART ANTIGEN REACTIVITY

4.2.2.1 Binding to Heart Sections: Immunoperoxidase Sandwich Assay

Hybridomas were screened against paraffin wax embedded sections of normal human heart (section 7.A.23).

Table 4.1 shows that a smaller percentage hybridomas showed positive binding to heart, than to group A streptococci. All of the hybridomas derived from patient M that were positive by immunocytochemistry (8%) also cross-reacted in ELISA with group A streptococci, whereas none from patient VR were cross-reactive.

4.2.2.2 Immunoblotting of Heart Extract and Sarcolemma

Immunoblotting of MA6 and MB4 against CHAPS detergent extracts of human heart (section 7.A.11) and against 3 M KCl extracts of cardiac sarcolemmal membranes (section 7.A.12) showed strong binding to a 43 kD constituent in both extracts (Fig.4.3). Both mAbs also bound less strongly to a 48 kD constituent in the CHAPS extracts. (MB4 stained some additional bands between 60 kD and 140 kD in the CHAPS extract).

4.2.2.3 Organ Non-Specificity of mAb Binding

Neither MA6 nor MB4 were specific for heart tissue antigens, as both reacted by immunoblotting with human kidney antigens in detergent extracts (to 100 kD, 94 kD and 90 kD constituents).

4.2.2.4 Nonspecific Binding to Mammalian Tissue Antigens

The specificity of MA6 and MB4 against mammalian antigens was further examined by reacting them against immunoblotted proteins derived from an SDS-PAGE molecular weight calibration kit (Pharmacia). Other investigators have noted irrelevant cross-reactive binding of mAbs to molecular weight standards (Ghosh and Campbell, 1986). The protein mixture included purified phosphorylase b (rabbit muscle), albumen (bovine serum), ovalbumen (egg white) and carbonic anhydrase (bovine erythrocyte).

Fig.4.4 shows that both mABs bound phosphorylase b and carbonic anhydrase. MA6 also reacted with albumen and ovalbumen.

Furthermore, ELISAs using myosin and phosphorylase b as solid phase antigens (section.7.A.6), confirmed strong reactivity of both mAbs to phosphorylase b (Table 4.3). Neither monoclonal bound myosin. Both these mAbs appeared therefore to lack specificity, or are possibly multispecific.

4.2.3 CROSS-REACTIVE BINDING OF HUMAN MABS TO HEART AND STREPTOCOCCAL AGS

Both mAbs were selected for the cross-reactivity they displayed between group A streptococci and human heart tissue antigens.

4.2.3.1 Immunoblotting

Cross-reactive binding of MA6 to streptococcal and heart antigens was additionally demonstrated by its reactivity in immunoblots, with constituents in both extracts of mol.wts. of about 43 kD, 45 kD and 83 kD (Fig.4.5). The binding was however weak and may have been nonspecific.

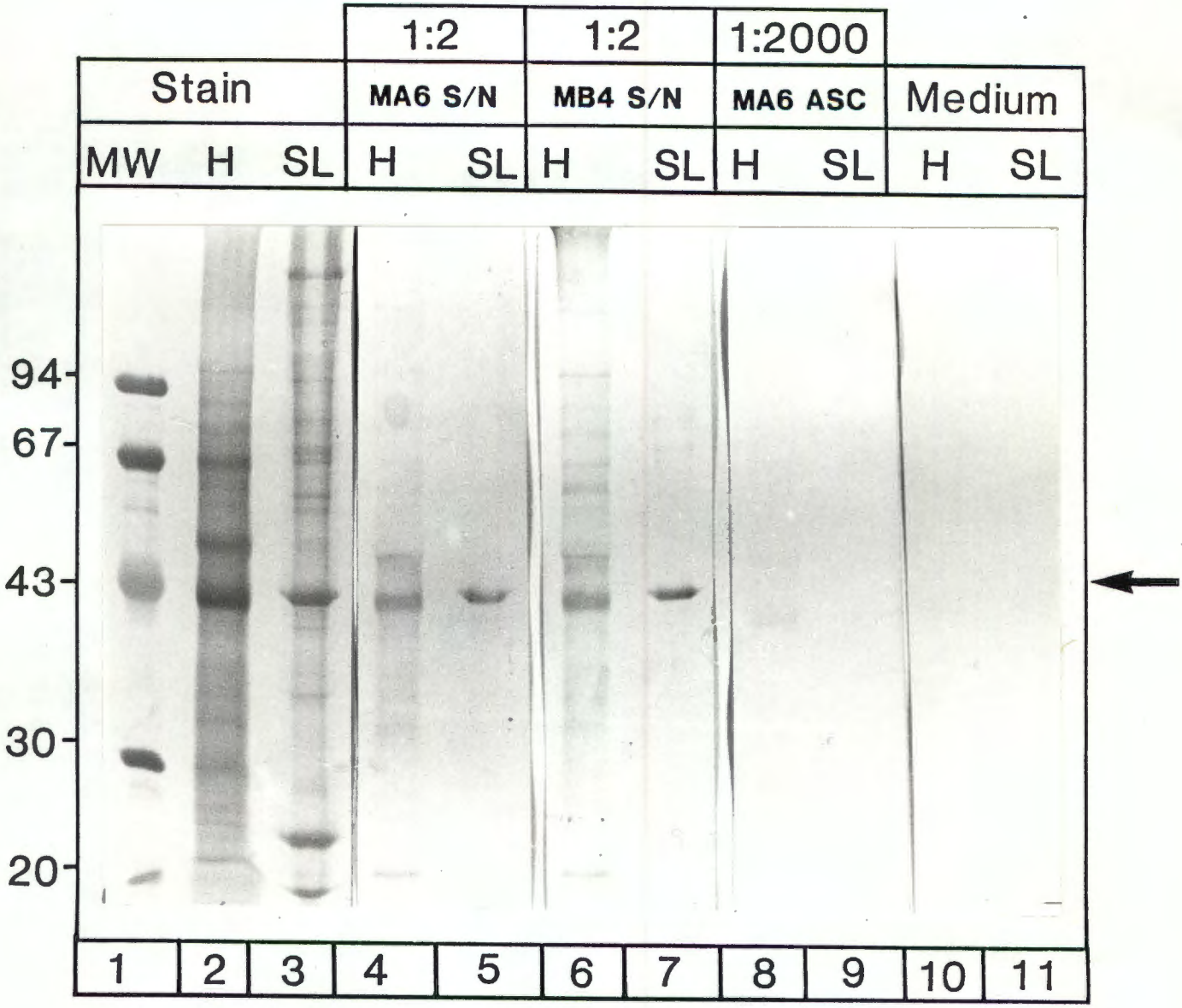


Fig. 4.3 Immunoblotting of human mAbs MA6 and MB4 against crude extracts of human heart and purified cardiac sarcolemmal membranes.

Crude CHAPS detergent extracts of human heart **(H)** (section 7.A.11) and purified human cardiac sarcolemmal **(SL)** membranes (section 7.A.12) were separated on 10% SDS polyacrylamide gels and immunoblotted against the human mAb MA6 and MB4 supernatants (S/N) and nude mouse ascitic fluids (ASC). A strongly reactive constituent of 43 kD present in both extracts was detected at concentrations of 1:2 in culture medium supernatants of both mAbs (lanes 4-7). The constituent was detected at concentrations of up to 1:1000 of ascitic fluid of the mAbs, but was no longer evident at dilutions of 1:2000 (lanes 8 & 9). Undiluted pure medium showed no binding (lanes 10 & 11). Amido Black staining of the extracts showed a prominent protein at 43 kD in both extracts, and numerous additional bands (lanes 2 & 3).

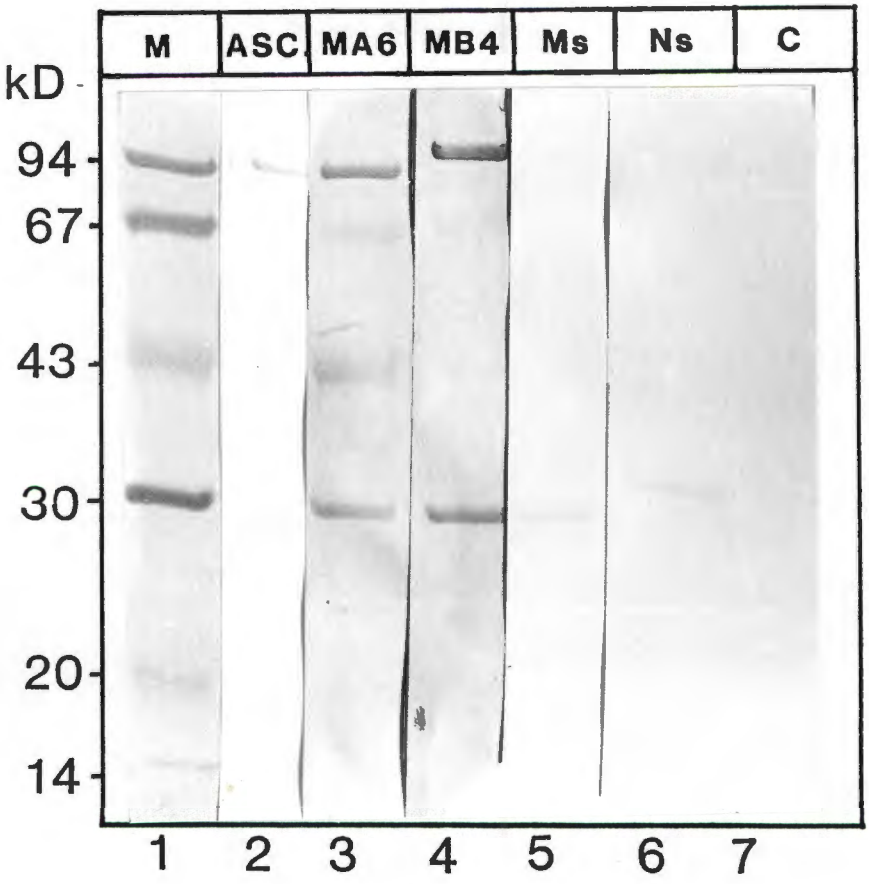


Fig. 4.4 Nonspecific cross-reactive binding of human monoclonal antibodies MA6 and MB4 to various proteins.

Purified proteins contained in a molecular weight calibration kit (Pharmacia) (phosphorylase b, albumen, ovalbumen, carbonic anhydrase, trypsin inhibitor) were separated by 10% SDS PAGE, transferred to nitrocellulose and reacted against the human mAbs MA6 and MB4 according to procedures described in section 7.A.9. Both mAbs reacted with phosphorylase b (94 kD) and carbonic anhydrase (30 kD), and MA6 also reacted weakly with albumen (67 kD) and ovalbumen (43 kD) (lanes 3 & 4). Serum from the PBL donor ARF patient (Ms) did not react with these proteins, nor did a pool of 5 normal sera (Ns) and the negative control (minus first antibody).

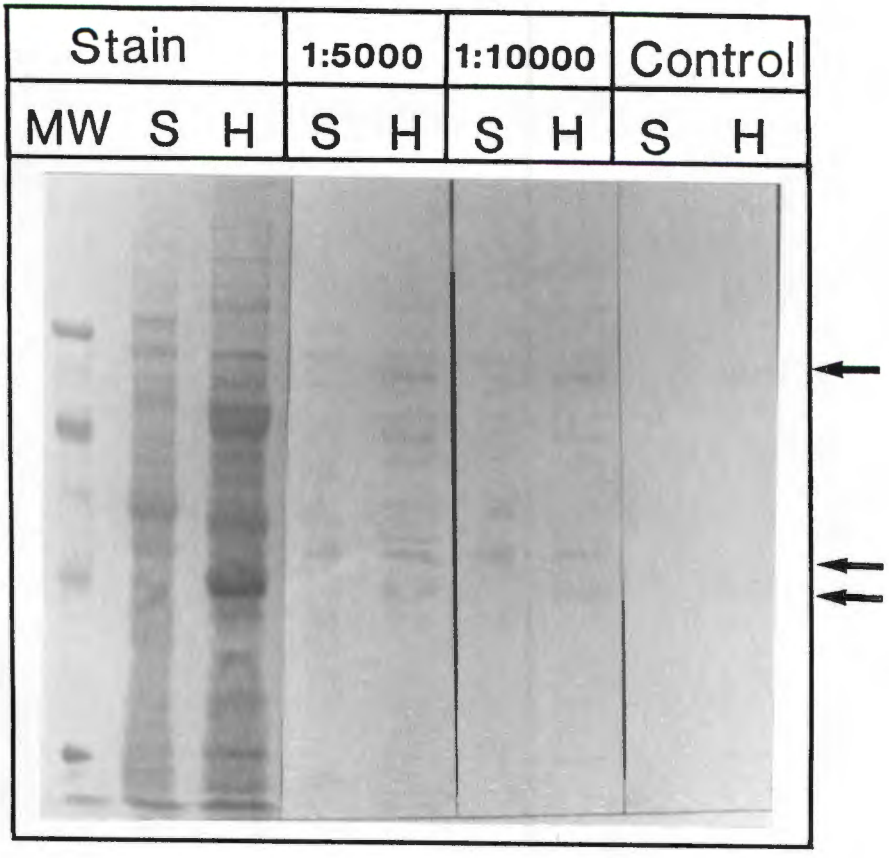


Fig. 4.5 Reactivity of human mAbs with crude extracts of human heart and with sonicates of group A streptococci.

Crude extracts of human heart **(H)** (section 7.A.11) and sonicates of group A streptococci **(S)** (section 7.A.30), were separated by 10 % SDS PAGE and immunoblotted against the human mAbs MA6 and MB4 at ascitic fluid dilutions of 1:5000 and 1:10000. Neither of the mAbs were specific for any particular constituent but showed binding to several bands in both these extracts, the most prominent of which occured at mol.wts. of about 43 kD, 45 kD and 83 kD.

4.2.3.2 ELISA Inhibition Assay

The inhibition of binding of MA6 (1:600 of ascites) to heart antigen in ELISA, was measured following absorption of the antibody with increasing concentrations of *S.pyogenes* homogenate.

Fig. 4.6 shows a proportional decrease in binding of MA6 to heart extracts in the ELISA, following pre-absorption of the mAb with a dilution range of *S.pyogenes* absorbant (undiluted: E_{470} 5.0) (section 7.A.30). It was calculated that a 1:8 dilution of absorbant inhibited binding by 74%, and a 1:16 by 31%, with no further inhibition after a 1:128 dilution of absorbant.

4.2.4 BINDING TO NUCLEAR ANTIGENS

Antibodies to nuclear antigens have been found in several autoimmune disease (Chapter 1 and section 2.2.4.1).

mAb MA6 was tested for reactivity against nuclear antigens in the HEp-2 cellular immunofluorescence system (Behring Diagnostics) (section.7.A.7), as well as by immunoblotting against nuclear extracts of Girardi and primary culture heart cells (section.7.A.15).

There was no evidence of binding to nuclear antigens in the HEp-2 immunofluorescence assay and binding to multiple bands in the immunoblotted nuclear extracts was seen.

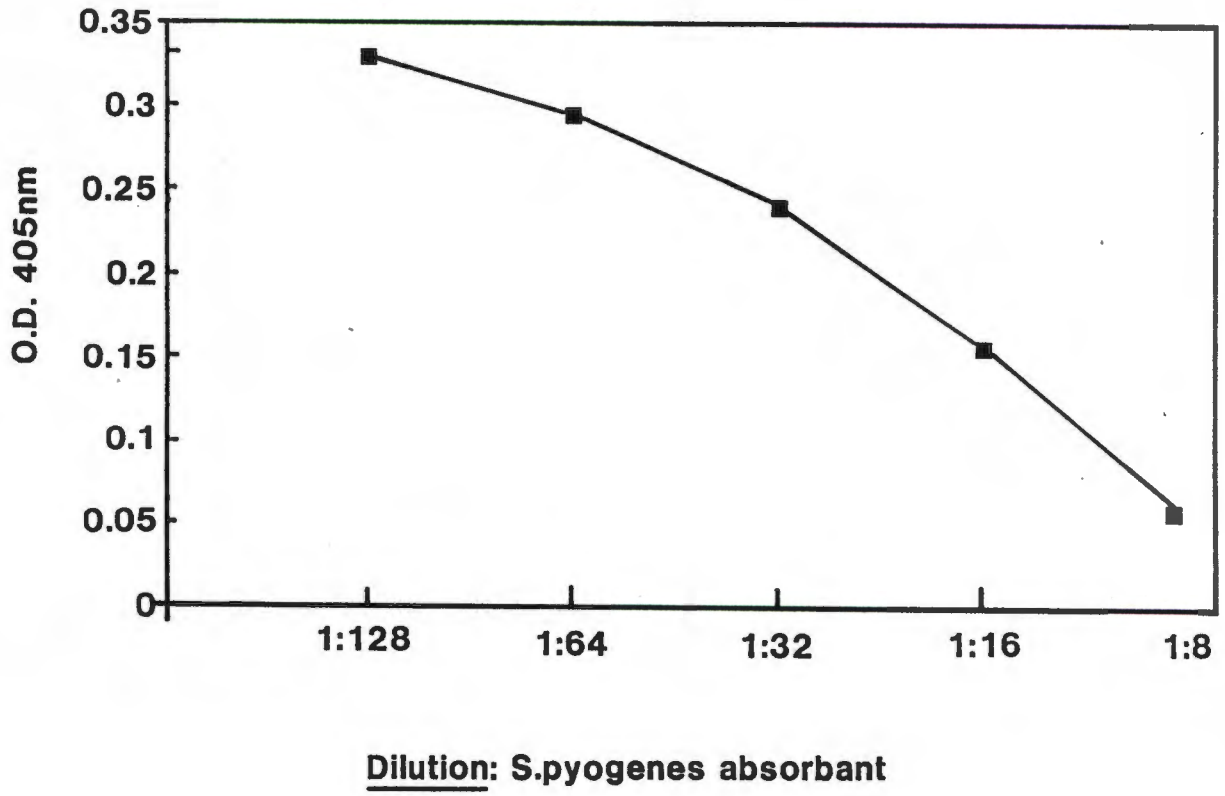


Fig. 4.6 ELISA inhibition assay: inhibition of binding of human mAb MA6 to human heart extracts in ELISA, following preabsorption with sonicates of group A streptococci

The graph shows a result of a typical ELISA inhibition experiment, in which a 1:600 dilution of MA6 was absorbed with a dilution range of a sonicate of *S.pyogenes* (undiluted concentration: E_{470} 5.0) and then incubated in ELISA against human heart extract (section 7.A.6.2). At a 1:8 dilution of the streptococcal absorbant, binding of MA6 to the heart extract was inhibited by 74%.

4.3 DISCUSSION

4.3.1 Characterization of Monoclonal Antibodies

4.3.1.1 Stability of Heterohybridomas

Because of their instability, many human hybridomas have not been adequately characterized and their specificity is therefore unclear. This seems particularly true of mAbs raised to autoantigens (James and Bell, 1987).

In this study, difficulty was experienced in producing stable antibody-producing heterohybrids, despite early and repeated subcloning. The use of PBL as cell fusion partners may have contributed to this effect, since these lymphocytes give rise to fewer antibody-secreting clones than lymphocytes from other tissues (Lagace and Brodeur, 1985). Peripheral blood appears to contain fewer antigen-specific B cells in an appropriate state of differentiation, and possibly the young age (4 years) of the PBL donor patient used in this study may have exacerbated this shortcoming.

Another source of instability in heterohybrids is that they appear to lose regulatory genes and segregate human chromosomes (Kozbor and Croce, 1985). The few human compared to mouse chromosomes found in the heterohybrids of the present study (Fig.4.1c) concurs with findings of other studies in which five times as many mouse as human chromosomes were found in heterohybrids (Raison et al., 1982). The lambda light chain phenotype of MA6 also fits in with the finding of Croce et al. (1980) that heterohybrids usually lose chromosome 2 (coding for the kappa light chains) and retain chromosome 14 (coding for the heavy chain).

Most studies however tend to favour a loss or defect in regulatory genes (or some other aspect of regulation) rather than a loss of structural genes, as the main reason for the instability of heterohybrids (Raison et al., 1982; Schwaber et al., 1984). The presence of such a regulatory defect was shown in the case of a non-secreting mouse/human heterohybrid that retained the heavy chain chromosome (No. 14) and could be stimulated (with lipopolysaccharide) to re-express immunoglobulin (Raison et al., 1982). The problem with many hybridomas appears to be one of defective antibody synthesis and secretion (Winger et al., 1983; Sikora et al., 1983), and examination of the cytoplasmic immunoglobulin of hybridoma MA6 showed some evidence of what may possibly be aberrant immunoglobulin synthesis.

The morphology of human hybridomas often shows structural abnormalities, such as poorly developed rough endoplasmic reticulum and the presence of free polyribosomes, which could be responsible for these defects in immunoglobulin production (Kozbor et al,, 1983; Sikora et al., 1983; Larrick et al., 1983). Although the morphology of hybridoma MA6 was not examined in this study, the 46 kD cytoplasmic protein in immunoprecipitates of the cell lysates, may represent a product of aberrant immunoglobulin synthesis. This protein was noticeably smaller than the 51 kD protein in the PWM control, thought to correspond to the heavy chain of IgG (Fig.4.2). Hybridoma cells have been known to express more than one antibody isotype, and cells expressing up to seven have been detected (Male et al., 1987). Whether this smaller molecule represents a product of defective IgG synthesis is not certain, but defects such as these may explain the instability of heterohybridomas.

The growth of the hybridomas only in nude (athymic) mice but not in BALB/c mice, may be due to the expression of the mouse H-2 haplotype but nonexpression of the human HLA antigens which would otherwise have led to rejection in nude mice (Raison et al., 1982).

Finally, the decrease with time in reactivity of MA6 antibody against *S.pyogenes* in ELISA could not be attributed to artefacts of prolonged storage of antigen-coated plates (Viljanen and Punnonen, 1989), since freshly coated plates displayed the same decreased reactivity.

4.3.1.2 Cross-reactivity/nonspecificity of Binding

These mAbs reacted with a wide range of antigens and were therefore probably nonspecific. Reasons for this nonspecificity may be the following. First, they were selected during screening for cross-reactivity between *S.pyogenes* and heart antigens. Second, the hybridomas were derived from a young patient whose antibodies might have been generally more cross-reactive than those of an older patient. Studies by Klinman et al. (1988) have shown that the expressed B cell repertoire of 1-to 5 week old mice produced antibodies generally more cross-reactive than those of older mice, and newborn mice have been reported to have very high frequencies of natural autoantibodies (Dighiero et al., 1985). If these findings pertain to humans, then the young age of the patient may have contributed to cross-reactivity of these mAbs. Third, these multivalent mAbs, because they were of the IgM class, would have a greater potential for nonspecific cross-reactive binding. Fourth, the nonspecificity of these mAbs may have been exacerbated by assays in which they were exposed to high densities of antigen (such as occur in crude extracts or in immunocytochemistry) with many potentially cross-reactive epitopes (Lampson and Fisher, 1985). Fifth, cross-reactivity could be the result of binding to an extremely common epitope.

4.3.2 Anti-Streptococcal Antigen Reactivity

The cross-reactivity of MA6 to other groups of streptococci (Table 4.2), is another indication of its lack of specificity.

Cunningham et al. (1984) found that although murine mAbs evoked by *S.pyogenes* also reacted with other groups of streptococci in ELISA, the reaction against group A streptococci was the strongest. This stronger reaction is perhaps attributable to the active immunization of mice with *S.pyogenes*, compared to natural infection in the case of the human mAbs. In this study the lower binding of monoclonal MA6 to group A streptococci, is reflected in the serum reactions (IgG and IgM) of the donor patient (M) which also showed higher reactivity to other groups of streptococci (Table 4.2). The human mAbs developed by Cunningham et al. (1988) were not tested for reactivity against other groups of streptococci. (Absorbance readings in the ELISAs of the present study were low compared to the those obtained by Cunningham and coworkers, since both the no-serum and the no-antigen control readings were subtracted from each value, and because peroxidase rather than the more sensitive alkaline phosphatase conjugates were used).

In addition to cross-reacting with other groups of streptococci, MA6 also displayed intense reactivity in immunoblotting with two *E.coli* antigens. Such cross-reactivity between mAbs (murine and human) and *E.coli* antigens (especially ß-D-galactosidase) has been noted by other investigators (Golding et al., 1987; Wallis et al., 1989).

4.3.3 Reactivity with Human Heart Antigens

The 43 kD constituent present in both crude heart extract and in sarcolemmal membranes with which both MA6 and MB4 reacted (Fig.4.3), may correspond to a constituent of the same mol.wt. in saline extracts of bovine heart that reacted with ARF patient sera (Zabriskie and Friedman, 1983). Possibly this band corresponds to actin (42 kD) since there is some evidence that ARF patients have anti-actin antibodies (Chapter 2). This constituent also occurs in a region of reactivity (40-and 43 kD) in Triton X-100 and SDS extracts of heart, with which rabbit immune sera to M5 and M25, as well as murine mAbs to M5 proteins, have been found to react (Cunningham and Russell, 1983; Cunningham et al., 1984; Sargent et al., 1987; Dale and Beachey, 1985a) (Chapter 3 and Table 3.1). (Neither MA6 nor MB4 reacted with M5 by immunoblotting, although they might have displayed reactivity in ELISA).

The fact that neither MA6 nor MB4 were specific for heart, but bound also kidney antigens, is further evidence of their nonspecificity. Serum antibodies in rheumatic fever however are not organ specific but bind components in joints, caudate nucleii, heart muscle, connective tissue and blood vessels (Zabriskie, 1985).

Likewise, binding of MA6 and MB4 to phosphorylase b, albumen and carbonic anhydrase may be irrelevant cross-reactive binding. Cross-reactive 4-7 amino acid sequence determinants would quite likely be present in these highly conserved proteins (Atassi, 1975: see Chapter 1). The cross-reaction with phosphorylase b however could possibly be important, since both in this study (Chapter 2) and in others (James Dale - personal communication to D W Beatty, 1987), ARF sera have been found to contain antibodies to this protein. Natural autoantibodies to albumen have been described by Dighiero et al. (1982) and others. There are no reports of autoantibodies to carbonic anhydrase. Thus, although autoantibody binding to these proteins is known, these mAbs are not specific for any of them.

These human mAbs do not therefore appear to fit in with either of the groups of reactivity displayed by human mAbs developed by Cunningham et al. (1988), namely those reacting with (1) myosin and DNA, or (2) myosin, keratin and/or actin. Possibly the strong binding of these mAbs to the 43 kD constituent in crude heart extracts and sarcolemmma (Fig.4.3), may be significant and merit further investigation.

4.3.4 Cross-reactivity: Group A Streptococcus and Heart

4.3.4.1 Immunoblotting

The recognition of constituents of similar mol.wt. in heart extract and *S.pyogenes* sonicates (Fig.4.5), is no proof of cross-reactive binding. Interestingly, the major constituents (43 kD 45 kD and 83 kD) do correspond in mol.wt. to components of *S.mutans* (42 kD, 46 kD and 82 kD) that react with rabbit sera raised against extracts of human heart (Ayakawa et al., 1985). *S.mutans* is known to cross-react with human heart (Van de Rijn et al., 1976), and Ayakawa et al. (1988) used murine mAbs to show that *S.mutans* and *S.pyogenes* share immunodeterminants. Cunningham et al. (1984) described a 43 kD component in *S.pyogenes* M5 homogenates to which murine mAbs evoked by M5 membranes bound; and ARF sera also recognise a 43 kD constituent of heart (Zabriskie and Friedman, 1983).

In summary, there is some evidence of constituents in extracts of heart and *S.pyogenes*, that are of similar mol.wt. and may contain cross-reactive epitopes.

4.3.4.2 ELISA inhibition Assay

The inhibition of binding of MA6 to heart extract antigen in ELISA by *S.pyogenes* absorbent, suggests a possible sharing of epitopes in these two extracts that are recognised by this mAb. Since these are crude extracts there is an increased likelihood, in this instance too, that these reactions may be nonspecific. Absorption experiments using purified proteins would provide a clearer indication of shared reactivity between such proteins and constituents in the heart extract.

4.3.5 Nuclear Antigen Reactivity

Neither MA6 nor MB4 were positive in the HEp-2 immunofluorescence assay, and therefore do not appear to be antinuclear antibodies, and the multiple binding pattern they displayed to heart cell nuclear antigens, appeared to be nonspecific.

4.4 CONCLUSIONS

1. Two human IgM mAbs displaying cross-reactivity between human heart and *S.pyogenes* antigens were isolated from heterohybridomas developed by the fusion of mouse myeloma cells and the lymphocytes from an ARF patient. Several difficulties pertaining to the instability of heterohybridomas were experienced in the process of cloning them.

2. The mAbs were not specific for group A streptococci but cross-reacted with other groups of streptococci as well as with *E.coli* proteins.

3. The mAbs were not specific for heart antigens but reacted with antigens in other organs as well.

4. Both monoclonals reacted strongly with 43 kD constituents in crude extracts of human heart and in purified cardiac sarcolemmal membranes. Antigens of a similar mol.wt. have been found by other investigators in different preparations of heart extract, using ARF sera, mAbs and rabbit immune sera raised to *S.pyogenes* antigens. This constituent may possibly therefore represent an autoantigen pathologically relevant to rheumatic fever, and may merit further investigation.

5. The reactivity of MA6 with phosphorylase b may be important since ARF sera were also found to contain antibodies reactive with this protein (section 2.2.2.4).

6. ELISA inhibition experiments using crude extracts of heart and *S.pyogenes* suggested the possibility of cross-reactive antigens in these extracts, but these findings should be verified using defined purified proteins. (Immunoblotting of MA6 against these extracts showed a multiple binding pattern of reactivity).

7. These mAbs were not specific for any particular antigen in nuclear extracts of heart cells, nor did they display reactivity with nuclear antigens by immunofluorescence.

8. These mAbs lacked specificity and their usefulness as probes for analyzing antigenic mimicry in rheumatic fever (or for screening cDNA expression libraries of heart cell genes - Chapter 5), is therefore in doubt.

5. CONSTRUCTION AND IMMUNOSCREENING OF λGT11 cDNA EXPRESSION LIBRARIES

5.1 INTRODUCTION 148

5.2 RESULTS 150
5.2.1 cDNA Synthesis and Construction of λgt11 Libraries 150
5.2.1.1 RNA Extraction 150
5.2.1.2 cDNA Synthesis 150
5.2.1.3 Cloning of cDNA into Bacteriophage λgt11 153
5.2.1.4 Analysis of cDNA Inserts 155
5.2.2 Screening of λgt11 cDNA Expression Libraries with Antibodies 155
5.2.2.1 Expression of Recombinant Fusion Proteins 158
5.2.2.2 Detection of Clones Reactive with ARF Patient Serum 158
5.2.2.3 Analysis of Positive Clones 161
5.2.2.3.1 Size Analysis cDNA Inserts 161
5.2.2.3.1 Size Analysis of Fusion Proteins 166
5.2.3 Reactivity of Fusion Proteins with Sera 166
5.2.3.1 Purified Plaque Assay: Specificity for ARF Sera 166
5.2.3.2 Fusion Proteins: Immunoblots against ARF and Normal Sera 170
5.2.3.2.1 Immunoblots of Bacterial Lysogen Lysates 170
5.2.3.2.1 Affinity Purification of Fusion Proteins 172
5.2.3.3 Plaque Selected Antibodies: Cross-reactivity with Streptococcal Antigens 172
5.2.4 DNA Sequence Analysis of Positive Clones 174

5.3 DISCUSSION 177
5.3.1 cDNA Synthesis and Construction of λgt11 Libraries 177
5.3.2 Screening of Libraries and Detection of Positive Clones 177
5.3.3 Characterization of Positive Clones 178
5.3.3.1 Specificity of Positive Fusion Proteins 178
5.3.3.1.1 Specificity of Purified Plaques for ARF Sera 178
5.3.3.1.2 Immunoblots of Bacterial Lysogen Lysates 179
5.3.3.1.3 Cross-reactivity of Plaque-Selected Antibodies with Streptococcal Antigens 179
5.3.3.2 cDNA Sequence of Positive Cloned Inserts 180

5.4 CONCLUSIONS 183

5.1 INTRODUCTION

This study presents a molecular approach to the identification of heart antigens implicated in rheumatic fever.

Compared to immunofluorescence by which Kaplan (1963) demonstrated heart-reactive antibodies in rheumatic fever, ELISA and immunoblotting (Chapters 2 and 3) are superior techniques. The latter methods are both exquisitely sensitive, and in immunoblotting the response to separate antigenic components can be measured. However, despite these advances, the heart antigens in rheumatic fever remain poorly defined, with the possible exception of myosin. Table 3.1 presents antigens of a wide range of mol.wts. that have been implicated in rheumatic fever. Results presented in the immunoblotting study (Chapter 3) showed evidence that ARF sera react with at least eleven major constituents present in a crude extract of human heart.

Molecular cloning offers a more precise approach to the definition and characterization of autoantigens (and specific epitopes) in rheumatic fever, and to the analysis of cross-reactivity with streptococcal antigens. The feasibility of using autoantibodies from patients in order to isolate cDNA, and ultimately genes, corresponding to poorly defined human antigens, has been demonstrated for several autoimmune and infectious diseases. Examples include autoimmune thyroid disease (Hirayu et al., 1987), bullous pemphigoid (Stanley et al., 1988), connective tissue diseases (Habets et al., 1987; Netter et al., 1988), Hashimoto's thyroiditis (Kaufman et al., 1989), enterococcal endocarditis (Burnie and Clark, 1989), Sjögren's syndrome (Chambers et al., 1988) and SLE (Chambers and Keene, 1985).

In rheumatic fever, the amino acid sequences of several of the streptococcal M proteins have been known for some time (Beachey et al., 1978). The antigenicity of these sequences and the homologies between them have been analyzed (Beachey et al., 1983; Miller et al., 1988; Hollingshead et al., 1987), and types M6, M12, M5 and M24 have been cloned (Fischetti et al., 1983; Scott and Fischetti, 1983; Spanier et al., 1984; Kehoe et al., 1985; Mouw et al., 1988). Poirier et al. (1985) were able to show that expressed polypeptides of the cloned M5 protein cross-react with human heart sarcolemmal membrane antigens.

Most of the molecular cloning analyses in rheumatic fever have concentrated on the streptococcal antigens rather than on the human cardiac antigens. Knowledge of the amino acid sequences of the streptococcal M proteins allowed for synthesis of oligonucleotide probes thereby greatly facilitating cloning of the genes. This probably explains why most of the effort has been concentrated in this area.

To identify the cardiac antigens, without knowledge of their protein sequence or structure, the best approach was to use antibodies to screen a cDNA expression library of human heart cell genes. The purpose of this study was to construct such a cDNA library, and to screen it for antigens cross-reactive with group A streptococcal antigens, using three types of antibody probe: (1) ARF patient sera; (2) murine and human monoclonal antibodies to *S.pyogenes* antigens; (3) rabbit hyperimmune sera to various *S.pyogenes* antigens. Of the three types of antibody probe, patient sera were considered the most relevant to the disease.

This study describes the construction of two λgt11 cDNA libraries derived from human heart mRNA sequences, their screening using an ARF patient serum, and the isolation and characterization of three positive clones.

5.2 RESULTS

5.2.1 cDNA SYNTHESIS AND CONSTRUCTION OF λGT11 LIBRARIES

5.2.1.1 RNA Extraction

RNA for cDNA synthesis was extracted from human heart tissue using the method of Chomczynski and Sacchi (1987).

Since it was important to construct a highly representative cDNA library for immunoscreening, careful attention was paid to isolating high yields of undegraded RNA. Heart tissue obtained at surgery was snap-frozen in liquid nitrogen within 30 seconds of excision and pulverized under liquid nitrogen. RNA was isolated as described in Materials and Methods (section 7.B.1.1). Instruments and utensils used in the isolation were meticulously treated against ribonucleases according to the recommendations of Blumberg (1987).

Only RNA preparations which showed no signs of degradation, as judged by the intactness of the 18S and 28S ribosomal RNA bands on formaldehyde-agarose gels (section 7.B.5.1) were considered for purification of poly $(A)^+$ RNA (section 7.B.1.2). The yields and purity of the RNA were measured spectrophotometrically. Only relatively pure extracts, with $A_{260/280}$ ratios between 1.8 and 2.0, were used.

Concentrations of RNA extracted from atrial appendage of heart were generally not as high per gram of tissue as could be expected. Extractions were therefore also made from a heart removed at transplant surgery (from a patient with no history or clinical evidence of rheumatic fever). Not only were higher yields obtained from this tissue, but pieces of tissue from different parts of the heart could be included in the extraction, thus potentially enhancing the range of protein-coding sequences in the cDNA library.

5.2.1.2 cDNA Synthesis

cDNA was synthesized by three different methods in this study. These were: (1) the S1 nuclease method, in which the 3'-end loop of the first strand is used as a primer for second strand synthesis before being digested away using S1 nuclease; (2) the RNAse H method, in which RNA in the RNA:DNA hybrid is nicked to create primers for second strand synthesis;(3) the random priming method, in which the first strand is primed randomly from several internal positions within the RNA molecule rather than from the 3'-end, as in the former two methods.

cDNA synthesized by these methods was used to construct two expression libraries in bacteriophage λgt11. *Library A* was a pool of three libraries each

constructed from cDNA synthesized by a different one of the three methods. Such a pooled library, it was thought, would possibly be more representative of the total population of heart mRNA sequences, and any bias towards a particular population of cDNA, as a result of its method of synthesis, would be reduced. *Library B* was constructed from cDNA synthesized by the RNAse H method (which was found to be the most efficient method), using mainly reagents and procedures prescribed by Promega Biotec (Madison, USA). Results of synthesized cDNA used in these two libraries are summarised in Table 5.1 below.

TABLE 5.1:

Yields of cDNA Used in Construction of λgt11 Libraries

cDNA METHOD	mRNA μg	1st cDNA strand ng	2nd cDNA strand ng	% mRNA into 1st strand	% 1st strand into 2nd	TOTAL cDNA ng
I. LIBRARY A:						
SI Nuclease	2	476	310	23.8%	65%	620
RNAse H	2	610	536	30.5%	88%	1072
Random primers	2	250	242	12.5%	96%	484
II. LIBRARY B:						
RNAse H	4.1	952	856	23.2	98	1712

cDNA was prepared for cloning into λgt11 by methylation with EcoR I methylase, the addition of EcoR I linkers and subsequent digestion with EcoR I as described in Materials and Methods (section 7.B.2). These steps in the cloning procedure are depicted in Fig.5.1.

Yields of cDNA were substantially reduced following chromatography through Sepharose CL-4B (to remove excess EcoR I linkers and cDNA molecules smaller than 500 bp in length). These reduced yields were due to removal of smaller molecules, as well as to probable losses incurred during manipulations of the preceding reactions. Table 5.2 shows amounts of cDNA available, following linker addition, for cloning into λgt11.

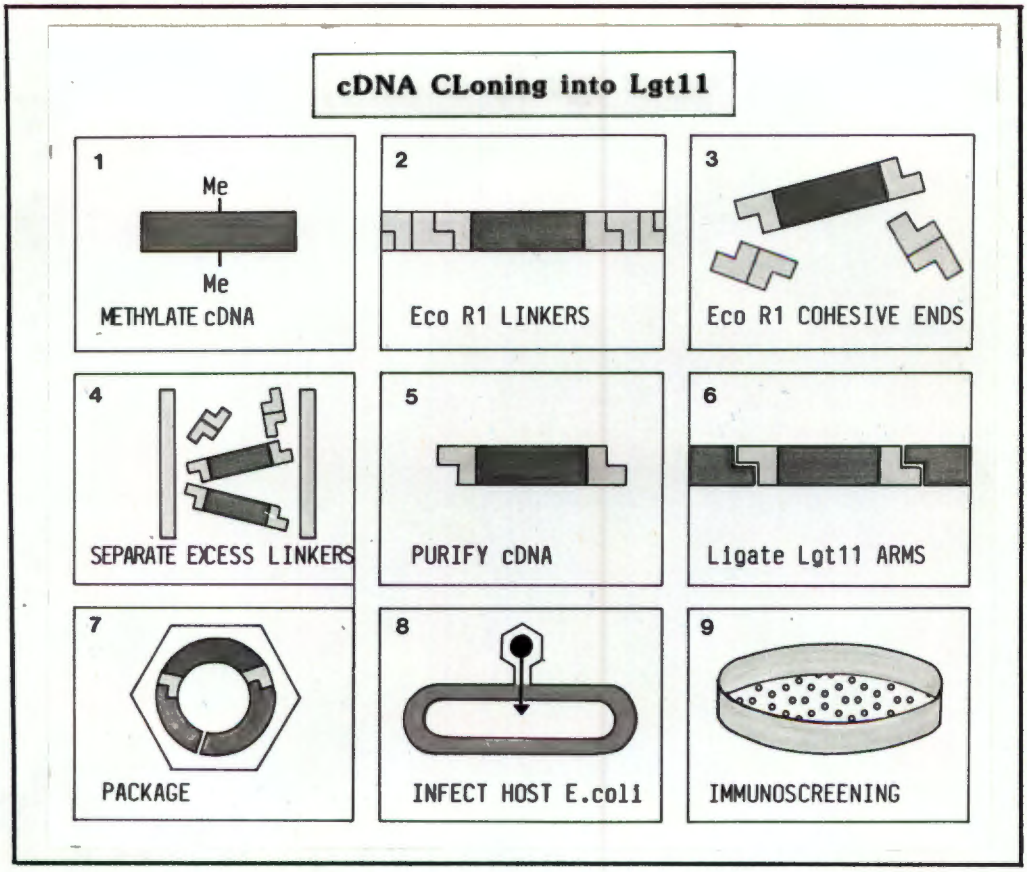


Fig. 5.1 Schematic presentation of cDNA cloning into λgt11 (Adapted from Amersham Research News Issue 5).

The procedures involved in the cloning are described in section 7.B.2 of Materials and Methods.

TABLE 5.2:

Amounts of cDNA used for Cloning Following EcoR I Linker Addition and Chromatographic Purification

cDNA METHOD	ng of cDNA
(Library A):	
S1 Nuclease	210
RNAse H	452
Random primers	194
(Library B):	
RNAse H	765

5.2.1.3 Cloning of cDNA into Bacteriophage λgt11

To determine the optimal cloning efficiency for each particular cDNA population, a range of quantities of each (25ng, 50ng, 100ng) was ligated into dephosphorylated λgt11 vector arms, according to the scheme (see Table 7.2.6) and procedures described in Materials and Methods (section 7.B.2.6). Ligation mixtures were packaged using Gigapack Plus packaging extracts (Stratagene), and the resultant libraries were titrated on agar plates in the presence of IPTG and X-gal for colour selection of recombinants (section 7.B.2.8). These cloning procedures are depicted in Fig.5.1.

Cloning efficiencies of the plated libraries were calculated as follows:

$$\text{Cloning Efficiency} = \frac{(\text{Number of recombinant plaques} \times 1000)}{\text{ng of cDNA used}}$$

Table 5.3 presents the cloning efficiencies of the optimal amounts of cDNA population ligated into λgt11.

TABLE 5.3:

λgt11 Library Cloning Efficiencies of Each cDNA Population

DNA Insert & amount	Library titre total pfu	% recombinants pfu/μg λgt11	Cloning efficiency pfu/μg cDNA
I. LIBRARY A:			
SI Nuclease (50 ng)	7.1×10^6	85%	1.4×10^8
RNAse H (50 ng)	8.5×10^6	89%	1.7×10^8
Random Primers (25 ng)	1.6×10^6	79%	6.4×10^7
II. LIBRARY B:			
RNAse H (50 ng)	8.1×10^6	94%	1.6×10^8
III.TYPICAL CLONING EFFICIENCIES OF CONTROLS:			
whole λgt11	6.1×10^8	-	-
λgt11 arms	5.1×10^3	-	-
Ligation control DNA (100ng)	1.2×10^7	84%	1.2×10^8
Cloning Control DNA (50ng)	9.1×10^6	78%	1.8×10^8

The control wild type λgt11 packaging reaction in all cases yielded virtually only blue non-recombinant plaques on plating. The relatively high titre of a typical reaction shown above was a reflection of the general efficiency of the packaging reaction (6.1×10^8 pfu/μg DNA). By comparison, packaging of the dephosphorylated λgt11 vector arms without any DNA insert, yielded low titres (typically between 10^3 and 10^4 pfu/μg arms) reflecting the generally low background of the cloning reactions.

The ligation control showed that the dephosphorylated λgt11 arms were efficient DNA cloning vectors, whereas the cloning control gave an indication of the efficiency for these cloning procedures of a non-cDNA blunt-ended

insert. (These control reactions were important in preliminary experiments for optimizing the cloning procedures).

5.2.1.4 Analysis of cDNA Inserts

Ten recombinant clones from each library were analysed for the range of their cDNA insert sizes. DNA was extracted from liquid (or plate) lysates (section 7.B.2.10.1), and separated on 1.5% agarose gels.

Fig. 5.2 (b) shows DNA extracted from recombinant clones in *library-A* and digested with Kpn I and Sac I. The EcoR I cloning site in λgt11 is located in a 2 kb Kpn I/Sac I fragment (see Fig.5.3). Clones containing inserts therefore show variously larger sizes of this fragment. The ten clones shown contained inserts ranging in size from 0.5 to 1.1 kb. Additional bands seen in some recombinants (lanes 6-9) are the consequence of partial digestion by the restriction enzymes.

Recombinant clones from *Library-B* were digested with restriction endonuclease Mlu I. Fig. 5.2 (a) shows ten such digested recombinant clones from this library separated on a 1.5% agarose gel and stained with ethidium bromide. The cloning site is contained in a 2.0 kb Mlu 1 fragment in the wild type λgt11 and thus varies in size among the recombinants (Fig. 5.3). The size range of cDNA inserts for this library was from 0.2 to 1.5 kb.

These analyses confirmed that both libraries contained cDNA inserts (derived from heart mRNA sequences) of a suitable size and range for immunoscreening.

5.2.2 SCREENING OF λGT11 CDNA EXPRESSION LIBRARIES WITH ANTIBODIES

Both libraries were screened with three types of antibody reagents:

(1) sera from patients with acute rheumatic fever and chronic rheumatic heart disease (used either separately or as pooled sera).

(2) rabbit hyperimmune sera to streptococcal antigens (against *S.pyogenes*, raised as described in section 7.A.1; and antisera to the proteins SM5, SM24 and M19, gifts from Dr J. Dale, University of Tennessee, USA).

(3) a murine monoclonal antibody to the purified streptococcal M protein pep M5 (designated IB7, a gift from Dr James Dale); and a murine mAb to the ARF-associated B cell alloantigen (designated D8/17, a gift from Dr John Zabriskie, Rockefeller University, New York). The human monoclonal antibodies developed in this study (Chapter 4) were not used as they lacked specificity.

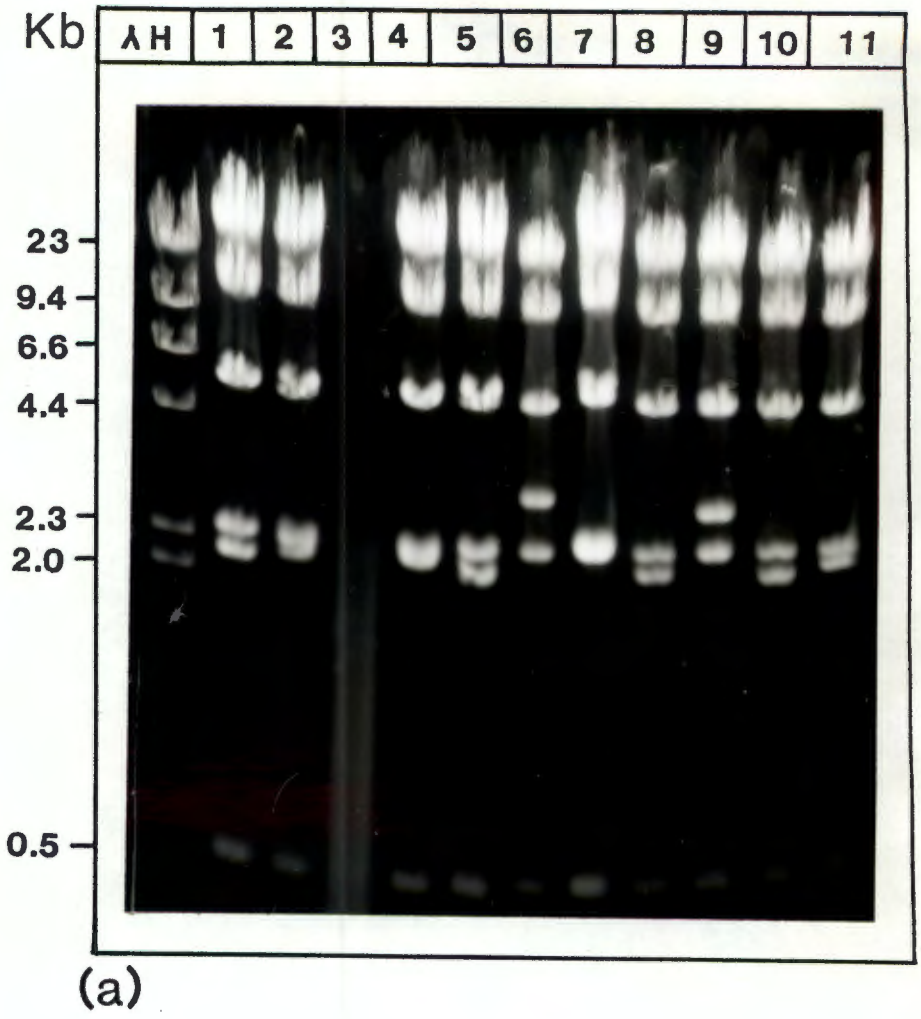
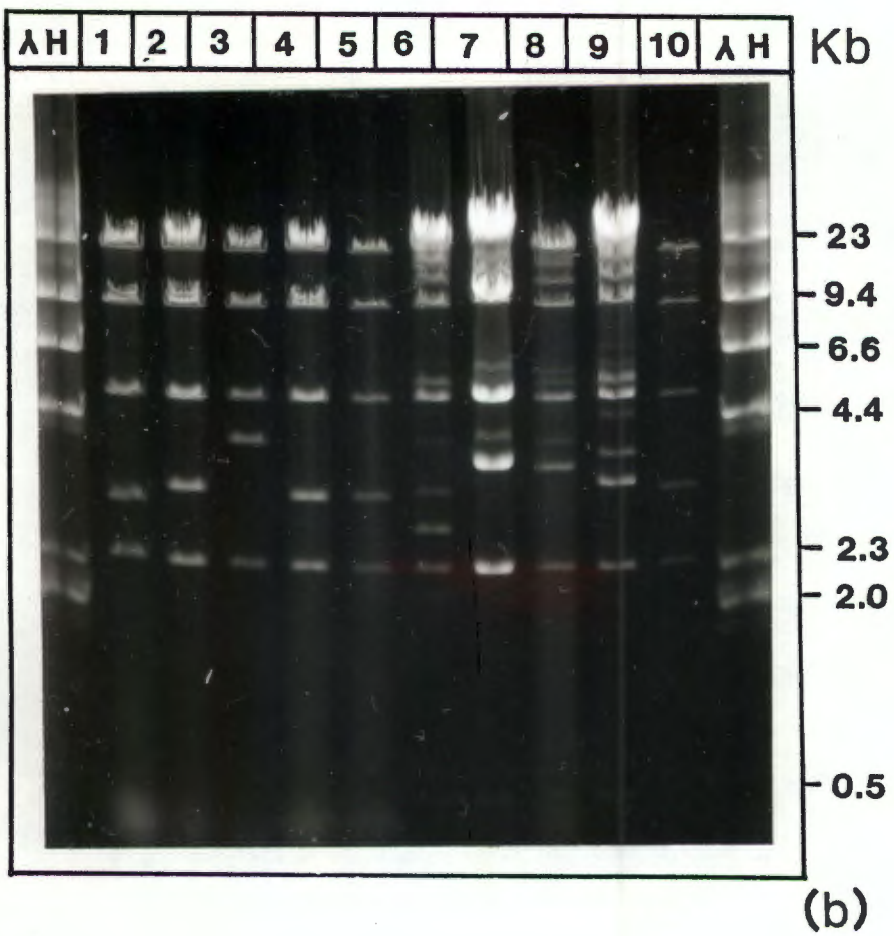


Fig. 5.2 Analysis of cDNA cloned insert sizes from kgt11 library-A and library-B

(a) *library-B*: ethidium bromide stained 1.5% agarose gel of Mlu I digested DNA extracted from 11 randomly selected recombinant clones in *library-B*. The DNA was extracted from the recombinant clones as described in section 7.B.2.10.1. The cloning site is contained in a 2 kb MluI site which thus varies in size among the recombinants (see Fig. 5.3). The size range of the cDNA inserts in the library was between 0.5 and 1.1 kb (λH:wild type lambda DNA cut with HindIII, used as markers).



(b) *library-A*: ethidium bromide stained 1.5% agarose gel of DNA extracted as in (a) above, from 9 randomly selected clones in *library-A* and digested with KpnI and SacI. The cloning site is located in a 2 kb KpnI/SacI fragment which is of variable size among the recombinants. The size range of cDNA inserts in the library was between 0.2 -and 1.5 kb. (The additional bands seen in lanes 6-9 are due to partial digestion by these low salt enzymes).

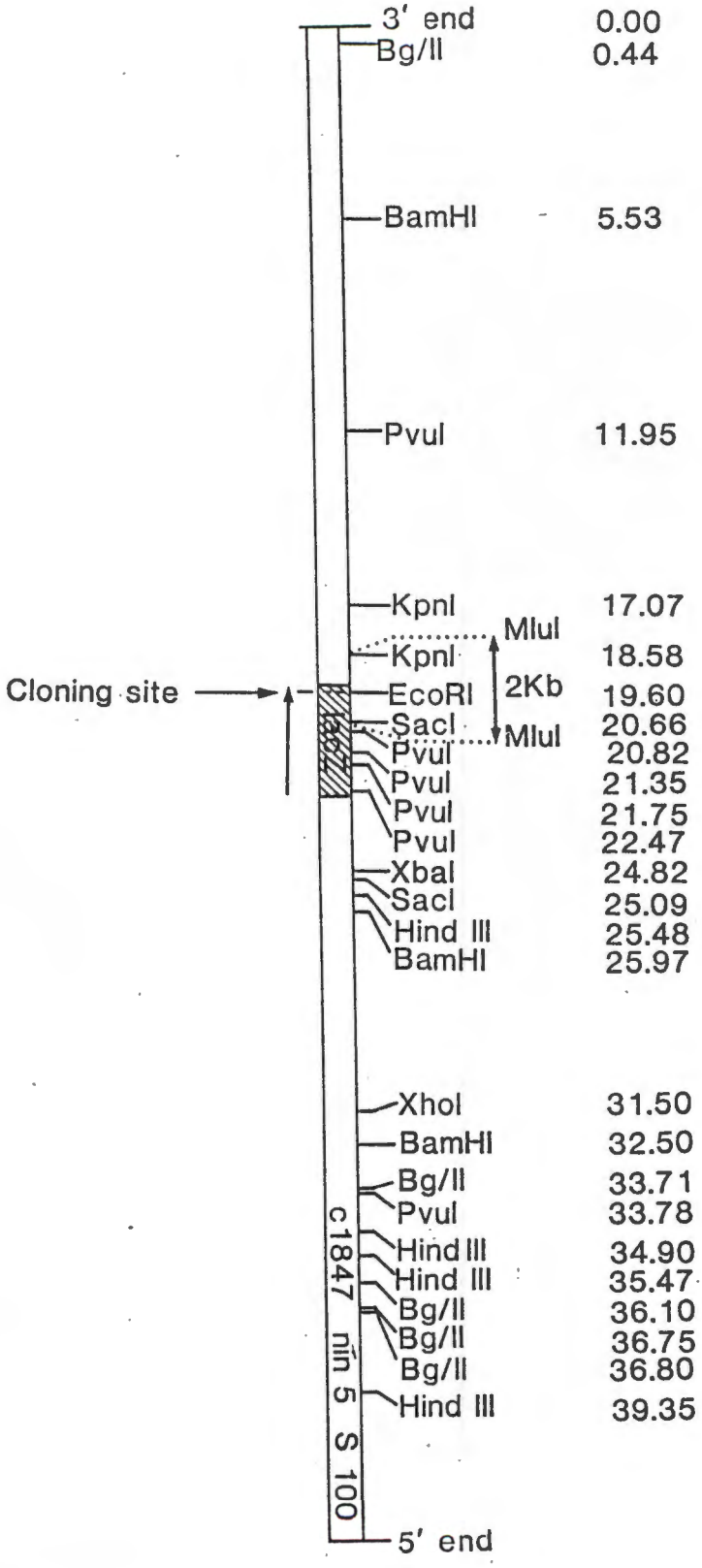


Fig. 5.3 Restriction map of λgt11.

Restriction endonuclease cleavage sites are designated in kilobase pairs from the 3' end. The EcoRI cloning site is indicated, and is flanked by the KpnI, SacI and MluI restriction sites used in size analysis of cloned cDNA inserts.

Of these antibody reagents, the patient sera were considered to be the most relevant to the disease. Three clones reacting positively with a particular ARF serum were purified to homogeneity and characterised. The detection and characterization of these clones are described here.

5.2.2.1 Expression of Recombinant Fusion Proteins

Prior to immunoscreening, both libraries were analyzed for expression of recombinant fusion proteins.

Ten recombinant clones were randomly selected from each library for isolation of lysogens (section 7.B.4.1). Phage were introduced as lysogens into *E.coli* strain Y1089 at a multiplicity of infection greater than 5. Following incubation overnight at 32°C, 25 clones of each recombinant were streaked onto each of two plates, one of which was incubated overnight at 32°C and the other at 43°C. Lysogenic clones were identified by their ability to grow at 32°C but not at 43°C.

Fig.5.4 shows the result of a representative recombinant incubated at these different temperatures. Whereas all colony streaks grew at 32°C, 10 out of 25 (40%) failed to grow at 43°C. These were identified as lysogens, and one of these from each of the ten original recombinants was selected for extraction of fusion proteins.

Fusion proteins were induced by raising the temperature of *E.coli* Y1089 lysogen cultures to 45°C for 20 minutes, then adding IPTG to 5 mM and incubating at 37-38°C for a further 45-60 minutes (section 7.B.4.1.2). Bacterial lysates of the clones were analyzed for the presence of fusion protein, by immunoblotting using a murine monoclonal antibody to ß-galactosidase.

Fig. 5.5 shows immunoblotted recombinant fusion proteins selected from *library-A*, indicating a size range of up to 162 kD compared to the 116 kD wild type ß-galactosidase protein.

These results indicated that the cDNA libraries were producing recombinant protein that was immunologically detectable.

5.2.2.2 Detection of Clones Reactive with ARF Patient Serum

A sensitive immunoscreening assay was established using an affinity-purified alkaline phosphatase conjugated goat anti-human IgG (H + L) second antibody system (section 7.B.3).

λgtII Lysogens

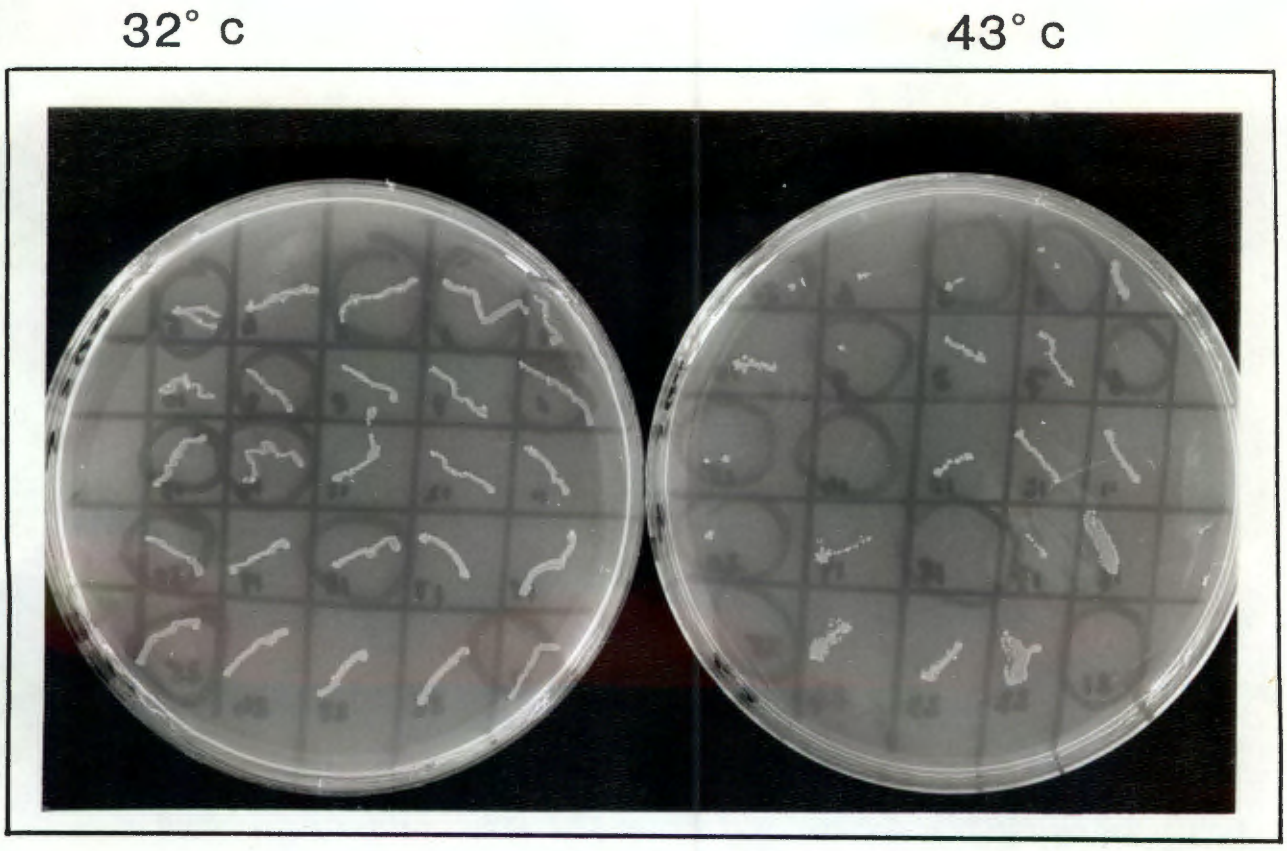


Fig. 5.4 Isolation of recombinant lysogens in *E.coli* Y1089.

Phage-competent Y1089 *E.coli* were prepared as described in section 7.B.4.1.1. Cells were infected at a m.o.i of 5 phage/cell and plated overnight at 32°C. Twenty-five colonies from the plate were randomly selected and streaked at similar locations onto two separate L-agar ampicillin plates. One plate was incubated at 32°C and the other at 43°C. Lyogenic clones were identified by their ability to grow at 32°C but not at 43°C. Such lysogens are ringed in the figure. Single lysogens were selected for isolating fusion proteins (section 7.B.4.1.2)

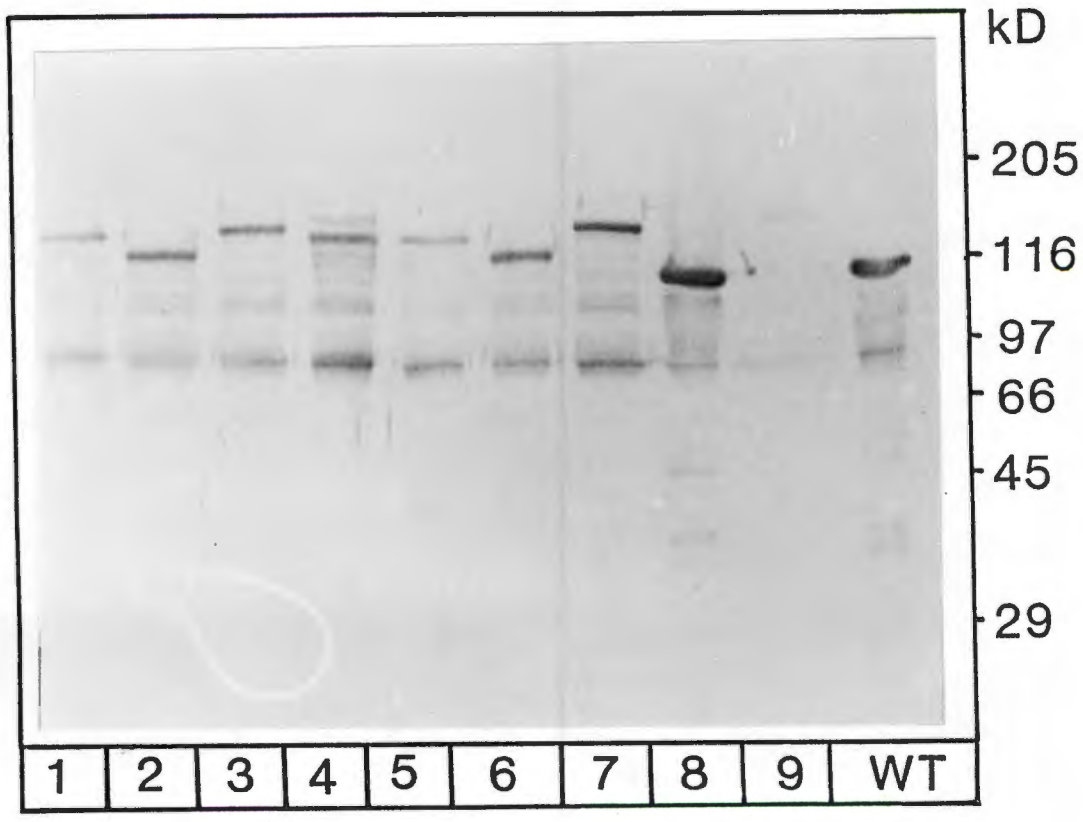


Fig. 5.5 Expression of recombinant fusion proteins in λgt11 cDNA libraries

Nine randomly selected recombinant clones from *library-A* were introduced as lysogens into *E.coli* Y1089 (section 7.B.4.1.1). Fusion proteins in recombinant lysogenic clones were induced with IPTG (section 7.B.4.1.2), and bacterial lysates of the lysogens were separated by SDS 7.5% PAGE, and immunoblotted against a murine mAb to ß-galactosidase (Promega). Positive binding was detected using a peroxidase conjugated Fab'_2 goat anti-mouse IgG (H+L) (Zymed) second antibody. Lanes 1-7 show fusion proteins from these clones of a larger mol. wt. than the 116 kD wild type (WT) non-recombinant ß-galactosidase protein. (The staining in lane 9 is faint since most of the protein was lost when the lysogen lysed during isolation).

Positive clones were detected using a highly suitable ARF serum (designated ARF-S1) at a dilution of 1:50. The patient was a twelve year old female with a *recurrent* attack of acute rheumatic fever, with severe carditis and an ASO titre of 1280 Todd units (see section 2.1.7).

Four positive clones were detected in screening twelve 150 mm plates containing about 50 000 plaques each. These clones were purified to homogeneity, but two of these clones (POS7 and POS8) were discovered to have identically-sized DNA inserts and fusion proteins, and partial sequencing of the cDNA suggested that these clones were identical. This particular positive is thus referred to in this study as POS7, POS8 or POS7/8. The other two positive clones are referred to as POS5 and POS6.

Fig. 5.6 shows the purification of one of these positive clones (POS6), from a single plaque to homogeneity. Positive binding is shown by plaques staining dark purple against the fainter plaque background. This contrasting signal to noise ratio permitted precise location of positive clones for purification.

5.2.2.3 Analysis of Positive Clones

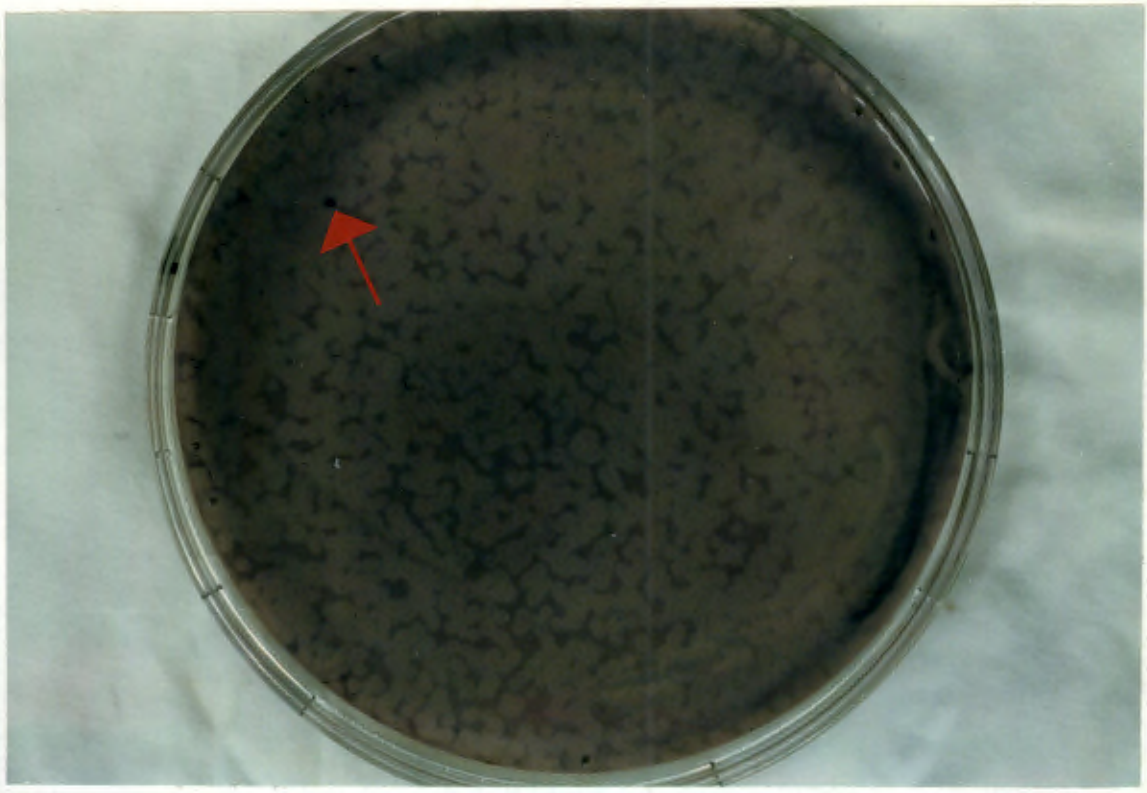
The size of the cDNA inserts, as well as the recombinant fusion proteins, in each of three positive clones was ascertained.

5.2.2.3.1 Size Analysis cDNA Inserts

Extracted DNA from each of the positives was digested with either Mlu I or with EcoR I, and fractionated on 1.5% agarose gels alongside wild type lambda DNA markers (digested with Hind III, Pvu II, or with Hind III and EcoR I).

Fig.5.7 (a) shows fractionation of the three positives digested with Mlu I indicating an insert size of 1 kb for POS5 and of 1.4 kb for POS6. The additional band visible in POS7/8 suggested that the insert contained an internal Mlu I site, and the size of the insert was therefore measured by EcoR I digestion of the DNA (see b below).

Fig. 5.7 (b) shows effective release of the cDNA inserts from each positive clone by EcoR I digestion of both λgt11 and (following subcloning of the insert for sequencing) of pUC 19 (section 7.B.4.2.1.1). This analysis confirmed the insert sizes of POS5 and POS6, and showed POS7/8 to have an insert of 1.1 kb.



a

Fig. 5.6 Isolation and purification of positive clones by immunological screening.

A positive clone (POS6) was detected in *library-A* using an ARF patient serum (ARF-S1: see section 5.2.2.2) using the screening procedures described in section 7.B.3. Recombinant phages were plated initially at a density of $5X10^4$ pfu/150 mm diameter plate (about 250 pfu/cm^2) onto 12 plates.

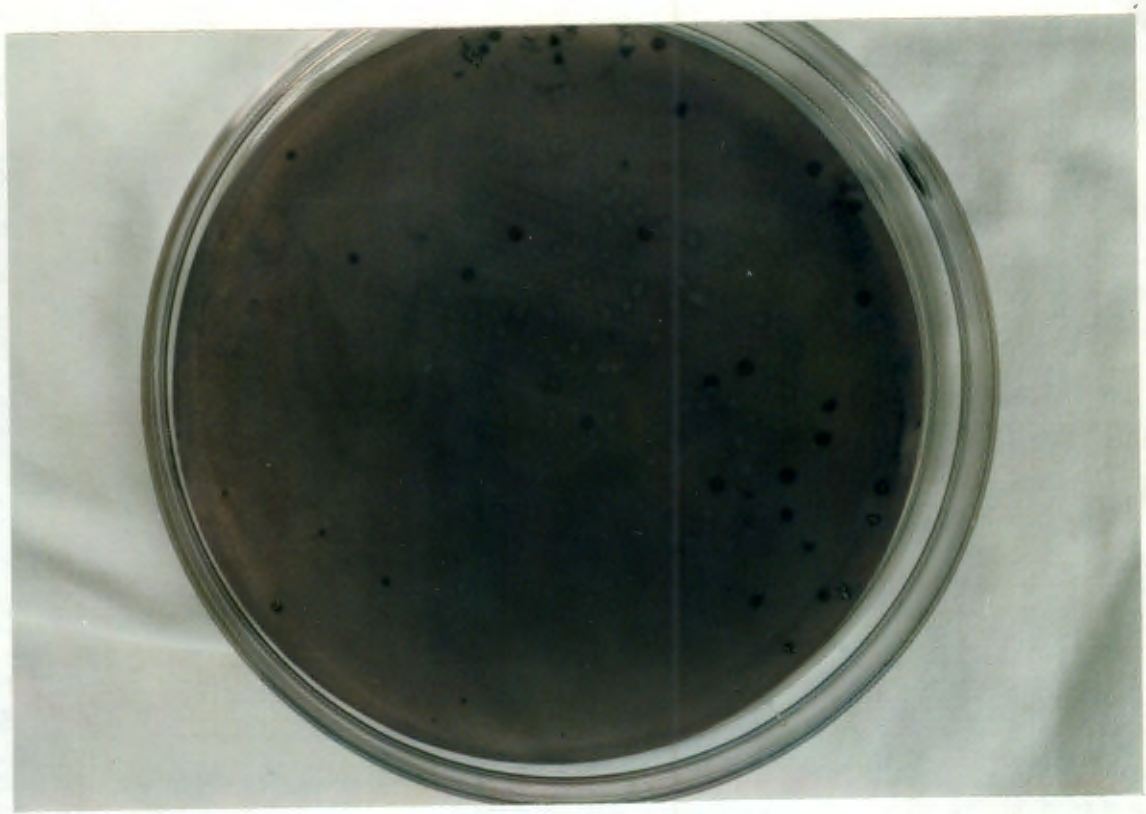
(a) a dark purple staining positive clone (arrow) was detected against the fainter background and cored out using a pasteur pipette.

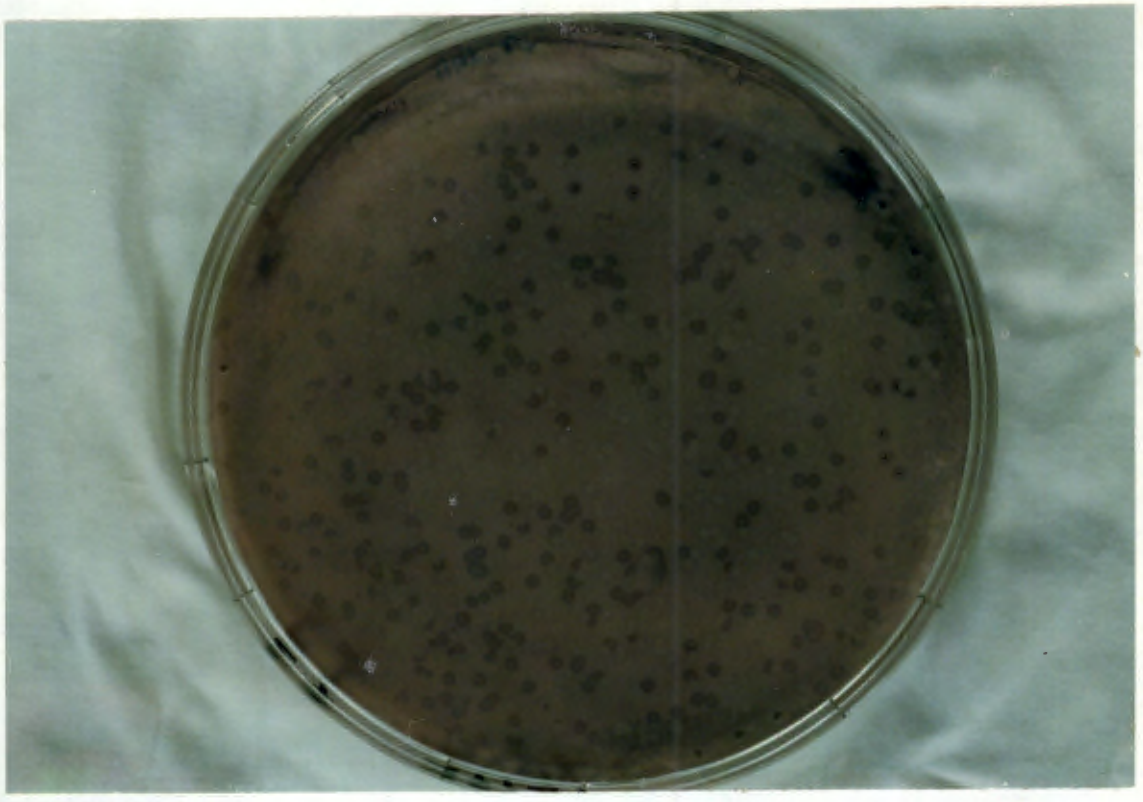
(b) Phage were eluted and replated at a density of about 200 pfu/plate and rescreened in order to isolate single clones for purification.

(c,d,e) The process of replating and rescreening was repeated until recombinant clones were purified to homogeneity, as demonstrated by the positive staining of all the plaques at high density screening **(e)**.

(continued over next 2 pages)

b

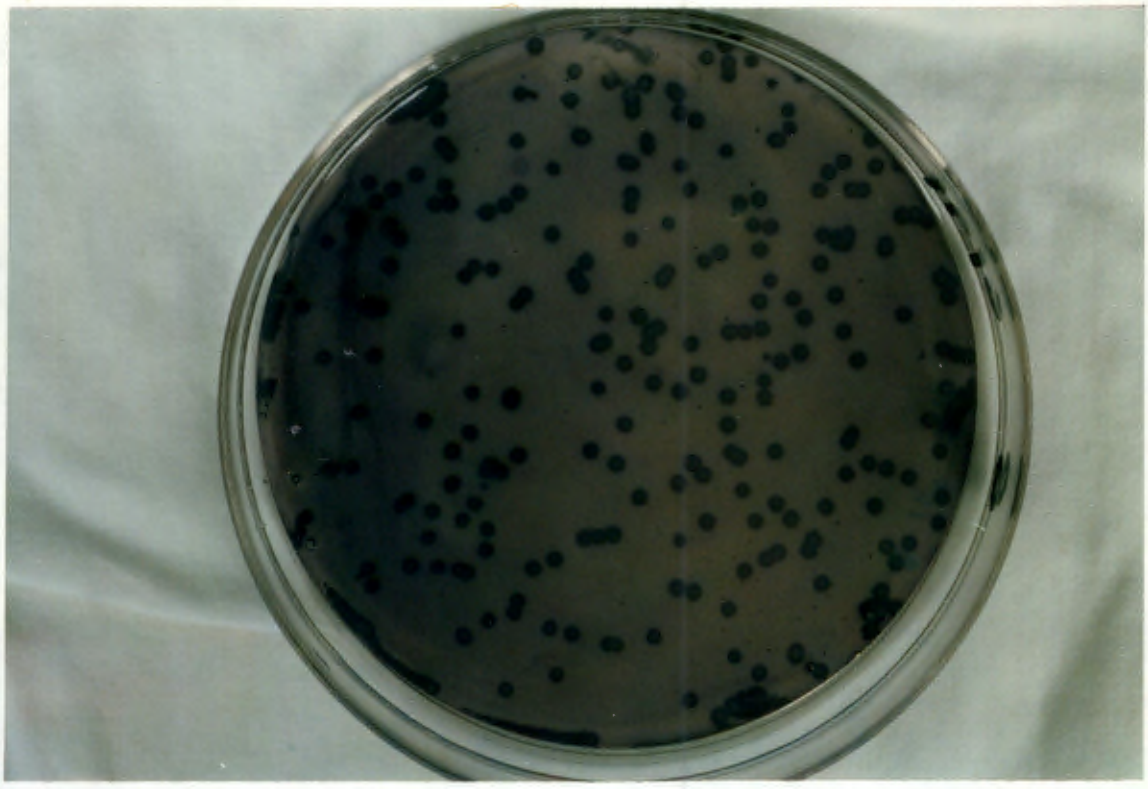




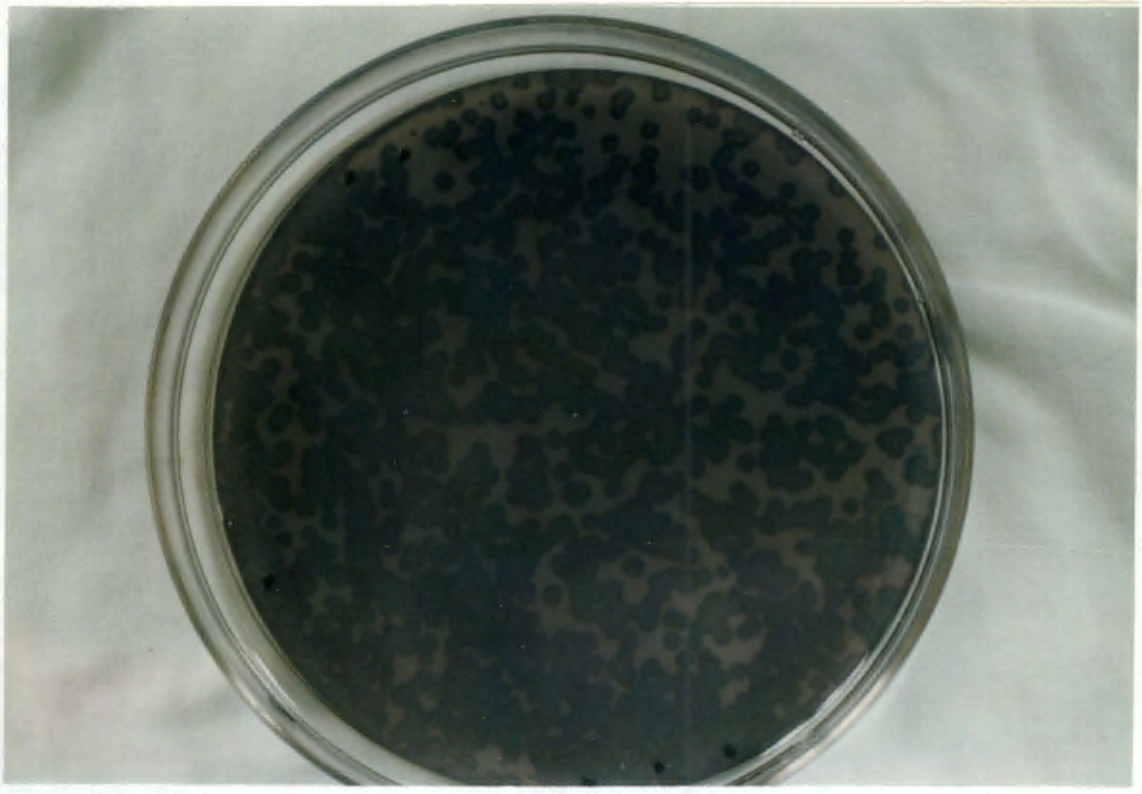
c

(continued from previous page)

(b) Phage were eluted and replated at a density of about 200 pfu/plate and rescreened in order to isolate single clones for purification.



d



e

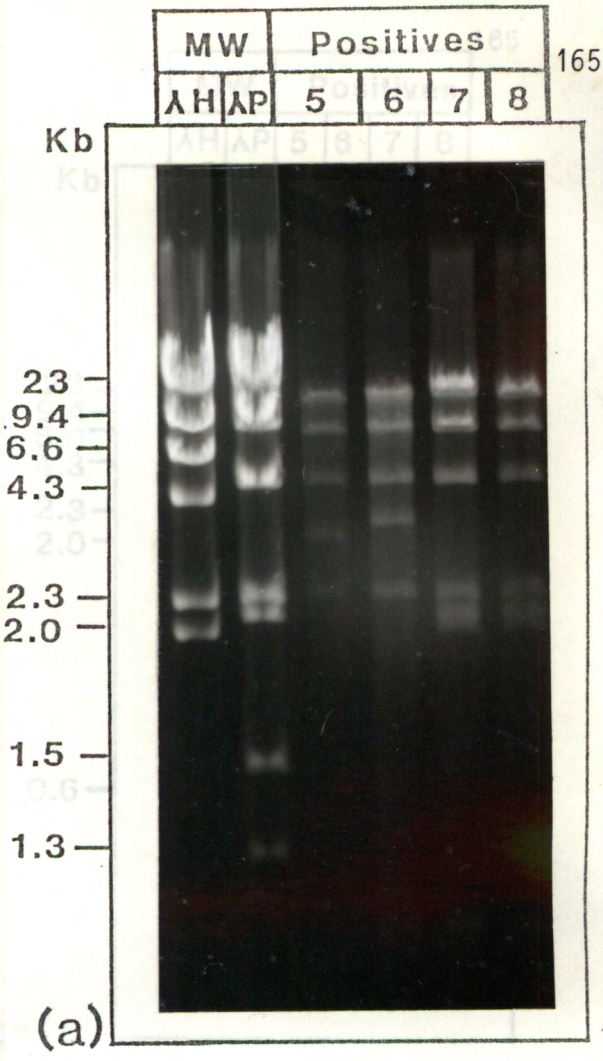
(continued from previous page)

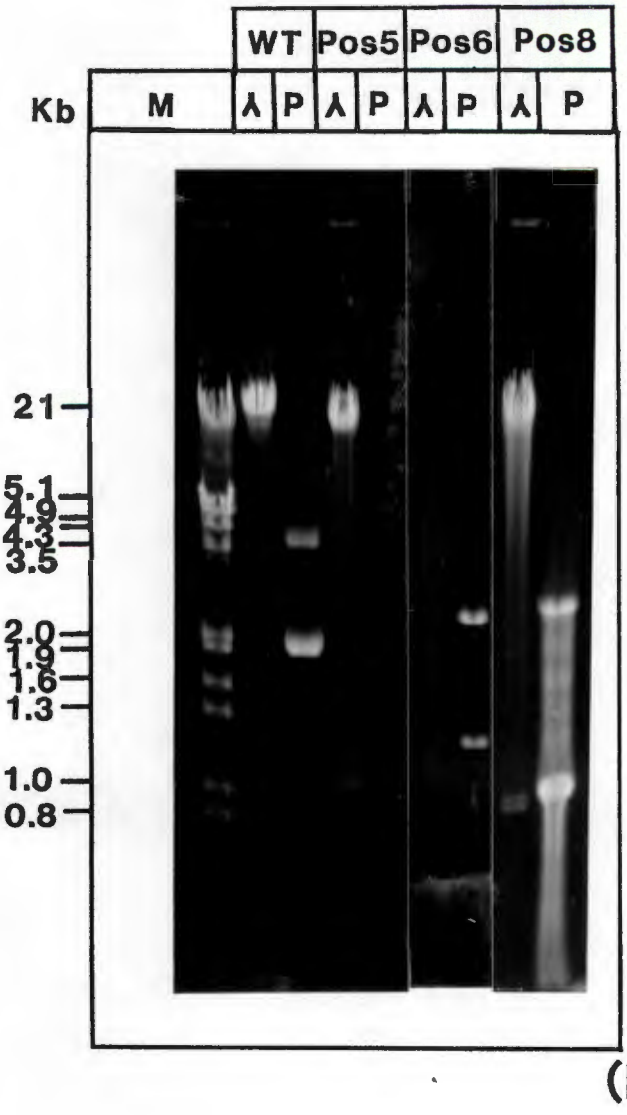
(c,d,e) The process of replating and rescreening was repeated until recombinant clones were purified to homogeneity, as demonstrated by the positive staining of all the plaques at high density screening **(e)**.

Fig. 5.7 Size analysis and subcloning of cDNA inserts of positive clones

DNA was extracted from positive λgt11 clones as described in section 7.B.2.10, and digested with MluI **(a)** and with EcoRI **(b)** to determine the size of the cDNA inserts, and to isolate the cloned EcoRI fragments for subcloning into pUC19. The digested DNA was fractionated on 1% agarose gels and visualised by ethidium bromide staining of the gel.

(a) MluI digested DNA from the positive clones 5,6,7 and 8, showing inserts of 1 kb (POS5) and 1.4 kb (POS6) larger that the 2 kb MluI fragment containing the cloning site. Positives 7 and 8 (POS7/8) show an additional band indicating an internal MluI site in the insert. The two positive clones were shown to be identical. Digestion with EcoRI of positive 8 (POS8) indicated an insert size of 1.1 kb (see **(b)** below).





(b) DNA from λgt11 positive clones digested with EcoRI and fractionated on a 1% agarose gel (λ lanes). The "P" lanes show inserts from the positive clones subcloned into pUC19 and released with EcoRI. (λ DNA inserts are only faintly visible).

λ	lambda DNA
P	pUC 19 DNA
WT	wild type lambda and plasmid DNA
λH	lambda DNA cut with HindIII
λP	lambda DNA cut with PvuII

5.2.2.3.2 Size Analysis of Fusion Proteins

The three positives were introduced as lysogens into *E.coli* strain Y1089 as described in Materials and Methods (section 7.B.4.1), and colonies of each were selected for extraction of fusion protein by their ability to grow at 32°C but not at 43°C (see Fig. 5.4).

Production of fusion proteins was induced with IPTG, and bacterial lysates of the clones were analyzed by immunoblotting against a murine anti-ß-galactosidase mAb, to measure the size of the fusion proteins.

Fig.5.8 (a) is an electroblot of the bacterial lysates stained with the protein stain *AuroDye forte* (Janssen Life Sciences). Additional protein bands, representing the fusion proteins, are evident in the lysates of the positive clones (lanes 3-6) compared to the non-recombinant wild type (lane 2).

Fig. 5.8 (b) is an immunoblot of these lysates reacted with the anti-ß-galactosidase mAb (dilution 1:10 000) and stained with a peroxidase conjugated goat anti-mouse IgG (H+L) (Zymed). The apparent mol.wts. of the fusion proteins were 145 kD, 120 kD and 138 kD, for POS5, POS6 and POS7/8 respectively. These correspond to cloned proteins of 29 kD, 4 kD and 22 kD larger than the wild type 116 kD ß-galactosidase molecule.

5.2.3 REACTIVITY OF FUSION PROTEINS WITH SERA

The specificity of the positive clones for ARF sera, as well as their cross-reactivity with streptococcal antigens, was tested in the following assays.

5.2.3.1 Purified Plaque Assay: specificity for ARF Sera

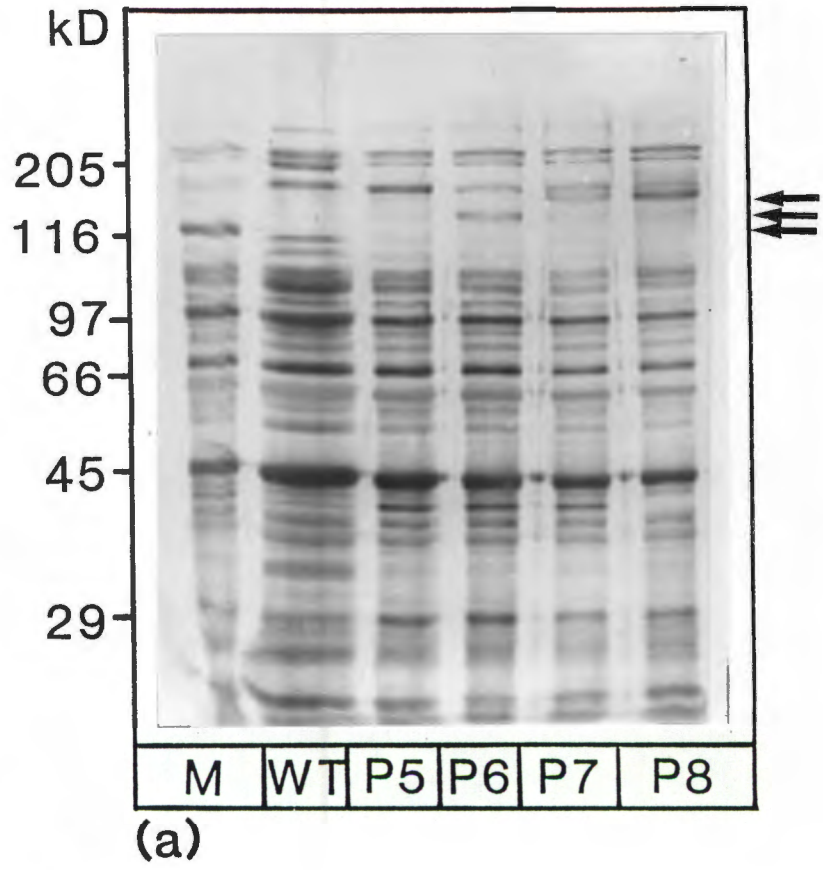
The specificity of the purified positive plaques for ARF sera was tested by preparing nitrocellulose lifts of the homogeneous plaques, and cutting them into 8 sectors, each of which was reacted with a different ARF or child normal serum. As controls, filters bearing wild type λgt11 or the irrelevant chicken ovalbumen clone (Clontech Laboratories) were likewise prepared and reacted against the ARF and normal sera. In this way, each of the three positives (and the control clones) were tested against 24 ARF and 24 normal sera.

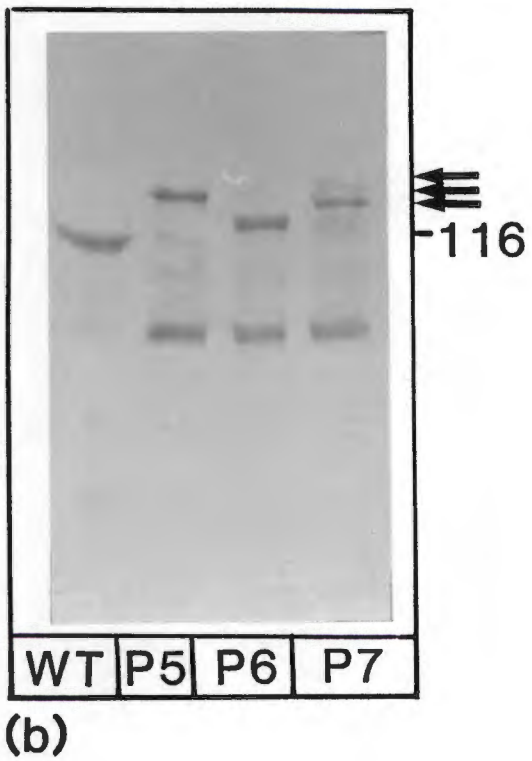
Fig.5.9 shows representative stains of three ARF sera and one normal serum against the clones: (a) a normal serum reactive with none of the clones; (b) an ARF serum reactive with only POS5; (c) an ARF serum reactive with the three positive clones but not with the controls; (d) an ARF serum reactive with both the positive and the control clones.

Fig. 5.8 Isolation and size analysis of positive clone fusion proteins

Fusion proteins of the positive clones (POS5, POS6, POS7 and POS8) were induced with IPTG (section 7.B.4.1.2), and lysates of the lysogens were fractionated on 7.5% SDS polyacrylamide gels and electroblotted onto nitrocellulose (section 7.A.9).

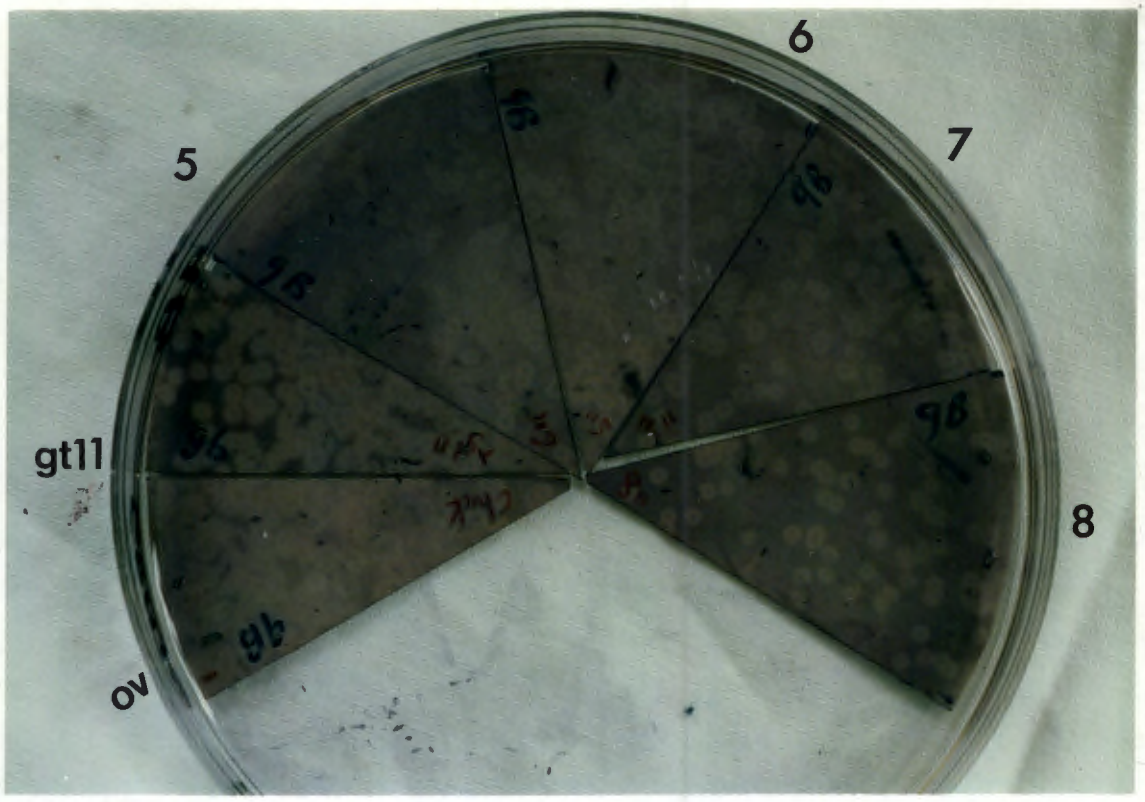
(a) *E.coli* Y1089 lysates immunoblotted and stained with *AuroDye forte* (Janssen). The arrows indicate the larger sized fusion proteins compared to the wild type (WT) and mol. wt. marker 116 kD ß-d-galactosidase protein.





(b) The extracts shown in (a) were immunoblotted against a murine monoclonal antibody to ß-d-galactosidase, and positive binding detected using a peroxidase conjugated second antibody as described in the legend to Fig. 5.5. The larger size of the fusion proteins compared to the wild type (WT) protein can be seen.

The sizes of the fusion proteins are: POS5 = 145 kD, POS6 = 120 kD, POS7/8 = 138 kD.



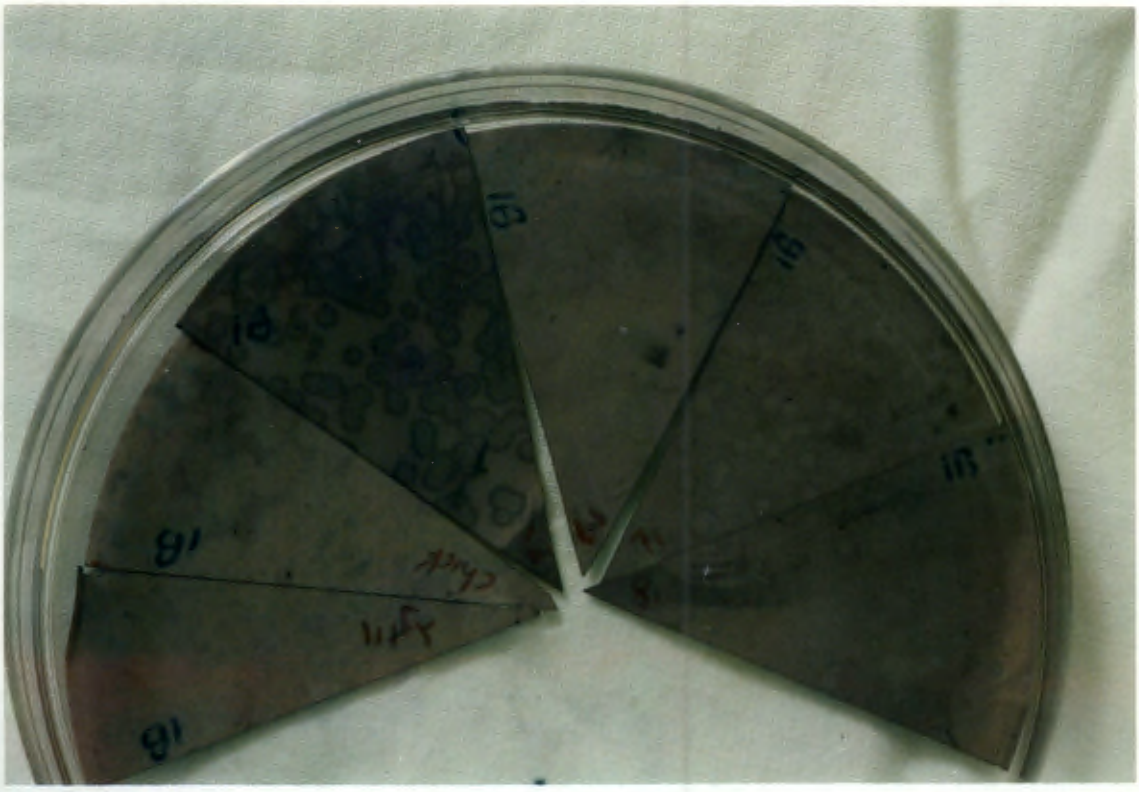
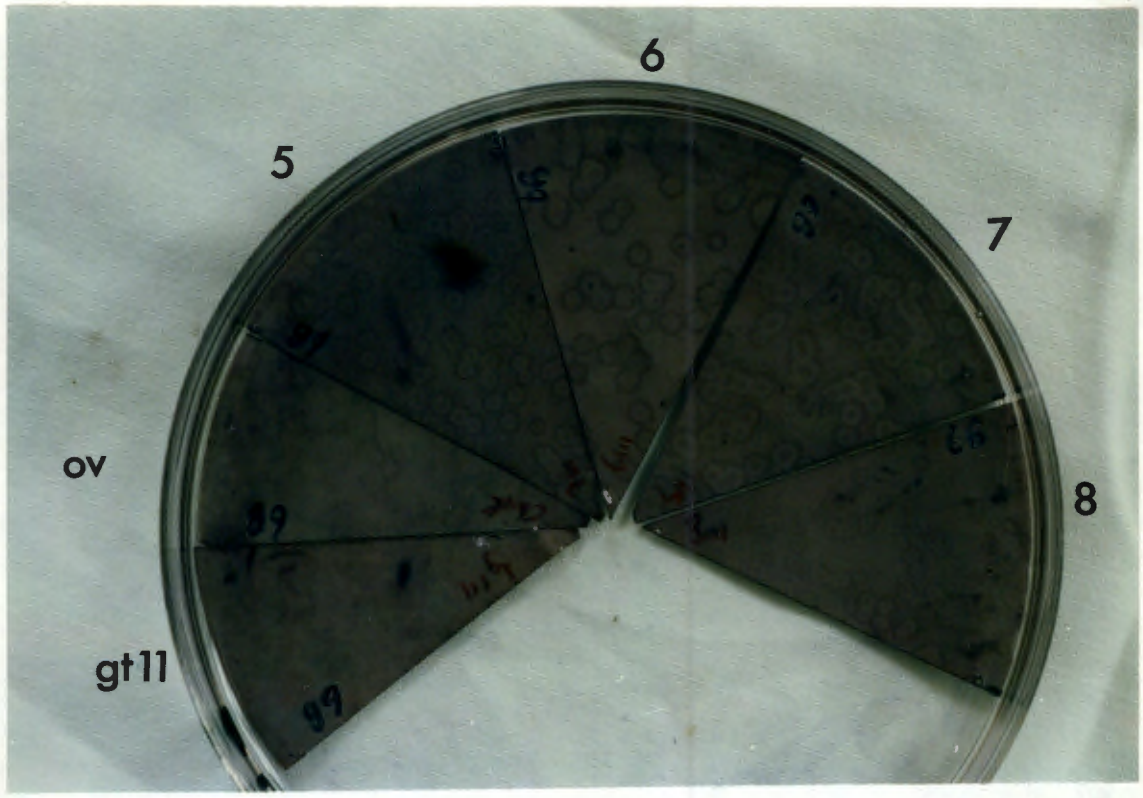


Fig. 5.9 Reactivity of ARF and normal sera against purified plaques of positive clones
Nitrocellulose lifts of purified plaques of POS5, POS6, POS7 and POS8 were prepared and cut into 8 sectors, each of which was reacted with a different ARF or normal serum according to procedures described in section 7.B.3.1. Similarly prepared filters bearing wild type λgt11 or an irrelevant chicken ovalbumen clone were used as controls and reacted against the same ARF and normal sera. 24 ARF and 24 normal sera were tested. (See Table 5.4)

(a) Reaction of a normal serum against the clones showing no reactivity with any of them.
(b) An ARF serum showing reactivity with only POS5.

(continued overleaf)



c

d

(Fig 5.9 *continued from previous page)*

(c) An ARF serum reactive with only the cloned proteins and not with the wild type or irrelevant ovalbumen clones.

(d) An ARF serum reactive with all positive clones and the control clones.

As shown in Table 5.4, none of the positives were specific (at the level of *plaque* reactivity) for only ARF sera since all of them reacted also with some normal sera. Certainly, a higher percentage of the ARF sera are reactive with the positive plaques, but this higher level of reactivity extends to the control clones as well.

TABLE 5.4:

Purified Plaque Assay: Specificity Analysis of Positive and Control Clones Against 24 ARF and 24 Normal Sera
(The number and percentage of reactive sera in each group are presented).

CLONE	wild type λgt11	oval-bumen	POS5	POS6	POS7
ARF sera (n=24)	1 (4%)	4 (17%)	21 (87%)	16 (67%)	20 (83%)
Normal sera (n=24)	0 (0)	2 (8)	11 (46%)	10 (42%)	14 (58%)

5.2.3.2 Fusion Proteins: Immunoblots Against ARF and Normal Sera

Since specificity was not evident at the level of plaque reactivity, the cloned antigens were electrophoretically separated for testing against ARF sera by immunoblotting. Conceivably, fusion protein epitopes thus exposed on nitrocellulose might be more readily available for detection by sera.

5.2.3.2.1 Immunoblots of Bacterial Lysogen Lysates

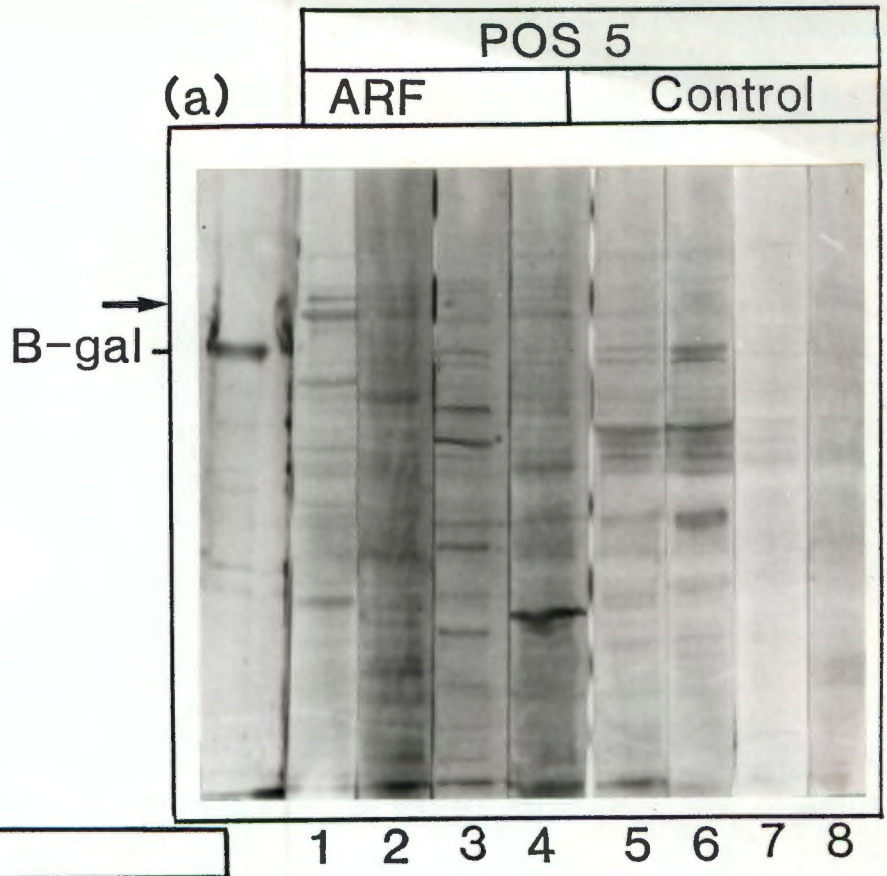
Lysates of IPTG-induced lysogens of the three positive clones, were separated on 7.5% SDS PAGE gels and immunoblotted against ARF and normal sera (dilution 1:100).

Each positive was reacted against ten ARF and ten normal child sera which had been pre-absorbed with purified ß-galactosidase (Sigma) at a concentration of 1 mg/ml (for 1 hour at 37°C followed by 18 hours at 4°C). Antibody binding was detected using a peroxidase conjugated goat anti-human IgG (H+L) affinity purified antibody (Zymed).

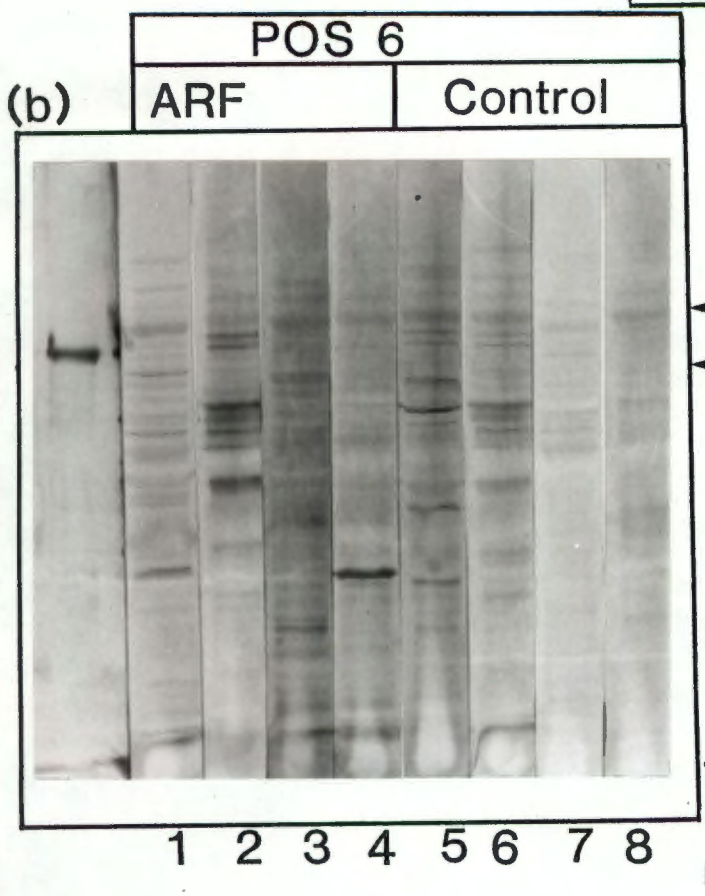
Fig.5.10 shows representative reactions of ARF and normal sera against the positive lysogen lysates. Despite pre-absorption with purified ß-galactosidase to remove reactivity with the bacterial portion of the fusion proteins, normal sera also reacted with these recombinant proteins.

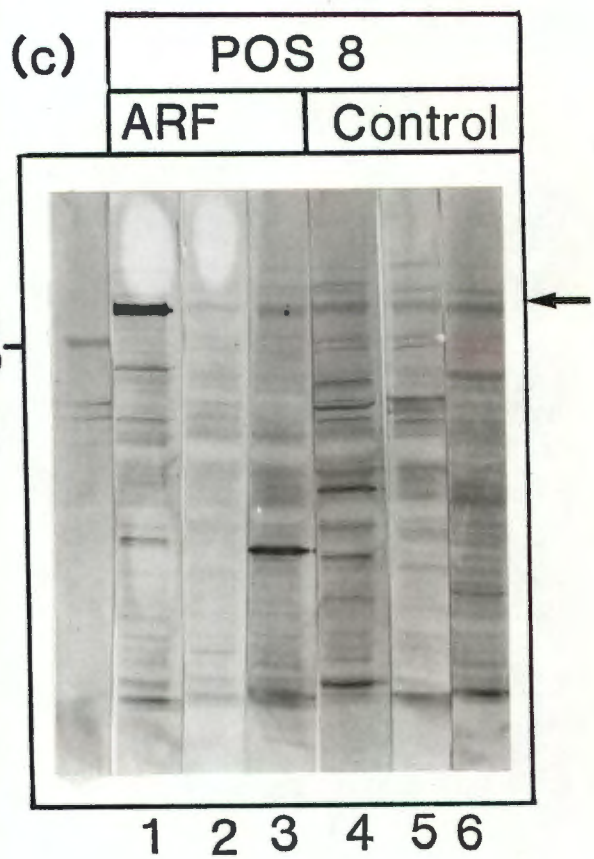
Fig. 5.10 Reactivity of ARF and normal sera against fusion proteins in lysates of the positive lysogen clones

Lysates of the IPTG induced lysogens of the three positive clones in Y1089 were immunoblotted (section 7.A.9) against 10 ARF and 10 normal sera.



(a) Representative immunoblots of 4 ARF and 4 normal sera against a lysogen lysates of POS5, indicating a somewhat more intense reaction of ARF sera compared to normal sera with the fusion protein (indicated by arrow).





(b) and **(c)** Immunoblots of ARF and normal sera against POS6 and POS8.

However, the reactivity of ARF sera against POS5 fusion protein is noticeably more intense than that of the normal sera, indicating that the absorptions may possibly not have been extensive enough.

The first lane of the ARF sera in each of the blots is that of the original screening serum, ARF-S1. Its reactivity with POS7/8 was much more intense than that of the other sera.

5.2.3.2.2 Immunoaffinity Purification of Fusion Proteins

Attempts to affinity purify the fusion proteins on columns containing anti-ß-galactosidase mAb attached to agarose beads (Protosorb LACZ column, supplied by Promega Biotech), were unsuccessful. Inclusion of nonionic detergent (0.05% Triton X-100) in all buffers to expose possibly hidden epitopes for binding to the column, were likewise unsuccessful in binding the fusion proteins.

5.2.3.3 Plaque Selected Antibodies: cross-reactivity with streptococcal antigens

To test whether antibody specific for purified positive plaques, cross-reacted with streptococcal antigens, a method similar to the "plaque antibody selection" technique of Ozaki et al (1986) was used.

Nitrocellulose lifts (150 mm) of each of the purified clones were prepared and reacted with the original screening serum, ARF-S1 (dilution 1:50), for 6 hours at 4°C. (Anti-*E.coli* antibodies had been pre-absorbed from the serum by pseudoscreening, as described in section 7.B.3.3). Filters were then washed three times for 15 minutes each, in TBS buffer (containing immunoscreening blocking solution minus goat serum). Specifically bound antibody was eluted from the plaques in 5 ml of 0.2 M glycine-HCl pH 2.5 for 2 minutes and then immediately neutralized in 750 µl of 2 M Tris pH 10. Eluted antibody was then reacted against immunoblotted streptococcal M proteins.

Fig.5.11 shows the results of immunoblotting these antibodies eluted from cloned heart fusion proteins, against streptococcal M proteins M5, M6, M19 and M24.

All eluted antibodies reacted with the same two bands (67 kD and 33 kD) in the M24 extract. Only antibody eluted from POS6 was reactive with M5 (30 kD). POS6-eluted antibody was also reactive with a protein in the M19 extract (31.5 kD) and with streptococcal M6 proteins (55 kD and 29 kD). POS7/8-eluted antibody was reactive with M19 (65 kD, 60 kD and 35 kD). No reactivity was observed with antibody eluted from the control wild type λgt11 or the irrelevant ovalbumen clones.

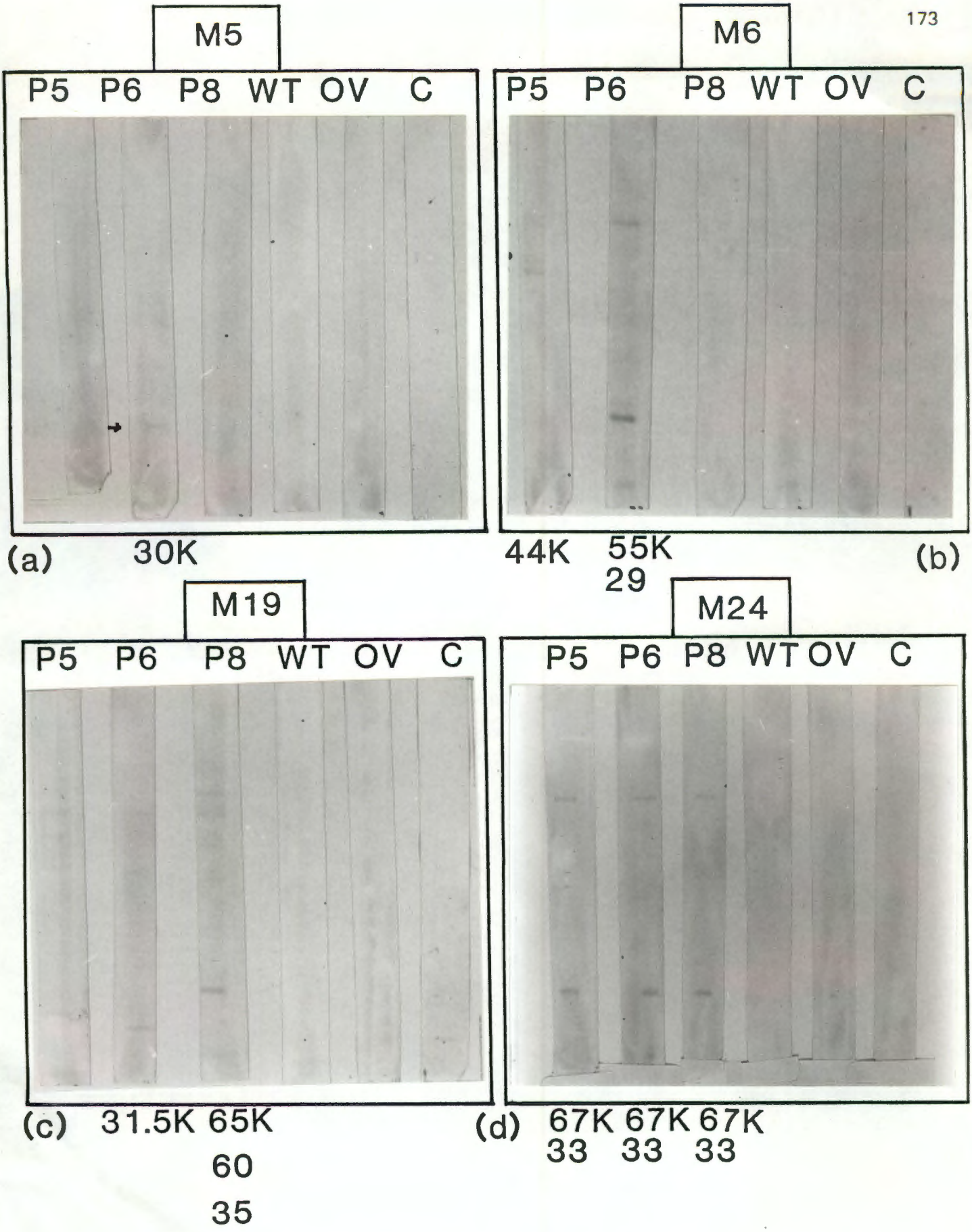


Fig. 5.11 Cross-reactivity between streptococcal M proteins and ARF serum (ARF-S1) affinity purified on plaques of positive clones

Nitrocellulose plaque lifts of each of the homogeneous positive clones were prepared and reacted with serum ARF-S1. Bound antibody was eluted as described in section 5.3.3, and reacted against immunoblotted streptococcal M proteins M5, M6, M19 and M24. As controls, antibody was also eluted from wild type λgt 11 plaques (WT) and from the irrelevant chicken ovalbumen clone (OV). Eluted antibodies cross-reacted with the M proteins with distinct patterns. (The mol.wts. of the reactive M protein constituents are given beneath each lane).

5.2.4 DNA SEQUENCE ANALYSIS OF POSITIVE CLONES

The positive cDNA inserts were released from λgt11 by EcoR I digestion (see Fig. 5.7 b), subcloned into pUC19 by in-gel ligation (section 7.B.4.2.1) and sequenced using the modified T7 polymerase, *Sequenase* (section 7.B.4.2.2). The full sequence of the POS6 cDNA insert was completed, while sequencing of POS5 and POS7/8 is still in progress.

Computor-assisted analysis of the cDNA sequences was performed using the University of Wisconsin Genetics Computor Group Sequence Analysis Software Package (Version 6) (Devereux et al., 1984, 1989). The *FastA* program of Lipman and Pearson (1985) was used to search the European Molecular Biology Laboratory (EMBL) and GenBank nucleic acid databases for similarity of sequences in the databases with the cloned DNA sequence. The National Biomedical Research Foundation (NBRF) protein bank was searched for homologies with the deduced amino acid sequence.

The most striking nucleic acid homology based on the above searches was with human cytokeratin 8, which showed virtually 100% identity in about a 1 kb overlap. A section of the cloned sequence is shown in Fig. 5.12, and the sequence showing identity with human cytokeratin 8 is depicted in Fig. 5.13.

Homology searches of the amino acid sequence of human cytokeratin 8 with sequences of other α-helical coiled-coil proteins putatively relevant to antigenic mimicry in rheumatic, disclosed homologies of between 40-and 50% with the heavy chain of human cardiac myosin, tropomyosin and streptooccal M5, M6 and M24 proteins.

A computor search of the NBRF protein bank, for the recently reported myosin cross-reactive pentameric sequence GLN-LYS-SER-LYS-GLN contained in pep M5 protein (Cunningham et al., 1989), demonstrated that this sequence was not contained in human cytokeratin 8 and hence not in the cloned protein.

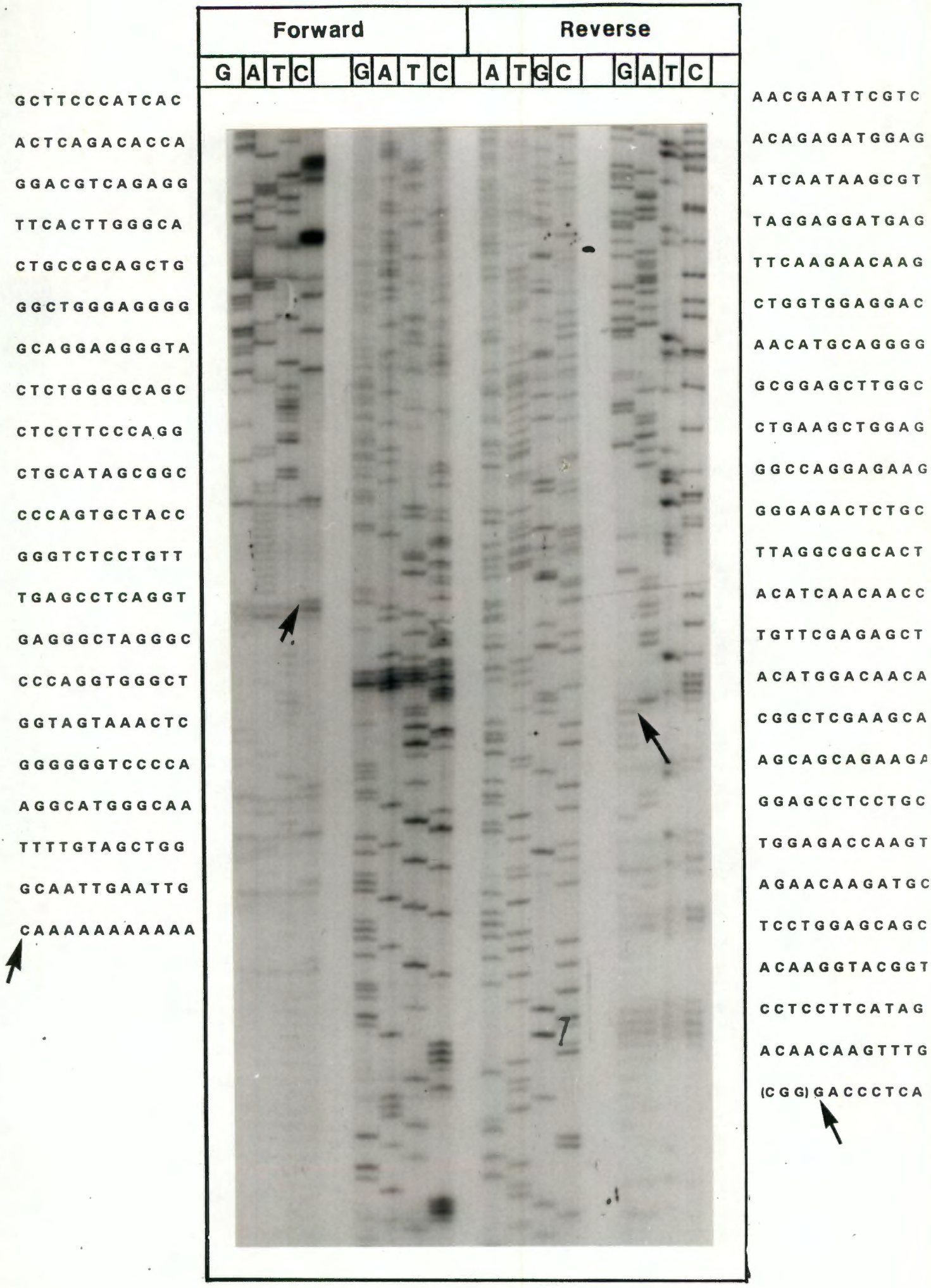
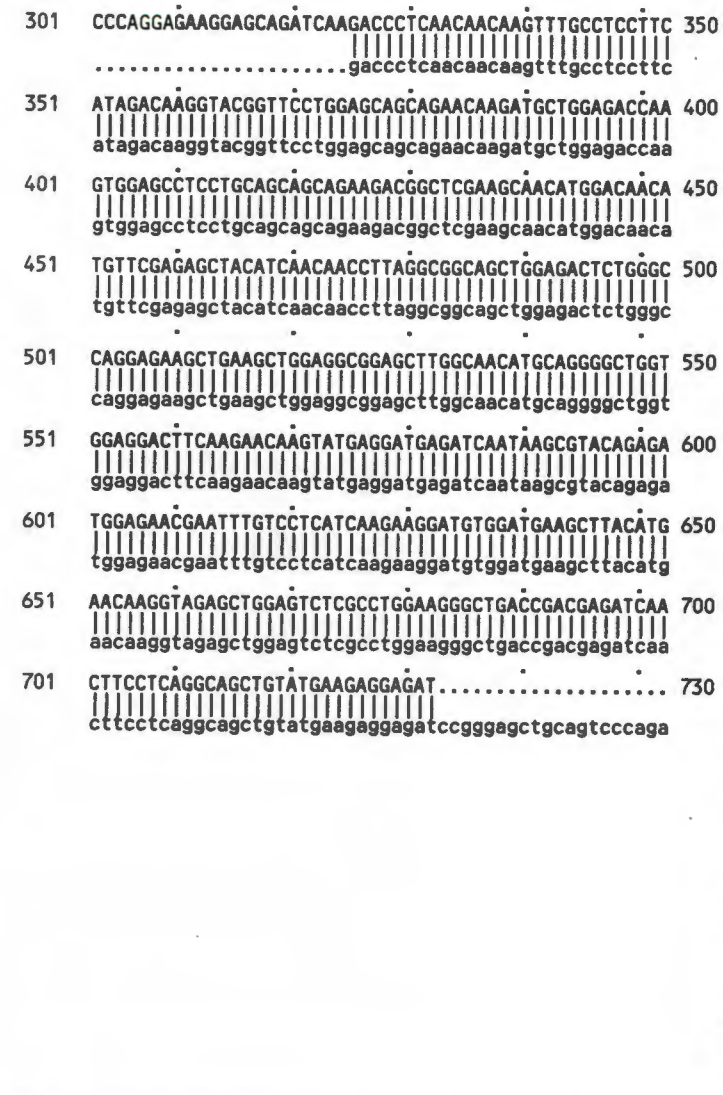
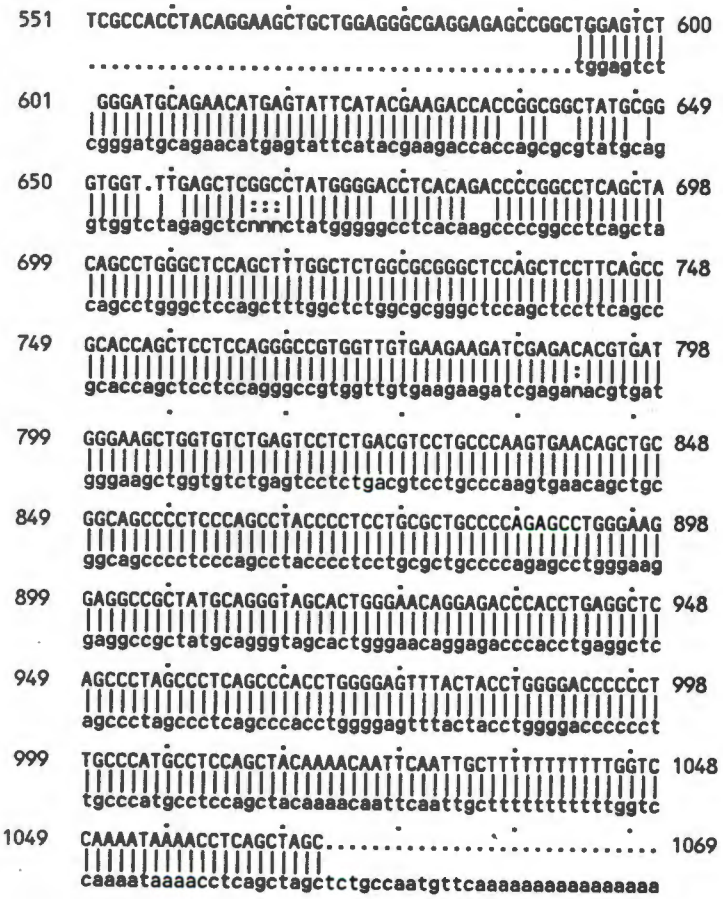


Fig. 5.12 Nucleotide sequence of POS6 showing section homologous with human cytokeratin 8. (Arrows indicate first bp read)



(a)



(b)

Fig. 5.13 Nucleotide sequence of POS6 (lower line) showing homology with human cytokeratin 8 mRNA (upper line). Two clones of cytokeratin 8 in the databases searched were homologous with POS6. **(a)** HUM8KER1 representing the 5' end of human keratin 8 mRNA, 100% homologous with POS6; **(b)** HUMKER8 representing the complete coding sequence of human cytokeratin 8 mRNA (derived from the human vulvar carcinoma cell line A-431) showing 98% homology with POS6.

5.3 DISCUSSION

5.3.1 CDNA SYNTHESIS AND CONSTRUCTION OF λGT11 EXPRESSION LIBRARIES

In this study, two efficient human heart cDNA expression libraries were constructed in λgt11, for the purpose of screening with antibodies relevant to rheumatic fever.

The efficiency of the libraries can be judged from the high number of recombinants obtained per microgram of cDNA cloned (78-94% recombinants in libraries containing about 10^8 pfu; see Table 5.3), as well as from the range of insert sizes in the two libraries (see Fig 5.2)

Efficient synthesis of cDNA was achieved by paying meticulous attention to the isolation and purification of undegraded mRNA so as to generate libraries containing as many as possible heart autoantigen-coding sequences. Table 5.1 presents the results of synthesis of the cDNA cloned in these libraries. Starting with 2 μg of poly $(A)^+$RNA, the amount of double-stranded cDNA obtained ranged between 484 ng and 1072 ng, the most efficient method of synthesis being the RNAse H method.

The rationale for pooling three libraries each constructed from cDNA synthesized by a different method, was to generate a library (*library-A*) that would avert any bias towards a particular population of cDNA as result of the method of synthesis. Such a library could therefore be potentially more representative of the available cardiac mRNA sequences.

5.3.2 SCREENING OF LIBRARIES AND DETECTION OF POSITIVE CLONES

Prior to screening the libraries, it was established that both were expressing antigenically detectable fusion proteins (Fig. 5.4).

The alkaline phosphatase antibody detection system used in screening, was the most sensitive of the various systems tried (peroxidase-conjugated antibodies; streptavidin-biotin-peroxidase complex; I-125 labelled protein A antibodies).

Mierendorf et al. (1987) showed that alkaline phosphatase was about ten times more sensitive than peroxidase-conjugated antibody detection systems, and could readily detect 20-50 pg of antigen. The system had the additional advantages, (1) that the reaction could be developed over many hours (thus enhancing its sensitivity), (2) that the signals were not subject to fading or bleaching, (3) that the contrasting signal to noise ratio of dark purple-staining plaques against a lighter background allowed the precise location of positive

plaques. By comparison, potential positives detected by the peroxidase antibody systems were subject to fading and proved difficult to locate and purify to homogeneity, whereas the protein A I-125 conjugates produced numerous false positives.

The ARF serum (ARF-S1) used to detect the three positives was carefully selected. Not only did the patient have a severe carditis and a high ASO titre, but (more importantly) she was in the acute phase of a second attack of ARF (clinically referred to as "acute-on-chronic"). Such a patient, it was thought, might have higher affinity anti-heart antibodies than either a patient with an initial attack of ARF or one with chronic rheumatic heart disease. Such high affinity antibodies would be ideal for screening. Rabbit hyperimmune sera and monoclonal antibodies are often preferred to patient sera in screening expression libraries since they frequently contain antibodies of higher affinity.

Although this ARF-S1 serum did not belong to the group of sera screened in the ELISA and immunoblotting assays (Chapters 2 and 3), it was shown by immunoblotting to react strongly with all eleven major bands in the CHAPS (section 3.2.1.1) detergent extracts of heart, and to react intensely with sections of human heart by immunocytochemistry (4+ staining in the biotin-streptavidin-peroxidase assay - section 7.A.18).

The use of this highly suitable patient serum for screening these cDNA expression libraries by the sensitive alkaline phosphatase antibody system, contributed to the detection and purification of the three positive clones.

5.3.3 CHARACTERIZATION OF POSITIVE CLONES

The following experiments to determine the specificity of the positive clones were performed before sequencing of the DNA was completed.

5.3.3.1 Specificity of Positive Fusion Proteins

5.3.3.1.1 Specificity of Purified Plaques for ARF Sera

The reactivity of ARF and normal sera against the purified positive plaques showed that (at the level of plaque reactivity) none of the positive clones were specific for only ARF sera but also reacted with about half of the normal sera (Table 5.4). Although a higher percentage of ARF sera were reactive with the positive plaques, some of these sera were also more reactive with the control λgt11 wild type and ovalbumen clones.

This overall higher reactivity could be indicative of a polyclonal B cell response in the ARF sera, and corresponds to their pattern of higher reactivity in the ELISAs against streptococcal and autoantigens (Chapter 2). It is possible,

however, that despite their reactivity with bacterial antigens, the ARF sera contain antibody subpopulations specific for the cloned antigens. In some studies, the specificity of positive clones has been established by extensively absorbing out such nonspecific antibodies to bacterial antigens (Hirayu et al., 1987).

Hirayu et al. (1987), in determining the specificity of a thyroid disease-related antigen, experienced great difficulty in removing nonspecific binding to bacterial proteins. Optimal absorption concentrations of *E.coli* lysates had to be determined for individual sera, in order to establish the specificity of clones. This contrasts with similar studies on immunoscreening for autoantigens, in which such nonspecific binding was not a problem (Habets et al., 1987; Stanley et al., 1988).

5.3.3.1.2 Immunoblots of Bacterial Lysogen Lysates

To test the reactivity of sera against the immunoblotted fusion proteins, sera were pre-absorbed with purified ß-galactosidase (Fig.5.9). The more intense reactivity of ARF sera (compared to normal sera) against POS5 fusion protein, suggests that more extensive absorptions against ß-galactosidase might have established the specificity of the clone.

All activity to ß-galactosidase could however not be removed using the available 1 mg/ml concentration of the protein. Even extensive absorptions against the wild-type λgt11 and *E.coli* proteins by pseudoscreening (section 7.B.3.3) failed to remove all anti-ß-galactosidase reactivity. The specificity of the positive clones for ARF sera could thus not be conclusively established.

The inability to affinity purify the fusion proteins on agarose columns of anti-ß-galactosidase mAbs (for the purpose of testing cell mediated responses of ARF patients to the cloned antigens) may have been due to peculiarities of the fusion protein imposed on it by the cloned polypeptide moiety. For instance, hydrophobic regions may cause portions of the protein to be membrane associated. However, inclusion of Triton-X100 in the buffers did not enhance the purification significantly. Possibly, the higher affinity column (Protosorb LACZ High Affinity Column - Promega Biotech) may have bound the fusion protein more efficiently for purification. (During this period however, Promega withdrew both columns from supply indefinitely).

5.3.3.1.3 Cross-reactivity of Plaque-Selected Antibodies with Streptococcal Antigens

The reactivity of antibodies eluted from the purified positive plaques against immunoblotted M proteins, suggests that the cloned antigens may contain epitopes cross-reactive with these strepotococcal antigens.

To establish whether this is true cross-reactivity (Berzofsky and Schechter, 1981), would require more sophisticated analyses of the epitopes involved. The different patterns of reactivity observed with the three eluted antibody populations (together with the fact that they did not react with the control clones), indicates that these reactions may be specific.

These are clearly preliminary findings, and the pathological significance to rheumatic fever of the antibodies and epitopes involved would require more detailed investigations, using functional biological assays.

5.3.3.2 cDNA and Deduced Amino Acid Sequence of Positive Inserts

The striking homology between POS6 and human cytokeratin 8 is interesting in the context of studies on antigenic mimicry in rheumatic fever. Keratin, which is antigenically related to cytokeratin (Kurki and Virtanen, 1984), is a member of the α helical coiled-coil family of proteins which have been found to have an greater potential for cross-reacitvity (Chapter 1). It falls into one of the two groups (myosin/keratin/actin and myosin/DNA) showing reactivity with human and murine mAbs evoked by group A streptococcal antigens (Cunningham and Swerlick, 1986; Cunningham et al., 1988) (see Chapters 3 and 4). Work by Fischetti and Manjula (1982) and Manjula et al. (1985) on the structure of streptococcal M proteins identified them as α helical coiled-coil proteins with a seven amino acid periodicity similar to that of myosin, tropomyosin and the desmin-keratin group of α-helical proteins. Recent work using murine mAbs has shown serologic cross-reactivity between streptococcal pep M5 and M6 proteins and tropomyosin, and some of these mAbs were also shown to cross-react with keratin, actin and myosin (Fenderson et al., 1989) (see Chapters 3 & 4 and Table 3.1). Homology searches in this study showed that human cytokeratin 8 is about 43% homologous with M protein and 42% with human cardiac myosin heavy chain.

Regarding the possible pathologic significance of the antigenic mimicry between streptococcal M proteins and these α-helical coiled-coil proteins, Manjula and Fischetti (1980) proposed that the resemblance between M protein and muscle regulatory proteins such as tropomyosin, may be responsible for the anti-phagocytic property of the streptococcal protein (section 3.3.6.1). One might speculate that antigenic mimicry between M proteins and cytokeratin may analogously impair immune cell function. Defects in cytoskeletal organization are known to be capable of disrupting cell function (Sturgess et al., 1980), and may be characteristic of some pathological processes (Runger-Brändle and Gabbiani, 1983).

Keratins are part of a group of five distinct types of intermediate filament constituting the cytoskeleton in almost all higher eucaryotic cells (reviewed in Steinert and Roop, 1988). Type I (acidic) and type II (neutral-basic) keratins occur in epithelial cells, are comprised of a group of about 20 different

polypeptides and are the most evolutionarily divergent intermediate filament type showing only 25-30% homology with the other three types (which share 60-70 % homology with each other) (Marchuk et al.,1984).

Antibodies to cytoplasmic proteins have often been associated with autoimmune diseases. Whereas antinuclear antibodies are considered important markers for some rheumatic diseases, antibodies to cytoplasmic structures have been associated with organ-specific autoimmune diseases (Kurki and Virtanen, 1984). There appears to be a high incidence of antibodies to cytoskeletal proteins in human sera suggesting that they form part of the natural autoantibody repertoire. Indeed, this may (at least in the case of POS6) explain the immense difficulty experienced in trying to absorb out from the sera nonspecific binding to the fusion proteins, in the process of trying to establish whether the clones were specific for ARF sera. Souroujon et al.(1988) demonstrated such natural autoantibodies to keratin in A/J mice. However, these autoantibodies tend to be of the IgM class, whereas anti-cytoskeletal antibodies in pathological conditions appear to be IgG (Kurki et al., 1983). The incidence of anti-cytokeratin antibodies in humans appears to be increased in diseases such as alcoholic liver disease and in rheumatic diseases, but what their role is remains unknown (Kurki et al., 1983). It has been suggested that they may contribute to the chronicity of the inflammation in these diseases (Kurki and Virtanen, 1984).

Antibodies binding to the cytokeratin fusion protein in the present study were detected using an IgG (H+L) second antibody and may therefore be such "pathological" autoantibodies. One might also speculate that the 67 kD reactive constituent detected in heart extracts in this study may be type II keratin (see Tables 3.1 and 3.2). In this study a constituent of this mol. wt. was found to be reactive with ARF patient sera, and in other studies constituents of this mol. wt. were reactive with mAbs and rabbit immune sera. Whether keratin is implicted in cross-reactivity between streptococcal antigens and human skin in group A streptococcal skin infections, such as in scarlet fever and psoriasis (Swerlick et al.,1986), is another interesting speculation.

In the final analysis, the detection of a human cytokeratin 8 autoantigen clone using an ARF patient serum in screening human heart cDNA libraries, raises the vexing question (see Chapter 1) as to the nature of autoantibodies in health and disease, and what role they play in antigenic mimicry in rheumatic fever. It could be argued that: (1) cross-reactivity between α-helical coiled-coil proteins like cytokeratin and streptococcal M proteins are irrelevant experimental artefacts due to the high propensity such proteins generally have for cross-reactive binding (Chapter1), and that is not indicative of antigenic mimicry; (2) that antibodies to the mammalian proteins form part of the natural/physiologic autoantibody repertoire and are pathologically irrelevant; (3) that they form part of the natural autoantibody repertoire, but that such autoantibodies can be triggered into being pathogenic; (4) that even though these proteins have a

high propensity for cross-reactivity and may form part of the natural autoantibody repertoire, they may nontheless still harbour epitopes (continuous or discontinuous) that are truly cross-reactive (Berzofksy and Schechter, 1981) with microbial epitopes and can lead to autoimmune disease through antigenic mimicry. Such epitopes could be precisely mapped.

Whatever the significance of cytokeratin 8 is to rheumatic fever is not clear but the identification of this cardiac autoantigen by molecular biological techniques is interesting both because of the implication of keratin in antigenic mimicry in rheumatic fever (using monoclonal antibodies and structural studies), and in view of its known implication as a conserved protein recognised by natural autoantibodies. Whether this heart antigen is pertinent to the pathogenesis or aetiology of rheumatic fever remains to be determined.

5.4 CONCLUSIONS

1. High quality intact poly $(A)^+$ RNA was isolated from normal human heart, and cDNA was synthesized from it by three different methods, referred to as the S1 nuclease, RNAse H and random primers methods.

2. The cDNA was cloned into λgt11 and two expression libraries of high efficiency were selected for screening with antibodies. *Library A* was a pool of three libraries each derived from cDNA synthesized by a different method; *library B* was derived from cDNA synthesized by the RNAse H method.

3. The libraries were screened with rheumatic fever patient sera, as well as monoclonal antibodies and rabbit immune sera raised against *S.pyogenes* antigens.

4. Using serum from an acute-on-chronic ARF patient with severe carditis and a high ASO titre, three positive clones were detected and purified to homogeneity.

5. The three clones were introduced into *E.coli* as lysogens, induced with IPTG, and lysates of the lysogens tested for reactivity against ARF and normal sera. Although control sera also reacted with the induced fusion proteins, the reactivity of ARF sera against some of the recombinants was shown to be stronger, following pre-absorption to remove non-specific binding.

6. Antibodies eluted from the purified plaques were tested against immunoblotted *S.pyogenes* M proteins, and showed different patterns of cross-reactivity.

7. The derived amino acid sequence of one positive clone (POS6) was virtually 100% homologous with human cytokeratin 8, an alpha-helical coiled-coil protein, and 40-50% homologous with the coiled-coil proteins myosin, tropomyosin and streptococcal M5 protein. Since streptococcal M proteins have been found, using monoclonal antibodies, to cross-react immunologically with alpha helical coiled proteins such as myosin, actin and keratin, this finding may be of significance to antigenic mimicry in rheumatic fever.

6. CONCLUSION

6.1 STUDY OBJECTIVES 185

6.2 NOVELTY OF THE APPROACH 185

6.3 SALIENT FINDINGS 185

6.4 DIFFICULTIES IN THE STUDY OF ANTIGENIC MIMICRY 189

6.5 RHEUMATIC FEVER AS A MODEL OF AUTOIMMUNITY AND IMMUNE FUNCTION 189

6.1 Study Objectives

The objective of this study was to identify cardiac antigens that may be implicated in the pathogenesis of rheumatic fever, and in addition to examine and compare the autoantibody profiles of rheumatic fever patients against a range of cardiac and nuclear antigens (and streptococcal antigens), with those of related-disease and normal subjects.

The investigations performed were based on the established hypothesis that antigenic mimicry between antigens of the group A streptococcus and human heart antigens, initiates an autoimmune response leading to cardiac damage in certain individuals.

6.2 Novelty of the Approach

The approach adopted was innovative in the following respects: (1) Autoantibody levels against these cardiac and nuclear antigens have not previously been measured and compared in these groups of rheumatic fever patients and control subjects; (2) Immunoblotting was used to compare the antibody isotype reactivity patterns of the patient and control sera against crude and purified cardiac and nuclear antigens. No previous reports have compared the reactivity of rheumatic fever patient sera against cardiac antigens by immunoblotting, although numerous investigations have used monoclonal antibodies and rabbit immune sera (see Table 3.1); (3) Lymphocytes of patients with acute rheumatic fever were used to isolate human monoclonal antibodies cross-reactive with group A streptococcal antigens. Previous reports have not used ARF patient lymphocytes; (4) λgt11 cDNA expression libraries of human heart genes were constructed and screened with ARF patient sera to identify cardiac antigens. No previous reports have adopted this approach.

6.3 Salient Findings

This study presents evidence that sera of patients with acute rheumatic fever and rheumatic heart disease contain raised levels of antibodies to various cardiac, nuclear and group A streptococcal antigens. The original finding by other investigators using immunofluorescence to show that patients with acute rheumatic fever have antibodies to heart (see Chapter 1), was confirmed in this study by ELISA, immunoblotting and by a sensitive biotin-streptavidin-peroxidase immunocytochemical assay.

ELISAs were used to measure antibody titres in rheumatic fever sera against crude heart extracts and purified cardiac. It was shown that acute and chronic rheumatic fever patients have elevated titres of antibody to crude extracts of heart antigens and to myosin, actin and phosphorylase b, whereas antibody levels to cardiolipin and type II collagen (and III and VI) were not significantly elevated in these patients. (Adult chronic rheumatic fever patients however showed some evidence of IgM antibodies to type II collagen). Regarding

nuclear antigens, in all the groups rheumatic fever sera antinuclear antibodies (using the HEp-2 cell substrate) were found although mostly only at low dilutions. Two ARF patients had antibodies to the Sm (Smith) or RNP (ribonucleoprotein) nuclear antigens. None of the rheumatic fever patients had anti-DNA antibodies. Antibody levels to group A streptococcal antigens were elevated in all groups of rheumatic fever patients (as expected) but were also found in the related-disease and normal control subjects. The raised antibodies to proteins M5 and M6 may indicate that these group A streptococcal serotypes are predominant among rheumatic fever patients in Cape Town.

A consistent, and important finding, in these investigations was that normal subjects in many cases also contained antibodies to the tested antigens. Although the titres of antibody were usually lower than in patient sera, the presence of these antibodies in normal sera may indicate that they form part of the natural/physiologic autoantibody repertoire. The relevance of natural autoantibodies to autoimmune disease and their relationship to pathogenic autoantibodies are unsolved questions, and hence the pathological significance of the autoantibodies measured in this study are difficult to assess. The finding that adult chronic rheumatic fever patients demonstrated the highest titres of antibody to several of these autoantigens, suggests that these antibodies may be a consequence of polyclonal B cell activation (possibly initiated by streptococcal antigens) and contributed to the chronicity of the systemic autoimmune disease.

Immunoblotting of these rheumatic fever and control sera against extracts of human heart, showed that the IgM isotype binding pattern of ARF sera to the eleven major constituents in the extract differed markedly in intensity and frequency of detection compared to the other sera. However normal sera also showed IgM binding although at lower intensity and frequency, indicating that these may be natural autoantibodies. Whether the ARF and normal sera recognise the same epitopes is not known. The dramatic shift to IgG anti-heart antibodies among the chronic rheumatic fever patients, may be a consequence of the natural progression of the disease from the acute to the chronic phase. Conceivably, this shift may represent a switch from natural/physiologic IgM autoantibodies to pathogenic IgG autoantibodies. IgM autoantibodies may potentiate the IgG response, and one might postulate that either the high titre or the nature of anti-heart IgM autoantibodies in certain hosts may be associated with increased susceptibility to the disease (see also section 3.4.1.1/2). IgM autoantibodies can however be pathogenic and are known to occur in certain diseases. Whether the anti-heart autoantibodies in rheumatic fever (either those deposited in heart tissues *in vivo*, or in the serum) are pathogenic, has never been established, and it is unfortunate that adoptive transfer experiments using the eluted antibody to prove this cannot be done without a suitable animal model of the disease.

The composition of the eleven heart extract constituents recognised by patient sera was not investigated, but immunoblot inhibition experiments using purified antigens might be used to identify some of them. Thus the 38-43 kD region of reactivity may contain actin (42 kD), the 67-70 kD band may be comprised of keratin and it would be particularly pertinent to other reports in rheumatic fever if the 33 kD doublet was shown to be comprised of the two chains of tropomyosin (35 kD).

Immunoblotting of purified human heart sarcolemmal membranes revealed a 200 kD constituent uniquely recognised by IgG antibodies in ARF sera. This constituent may be myosin, which has been found associated with sarcolemmal membranes, and immunoblot inhibition experiments such as performed by Dale and Beachey (1985a) are being planned to verify this.

Such inhibition experiments are nonetheless severely limited in their capacity to identify relevant cardiac antigens. Epitope mapping, such as recently applied by Cunningham et al. (1989), offers a more sophisticated approach. Due to the immense scope that exists for cross-reactivity between antigens (Atassi, 1975) and due to the "operational nature" of solid phase binding assays (Van Regenmortel, 1989), evidence of antigenic mimicry between cardiac and streptococcal antigens based on immunoblotting and ELISAs alone cannot be accepted as definitive. Thus the cross-reaction of the antibodies eluted from the 38-40 kD heart extract constituent with streptococcal antigens in a second immunoblot (section 3.2.2), cannot be accepted as proof of molecular mimicry between these antigens.

Shared epitopes between microbial and host antigens may be fortuitous and are no proof of an evolutionary relationship between the molecules, nor of the pathological relevance of the cross-reaction. It is somewhat puzzling that the numerous investigations into the cross-reaction between myosin and streptococcal M5 protein, have seldom been supported by hypotheses explaining how such antigenic mimicry might initiate a pathologic autoimmune response leading to the cardiovascular damage. Antibodies cross-reactive with the Ca^{2+}ATPase enzyme associated with the light chains of myosin offers an attractive hypothesis which might account for the muscle damage seen in rheumatic fever, and this study presents evidence of one ARF patient with high titres of antibody to the enzyme. Antibodies against this enzyme are known to occur in Chagas disease in which anti-heart antibodies have been found (Rose et al., 1988).

There are no previous reports in rheumatic fever in which patient lymphocytes have been used to develop human mAbs. Unlike murine mAbs, human monoclonals can offer an inside-view of the antibody repertoire relevant to the disease. Shortcomings of the present study would appear to be that the lymphocytes were derived from too young a patient (which may have contributed to the nonspecific cross-reactive nature of the mAbs) and the use

of peripheral blood lymphocytes rather than tonsillar lymphocytes which may have contributed to the instability of the hybridoma clones (section 4.1.2). The IgM mAbs isolated may be part of the natural autoantibody repertoire in these patients. This does not necessarily preclude them from being of relevance to rheumatic fever, since the relationship between natural and pathogenic autoantibodies appears to be complex and has yet to be elucidated (Chapter 1). These mAbs do not fall into either of the groups of reactivity (myosin/DNA or myosin/keratin/actin) described in a previous report on human mAbs in rheumatic fever (Cunningham et al., 1988). Possibly the most relevant result from this aspect of the study, is the binding by MA6 of a 43 kD constituent in both crude extracts of heart and in purified sarcolemmal membranes, since cardiac antigens in this mol.wt. region have been identified in several other studies using mAbs and rabbit immune sera (see Table 3.1).

The construction of two highly efficient λgt11 cDNA expression libraries of human heart genes, and the screening of them using monoclonal antibodies, rabbit immune sera and patient sera, offered the most productive and precise approach to the definition of cardiac antigens, and potentially to the identification of the cross-reactive epitopes in rheumatic fever. Patient serum containing high titres of antibodies to both heart autoantigens and to *S.pyogenes*, was considered the most relevant reagent for analysing antigenic mimicry. Serum ARF-S1 from an ARF patient with high titres of antibodies to cardiac and streptococcal antigens, was used to isolate three positive clones, using a sensitive alkaline phosphatase second antibody detection system. One of the clones identified was virtually 100% homologous with human cytokeratin 8, and 40-50% homologous with the α-helical coiled-coil proteins myosin, tropomyosin and streptococcal M proteins, all of which have been strongly implicated in antigenic mimicry in rheumatic fever. Keratin, which is antigenically related to cytokeratin, has been identified as a member of one of the two groups of host antigens (myosin/DNA and myosin/keratin/actin) binding murine and human mAbs cross-reactive with group A streptococcal antigens.

Antigens with regular repeating structures such as these coiled-coil proteins are known to have a greater potential for binding cross-reactive antibodies, and one might argue that these cross-reactions are fortuitous and irrelevant to the disease. However, the streptococcal M protein possesses such a coiled-coil structure, and the host antigens it putatively mimics could be expected to have a similar structure.

It is also known that intermediate filaments such as cytokeratin and vimentin are major targets for autoantibodies in rheumatic diseases, and also occur in normal subjects (Kurki et al., 1983). It could be argued that there is no biological mimicry between cytokeratin (or other coiled-coil proteins) and *S.pyogenes*, and that the cross-reactivity is a fortuitous consequence of autoantibody production in the host. This leaves unanswered the question as to the function and the relationship between physiologic and pathogenic

autoantibodies. In any event, autoantibodies directed against intermediate filaments such as cytokeratin offer some attractive hypotheses as to how cellular function might be impaired and initiate autoimmune disease.

Characterization of the other two positive clones detected in these libraries, as well as screening with other patient sera, is in progress and may disclose more about the nature of cardiac autoantigens in rheumatic fever. It will then be possible to determine whether these autoantigens share epitopes with the well-characterised streptococcal M proteins (or other *S.pyogenes* antigens), and perhaps to determine whether antibodies to these epitopes are cytotoxic.

6.4 Difficulties in the Study of Antigenic Mimicry

Some of the difficulties encountered in this study of antigenic mimicry in rheumatic fever might be summarised as follows: (1) the limitations of solid phase binding assays in which assay conditions have a determining influence on whether binding is observed or not, and hence on whether particular residues are scored as being part of the epitope (Van Regenmortel, 1989); (2) the frequent unavailability of adequate positive control sera, other than rabbit immune and human related-disease sera, for measuring and evaluating the relevance of cross-reactive autoantibody binding; (3) the presence of natural/physiologic autoantibodies in normal human and animal sera which makes it difficult to ascertain whether the patient autoantibodies are pathogenic; (4) the immense scope that exists for cross-reactivity within the antigenic universe, considering that determinants as short as 4-7 amino acids can be recognised by antibodies (Atassi, 1975); (5) the lack of an animal model of rheumatic fever to assess the pathogenicity of cross-reactions (associated with this is the difficulty of determining which of the many streptococcal products are pathologically important to the disease); (6) the problem of experimentally establishing cause and effect within a complex biological system (perhaps demonstrated in rheumatic fever by the inability as yet to establish whether the cardiac autoantibodies are the cause or the consequence of the disease).

6.5 Rheumatic Fever as a Model of Autoimmunity and Immune Function

The study of this complex relationship between microbe and host, and of antigenic mimicry as a possible pathogenic mechanism leading to autoimmune disease and cardiovascular damage in rheumatic fever, may offer some valuable clues to the understanding of other infectious and autoimmune diseases and lead to a deeper understanding of physiologic autoimmunity (Chapter 1) and the natural functioning of the immune system.

7. MATERIALS AND METHODS

7.A AUTOANTIBODIES AND IMMUNOBLOTTING (Chapters 2, 3) 192
7.A.1 Raising Rabbit Hyperimmune Sera 192
7.A.2 ASO and DNAse B Antibody Titres 192
7.A.2.1 Anti-streptolysin O antibodies (ASO) 192
7.A.2.2 Anti-DNAse B antibodies (DNAseB) 192
7.A.3 Group A Streptococcus ELISA 192
7.A.4 Actin ELISA 193
7.A.5 Collagen Type II ELISA 193
7.A.6 ELISAs to Cardiac Autoantigens 194
7.A.6.1 Myosin 194
7.A.6.2 Heart Extract 194
7.A.6.3 Phosphorylase b 195
7.A.7 Antinuclear Antibodies 195
7.A.8 Bacterial Cell Cultures 195
7.A.9 Immunoblotting 195
7.A.10 SDS Polyacrylamide Gel Electrophoresis 196
7.A.11 Heart Antigen Extract 197
7.A.12 Sarcolemmal Membrane Purification 197
7.A.13 Affinity Purification of Antibody on Immunoblots 198
7.A.14 Nuclear Extracts from Cultured Heart Cells 198
7.A.15 Primary Culture of Human Heart Cells 199
7.A.16 Processing of Tissue for Immunocytochemistry 199
7.A.16.1 Paraffin Wax Sections 199
7.A.16.2 Frozen Sections 199
7.A.17 Immunofluorescence on Tissue Sections 200
7.A.18 Immunocytochemistry: Biotin-streptavidin-peroxidase 200

MONOCLONAL ANTIBODIES (Chapter 4) 201
7.A.19 Cell Culture 201
7.A.20 EAC Rosettes: B Cell Purification 201
7.A.21 Monoclonal Antibody Cell Fusions 202
7.A.22 Hybridoma Screening against Heart and *S.pyogenes* 202
7.A.23 Immunoperoxidase Sandwich Assay 202
7.A.24 Production of Ascites 203
7.A.25 Cryopreservation of Hybridoma Cells 203
7.A.26 Karyotyping of Hybridomas 203
7.A.27 Immunoprecipitation of Hybridoma Immunoglobulin 203
7.A.28 Indirect Immunofluorescence: Cell Surface Antigens 204
7.A.28.1 Cell surface immunoglobulin 204
7.A.28.2 $CD5^+$ Antigen 204
7.A.29 Tissue Absorptions 204
7.A.30 Bacterial Antigen Sonicates 205
7.A.31 Bacterial Detergent Extracts: *S.pyogenes* and *E.coli* 205

7.B λGT11 CDNA LIBRARIES: CONSTRUCTION AND IMMUNOSCREENING (Chap. 5) 206

7.B.1 Preparation of RNA and Synthesis of cDNA 206
7.B.1.1 RNA Extraction 206
7.B.1.2 Poly (A)+ RNA Isolation 206
7.B.1.3 Synthesis of cDNA 207
7.B.1.3.1 S1 Nuclease Method 207
7.B.1.3.2 RNAse H Method 208
7.B.1.3.3 Random Hexanucleotide Priming Method 210
7.B.1.3.4 Calculation of cDNA Reaction Yields 210
7.B.2 Cloning of cDNA into λgt11 Expression Vector 210
7.B.2.1 T4 DNA Polymerase Blunt End Repair of cDNA 210
7.B.2.2 Methylation of Internal EcoR I Sites 210
7.B.2.3 Ligation of EcoR I Phosphorylated Linkers to cDNA 211
7.B.2.4 EcoR I Digestion of Linkered cDNA 211
7.B.2.5 Separation of Excess Linker Molecules 211
7.B.2.6 Ligation of EcoR I-Ended cDNA into λgt11 211
7.B.2.7 Packaging of λgt11 Recombinants 212
7.B.2.8 Plating and Titration of λgt11 Libraries 213
7.B.2.9 Evaluation of Libraries 213
7.B.2.10 Lambda DNA Extraction 214
7.B.2.10.1 DNA Extraction from Liquid Lysates 214
7.B.2.10.1.1 Liquid Lysate 214
7.B.2.10.1.2 DNA Extraction 214
7.B.2.10.2 Plate Lysates 215
7.B.3 Immunoscreening of λgt11 Libraries 215
7.B.3.1 Antibody Screening Procedure 215
7.B.3.2 Primary Antibody Dilution and Treatment 216
7.B.3.3 Removal of E.coli Cross-reactivity 216
7.B.3.4 Affinity Purification of Antibody on Western Blots 216
7.B.3.5 Purification of Positive Clones 216
7.B.4 Analysis of positive clones 217
7.B.4.1 Analysis of Fusion Proteins 217
7.B.4.1.1 Isolation of Recombinant Lysogens 217
7.B.4.1.2 Fusion Proteins 217
7.B.4.2 Analysis of DNA Positive Inserts 218
7.B.4.2.1 Subcloning of λgt11 Inserts into pUC19 218
7.B.4.2.1.1 In-gel Ligation of Insert into pUC19 218
7.B.4.2.1.2 Plasmid DNA Isolation 218
7.B.4.2.1.3 Small Scale (Rapid) Preparation 218
7.B.4.2.1.4 Large Scale Preparation 219
7.B.4.2.1.5 Preparation of Competent *E.coli* Cells 219
7.B.4.2.1.6 Plasmid DNA Transfection of *E.Coli* Cells 220
7.B.4.2.2 Sequencing of DNA 220
7.B.5 General Nucleic Acid Techniques 220
7.B.5.1 Formaldehyde Agarose Gel Electrophoresis of RNA 220
7.B.5.2 Agarose Gel Electrophoresis of DNA 221
7.C BUFFERS AND SOLUTIONS 222

7.A AUTOANTIBODIES AND IMMUNOBLOTTING (CHAPTERS 2, 3)

7.A.1 Raising Rabbit Hyperimmune Sera

Antisera were raised in female New Zealand White rabbits to the following antigens: (a) sonicates of group A streptococci (section 7.A.30); (b) purified cell walls and membranes of group A streptococci; (c) human heart detergent extract (section 7.A.11).

(a) *Group A streptococcal sonicates.* 500 µl of sonicate (50 µg/ml) emulsified in Freund's incomplete adjuvant (FIA) (1:1), was injected intramuscularly (i.m.) into each limb of the rabbit. Six weeks later, 250 µl per limb of sonicate was injected i.m., and then at 4-week intervals booster injections in FIA were administered s.c. at six sites along the back. Test bleeds were done very 2 weeks.

(b) *Streptococcal cell wall and membrane.* Antisera were raised by the same procedure as for streptococcal sonicates (above) except that antigens were emulsified in FCA for the first injection.

(c) *Heart extract.* Antisera were raised as for (a) above, but instead of s.c. injections, a final boost was given intravenously to the marginal ear vein 5 days before the final bleeding.

7.A.2 ASO and DNAse B Antibody Titres

7.A.2.1 Anti-streptolysin O antibodies (ASO)

Anti-streptolysin O antibody titres were measured using Streptolysin O Reagents supplied by Technicon (New York, U.S.A.), according to instructions.

7.A.2.2 Anti-DNAse B antibodies (DNAseB)

Anti-DNAse B antibodies were measured using the Streptonase-B kit supplied by Wampole Laboratories (New Jersey, USA), according to instructions.

7.A.3 Group A Streptococcus ELISA

Streptococci were grown in chemically defined medium (Van de Rijn and Kessler, 1980), washed four times in sterile PBS pH 9 (PBS-9), suspended to a concentration of $A_{660} = 0.6$, and then 50 µl per well (2.5×10^6 cfu) was added to polyvinyl chloride microtitre plates (Falcon). The bacteria were fixed by centrifuging the plate for 1 minute at 100 X g before adding 0.25% glutaraldehyde for 5-10 minutes. Plates were washed three times with sterile PBS-9, and nonspecific protein binding sites blocked with 1% BSA, 100 mM

glycine, 1% goat serum, for 1 hour at room temperature. The plates were rinsed once, sealed and stored at 4 °C until used (within 1 week). Patient sera were tested in the ELISA at an optimal dilution of 1:100 in PBS pH 7.4 for 2 hours at room temperature. The plates were washed 6 times with PBS-7.4 containing 0.01% gelatin and 0.05% Tween 20, before addition of the peroxidase conjugated antibody, goat anti-human IgG (H+L) (Cappel), for 1 hour at room temperature. After washing 10 times, the reaction was developed with ABTS for 1 hour, and the optical density was measured at 405 nm in an SLT ELISA reader. A rabbit hyperimmune serum to *S.pyogenes* was used as a method control (section 7.A.1).

7.A.4 Actin ELISA

Actin from rabbit heart (Sigma) at a concentrations of 10 µg/ml in bicarbonate buffer pH 9.5, was coated onto polyvinyl chloride microtitre plates (Falcon) (50 µl/well), and the sealed plates incubated overnight at 4°C. The plates were then washed 3 times with PBS-7.4, and nonspecific protein binding sites were blocked with 1% BSA, 1% goat serum, 100 mM glycine for 1 hour at room temperature. The plates were rinsed 3 times with PBS, and sera were added in duplicate at dilutions of 1:10, 1:100 and 1:1000, and incubated in a moist chamber for 2 hours at room temperature. After washing 6 times with PBS-7.4, peroxidase conjugated Fab'_2 goat anti-human IgG (H+L) (Cappel) antibody was added for 1 hour. The plates were washed 10 times, developed with ABTS, and the optical density was measured at 405 nm in an SLT ELISA reader. A rabbit anti-actin antiserum (Biomakor) was used as a method control.

7.A.5 Collagen Type II ELISA

Antibodies to type II collagen were measured using an ELISA method adapted from Boissier et al.(1987). NUNC Immuno Maxisorp plates were coated with bovine type II collagen (Southern Biotechnology Associates) at a concentration of 10ug/ml in sterile PBS (50 µl /well), and incubated overnight at 4°C. The plates were rinsed 3 times with sterile TBS (0.1M Tris, 0.15 M NaCl) and nonspecific antibody binding sites were blocked by adding 0.25% BSA for 1 hr at room temperature. Plates were rinsed as above and first antibody diluted 1:100 in TBS was added (50µl/well) and the plates were incubated overnight in a moist chamber at 4°C. After rinsing 5 times with TBS, peroxidase conjugated Fab'_2 goat anti-human IgM (µ) (Cappel) (dilution 1:1000) was added for 1 hr at room temperature. The plates were washed 10 times, developed with ABTS for 1 hr and the optical density read at 405 nm in an SLT ELISA reader. Goat antiserum to bovine type II collagen (South Biotechnology Associates) was used as a method control.

7.A.6 ELISAs to Cardiac Autoantigens

The following are brief descriptions of ELISAs developed for measuring antibodies to myosin, crude heart extract and phosphorylase b. The ensuing ELISAs were performed using variations of the following basic protocol.

Polyvinyl chloride (PVC) flat bottom plates (Falcon) were coated with the antigens appropriately diluted in bicarbonate buffer pH 9.5, and incubated overnight at 4°C. Alternate rows on the plate received no antigen and were incubated with buffer alone (no-antigen control). The plates were rinsed with PBS pH 7.4 containing 0.01% Tween 20 (*PBS-Tween buffer*) and nonspecific antibody binding sites on the plastic were blocked using a solution of 1% BSA, 1% goat serum, 100 mM glycine (*blocking buffer*) for 1 hour at room temperature. Plates were washed once, and first antibody at dilutions of 1:100, 1:1000 and 1:10000 in *PBS-Tween buffer* were added in duplicate to the wells (100 µl/well) and incubated for 2 hours at room temperature. A set of wells received no first antibody and were incubated with only diluent buffer (no-serum control). Plates were washed 6 times and a peroxidase conjugated Fab'_2 goat anti-human IgG (γ) antibody (Cappel) diluted 1:500 in PBS-Tween buffer (100 µl/well) was added for 1 hour at room temperature. Plates were washed 10 times, and *ABTS/H_2O_2 substrate solution* (100 µl of 40 mM ABTS added to 10 ml citric acid pH 4.0 with 100 µl of 30% H_2O_2) was added for 1 hour at room temperature. Optical density was measured at 405 nm in an SLT ELISA reader. Both the no-antigen and the no-serum controls were subtracted from each value, and the average of the duplicates calculated for each dilution. Various rabbit and human sera mentioned below were used as positive controls.

7.A.6.1 Myosin

Bovine muscle myosin (Sigma) was coated onto plates at a concentration of 5 µg/ml and a rabbit antiserum to whole myosin (Sigma) together with a goat anti-rabbit IgG peroxidase conjugated antibody (Cappel) was used as a method control.

7.A.6.2 Heart Extract

Plates were coated with CHAPS crude extracts of human heart (section 7.A.11) at a concentration of 5 µg/ml and the rabbit antiserum raised to this extract (section 7.A.1) was used as a control. In addition an ARF serum found to react strongly with the extract in this assay (and in immunocytochemistry; section 7.A.18), was included on each plate to control for interassay variability.

7.A.6.3 Phosphorylase b

Phosphorylase b (Sigma) was coated onto plates at a concentration of 20 μg/ml and the rabbit antiserum raised to the crude extract of heart (section 7.A.11), which reacted relatively strongly with this protein compared to a pool of five nonimmune rabbit sera, was used as a control. An ARF serum demonstrating a relatively high titre of antibodies to this protein, was included on each plate to control for interassay variability.

7.A.7 Antinuclear Antibodies

Antinuclear antibodies were measured by indirect immunofluorescence against the human epithelial cell line HEp-2 as a substrate, using the Behring AFT HEp System (Behring Diagnostics). Sera diluted 1:40 in PBS were added to slides and incubated in a moist chamber for 30 minutes at room temperature. The slides were rinsed and then washed with PBS in a Coplin jar with gentle stirring for 10 minutes. Excess moisture was wiped off and the FITC-conjugated labelled goat anti-human IgG (H+L) antibody conjugate was added, for 30 minutes at room temperature. The slides were washed as before, mounted in glycerol, and examined under a Nikon Optiphot microscope (PlanApo 60 X / 1.4 oil lens) with camera attachment (FX-35A and Kodak 400 ASA colour film). Sera from SLE patients were used as a positive control. Sera demonstrating more than one pattern of binding were titrated.

7.A.8 Bacterial Cell Cultures

Group A streptococci (*S.pyogenes*) were cultured from a throat swab of a patient with acute rheumatic fever. This culture was grown in Todd-Hewitt broth or in chemically defined medium (Van de Rijn and Kessler, 1980) and was used as the solid phase antigen in the Group A Streptococcal ELISA (section 7.A.3) as well as for preparing cell sonicates (section 7.A.30).

A panel of streptococcal group serotypes comprised of group B (*S.agalactiae*), group C (*S.equisimitis*), group D and group G (*S.auginosi*) were obtained from the South African Institute for Medical Research (SAIMR) in Johannesburg, and from the Department of Bacteriology at Red Cross Children's Hospital in Cape Town, South Africa. *Escherischia coli* were obtained from the American Type Culture Collection (ATCC: 25922). These bacteria were maintained in TSB broth.

7.A.9 Immunoblotting

Proteins were separated by SDS polyacrylamide gel electrophoresis (section 7.A.10) and transferred to nitrocellulose by electroblotting. The gel was equilibrated for 30 minutes in transfer buffer (0.05 M Tris, 0.3 M glycine, 20% v/v methanol) before transfer onto nitrocellulose (Schleicher and Schuell BA85, 0.45 μm) in a "sandwich" comprised of layers of 3MM Whatman paper and

scotchbrite pads. Transfers were done at 600 mA constant current for 3 hours at 4°C, or else overnight at 100 mA. Duplicate blots were made to assess the efficiency of the transfer, by staining the membrane with either Amido Black or with *AuroDye forte* (Janssen Life Sciences). Nonspecific protein binding sites on the unstained membrane were blocked with 2% BSA, 1% goat serum, 0.05% Tween 20 in TBS buffer (20 mM Tris-HCl, 0.9% NaCl, pH 7.5) for 1 hour at room temperature. The blots were briefly rinsed in TBS, and incubated with the first antibody diluted in 0.2% BSA, 0.1% goat serum, 0.05% Tween 20, for 2 hours at room temperature, with shaking. After three 10-minute washes in TBS, the blots were incubated with the peroxidase conjugated second antibody for 1 hour at room temperature, washed again as before, and developed with 4-chloro-1-napthol (section 7.C).

7.A.10 SDS Polyacrylamide Gel Electrophoresis

Separating gel 10%:

For a 10% separating gel the following solution was mixed:

acrylamide:bisacrylamide (39:1)	8.0 ml
1 M Tris-HCl pH 8.8	9.25 ml
7.5 % glycerol	0.75 ml
Distilled water	13.6 ml

This mixture was degassed, and the following added and mixed:

10 % SDS	400 µl
TEMED	32 µl
10 % ammonium persulphate	320 µl

The gel was cast in a Hoefer 600 SE vertical slab gel apparatus, and allowed to set (covered with a thin layer of water) before addition of the stacking gel.

Stacking Gel (4%):

For a 4% stacking gel the following solution was mixed:

acrylamide:bisacrylamide (39:1)	3.0 ml
0.125 M Tris-HCl pH 6.8	26.7 ml

The solution was degassed before the following were added and mixed:

10% SDS	300 µl
TEMED	180 µl
10% ammonium persulphate	37.5 µl

The stacking gel was poured on top of the set separating gel, a comb was inserted and the gel left to set at room temperature.

Protein samples (20-50 μg protein) were prepared in an equal volume of *sample buffer* comprised of 48 mM Tris-HCl, 2.5% SDS, 20% glycerol, 0.25% bromophenol blue and 5% ß-mercaptoethanol. Samples were boiled for 3-5 minutes and loaded immediately. The gel was run in *SDS-PAGE running buffer* (0.025 M Tris-HCl, 1.5% glycine, 0.1% SDS, pH 8.3). The gel was run at 25 mA until samples had just entered the separating gel and thereafter at 35 mA. The gel was stained in 0.1% Coomassie brilliant blue (R250) in 33% methanol and 11% trichloroacetic acid (w/v). Destaining was done in 20% ethanol, 0.7% acetic acid.

7.A.11 Heart Antigen Extract

Heart tissue was obtained either at transplantation surgery or at autopsy (within 8 hours of death) and stored in liquid nitrogen. The tissue was ground to a powder under liquid nitrogen using a mortar and pestle, and then homogenized on ice using an Ultra Turrex homogenizer at maximal setting (for three 30-second bursts) in *heart extract homogenization buffer* (20 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM PMSF pH 8.0). The homogenate was centrifuged at 800g for 10 minutes and the supernatant filtered through a double layer of cheesecloth. The filtrate was centrifuged at 800g for 5 minutes, passed through cheese-cloth as before, and centrifuged at 10 000g for 60 minutes in a Beckman L8-70 ultracentrifuge. The pellet was resuspended (1 ml/gram of original tissue) in *heart extract solubilization buffer* (20 mM Tris-maleate, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM PMSF, 1 mM thymol, pH 7.25) containing 5 mM CHAPS detergent (Sigma). The solubilization proceeded for 1 hr at 4°C on a Coulter roller. The extracted proteins were centrifuged at 10 000g for 5 minutes, and the supernatant aliquoted into sterile tubes and stored at -70°C.

7.A.12 Sarcolemmal Membrane Purification

Sarcolemmal membranes were purified from human and rabbit heart essentially by the method of Van de Rijn et al. (1977). 10 g of heart tissue (obtained at surgery or postmortem) was pulverised under liquid nitrogen using a mortar and pestle and then homogenised in 50 ml of 0.05 M $CaCl_2$ using an Ultra Turrex homogenizer (two 30-second bursts). The slurry was washed three times in cold PBS pH 7.2 by centrifugation at 2000g for 10 minutes at 4°C. The final pellet was resuspended and incubated for 3 hours at 4°C in PBS pH 7.2 containing 5 mM $CaCl_2$, 20 μg/ml DNAse I, 20 μg/ml RNAse. The suspension was centrifuged at 14 000g for 30 minutes in an SW27 rotor (Beckman L8-70 Ultracentrifuge), and the pellet was suspended at 100-fold weight/volume concentration in distilled water containing 1 mM PMSF for 24 hours at 4°C, with stirring. The slurry was centrifuged at 14 000g for 30 minutes, and the pellet frozen in liquid nitrogen and lyophilized. The membrane extraction was done by incubating 1 g of lyophilized heart extract with 100 ml of 3 M KCl for

18 hours at 4°C. The extract was centrifuged at 10 000g for 30 minutes, the supernatant was kept, and the pellet re-extracted with 50 ml of 3 M KCl for 8 hours at 4°C, followed by centrifugation as before. The second supernatant was also kept, and the pellet extracted with 25 ml of 3 M KCl for 16 hours at 4°C, followed again by centrifugation. The third supernatant was pooled with the first two, dialyzed extensively against 0.01 M ammonium bicarbonate, and lyophilized.

7.A.13 Affinity Purification of Antibody on Immunoblots

A modification of the three-step procedure for elution of antibody from nitrocellulose bands described by Smith and Fisheer (1984) was found to be more effective than the single step low pH elution originally described by Olmsted (1981).

Protein extracts were separated in a single well by SDS PAGE, transferred to nitrocellulose, and the relevant polypeptide bands were located by developing vertical strips from either side of the nitrocellulose. The excised strips were incubated in TBS buffer (20 mM Tris-HCl, 0.9% NaCl) containing 2% BSA and 0.05% Tween 20 for 1 hour at room temperature, followed by brief rinsing. The strips were incubated with first antibody diluted 1:50 in TBS for 2 hours at room temperature, then washed 3 times for 10 minutes each in TBS/0.05% Tween 20, and the bound antibody was eluted in three, using (1) 500 μl of 5 mM glycine-HCl pH 2.3 and 500 mM NaCl, for 3 minutes, followed by immediate neutralization of the eluate in 50 mM Na_2HPO_4 and 2% BSA; (2) 500 μl of 0.5% Tween 20 in TBS for 3 minutes; (3) 500 μl of 3 M KSCN, 150 mM KCl, 10 mM $NaPO_4$, for 3 minutes. Between each step, the remaining nitrocellulose strip was immediately rinsed in TBS, and the eluates were pooled. To accumulate sufficient eluate for probing secondary immunoblots, additional elutions were done from new antigen transfers.

7.A.14 Nuclear Extracts from Cultured Heart Cells

Crude extracts of nuclear proteins were prepared from both Girardi heart cells (ATCC: CCL-27) and from a primary cultures of human heart (endothelial-like) cells (section 7.A.15). Cells were grown to confluence and harvested by scraping with a rubber policeman, washed with cold PBS and centrifuged at 2000 rpm for 10 minutes at 4°C. The pelleted cells were suspended in 5 cell volumes of PBS and centrifuged again as before. The cells were resuspended in 5 volumes of *Dignam buffer A* (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT), left on ice for 10 minutes and again centrifuged. The cells were suspended in 2 volumes of *buffer A* and then lysed with 10-15 strokes of a Dounce homogenizer. Homolysis of the cells was checked microscopically. The homogenate was centrifuged as before to pellet the nucleii, the supernatant was discarded and the pellet recentrifuged at 25 000g for 20 minutes to remove residual cytoplasmic debri. The pellet containing the nucleii was then suspended in 3 ml/10^9 cells of *Dignam buffer C* (20 mM

HEPES pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% v/v glycerol, 0.5 mM DTT, 0.5 mM PMSF). Nucleii were homogenised by 10 strokes of a Dounce homogenizer, the suspension gently stirred on ice for 30 minutes, and then centrifuged at 25 000g for 30 minutes. The supernatant was dialysed for 5 hours against 50 volumes of *Dignam buffer D* (20 mM HEPES, 0.1 M KCl, 0.2 mM EDTA, 20% v/v glycerol, 0.5 mM DTT, 0.5 mM PMSF). The dialysate was centrifuged at 25 000g for 20 minutes, and the supernatant stored in 50 μl aliquots at -80°C.

7.A.15 Primary Culture of Human Heart Cells

A primary culture of cells derived from heart tissue was developed using the method of Kasten (1973). Heart tissue obtained at surgery (Fallot's tetralogy) was washed in Minimum Essential Medium (MEM), weighed, cut into 1 mm^2 pieces and digested with 0.125% trypsin in an Erlenmeyer flask for 10 minutes at 37°C with stirring. The supernatant was removed and 20 ml of fresh trypsin per gram of original tissue was added for 15 minutes at 37°C. The digest was centrifuged at 1300 rpm and the supernatant decanted and inactivated with 2 ml MEM. The trypsinization was repeated on the remaining digest, and the two supernatants were pooled. The entire process was repeated again on the residual tissue and the two sets of supernatants were pooled, washed twice in fresh MEM and the cell concentration adjusted to about 6 X 10^5 cells/ml. Cells were plated into petri dishes at 1.5 X 10^5 cells/ml and maintained in MEM containing 10% FCS, 20 mM HEPES and antibiotics (section 7.A.19). The differential attachment technique recommended for obtaining higher yields of myocardial cells was not successful, and electron microscopy showed that the cells were endothelial in morphology.

7.A.16 Processing of Tissue for Immunocytochemistry

All slides were first thoroughly cleaned in chromic acid followed by methanol, and rinsing in water.

7.A.16.1 Paraffin Wax Sections

Tissue obtained from surgery was fixed in Carson's formal buffered saline (40% formaldehyde, 120 mM $NaH_2PO_4.H_2O$, pH 7.2). It was then processed to wax (Shandon Processor 2LE) and 4 μm sections were cut on a microtome (Reichert-Jung 2040), picked up on glass slides and incubated at 65°C for 20 minutes. Slides were stored at room temperature.

7.A.16.2 Frozen Sections

Tissue excised at surgery was immediately frozen in liquid nitrogen, and 4 μm thick cryostat sections were cut, picked up on glass slides and stored at -20°C.

7.A.17 Immunofluorescence on Tissue Sections

Sections of frozen tissue were cut and lifted onto glass slides, as described (section 7.A.16.2). Nonspecific antibody binding sites were blocked by incubating sections for 30 minutes in a moist chamber with 20% goat serum in PBS (if the second antibody was raised in goat). The blocking solution was blotted off gently and the first antibody, optimally diluted in PBS, was added to sections and incubated for 30 minutes in a moist chamber at room temperature. Slides were rinsed, avoiding cross-contamination between wells, and then immersed in a bath of fresh PBS for 10 minutes with gentle stirring. Excess moisture was removed from around the wells, while ensuring the sections remained moist, before addition of the FITC-conjugated second antibody for 30 minutes. Slides were rinsed and washed as before, mounted in sterile 30% glycerol in PBS, and examined and photographed using a Nikon Optiphot microscope with camera attachment (Nikon FX-35A).

7.A.18 Immunocytochemistry: biotin-streptavidin-peroxidase

Paraffin wax sections of human heart tissue (section 7.A.16.1) were dewaxed in xylene, rehydrated and washed in a bath of PBS pH 7.4 with gentle stirring. Endogenous peroxidases in the tissue were inhibited by immersing the slides for 10 minutes in 0.3% H_2O_2 in methanol followed by rinsing and washing for 5 minutes in bath of tap water. To expose cross-linked antigens, sections were immersed for 12 minutes in a freshly prepared 37°C solution of 0.1% trypsin (Sigma type II) in 0.1% $CaCl_2$ pH 7.8. Slides were then immediately rinsed under cold tap water, transferred for 5 minutes to a bath of distilled water at 4°C, and washed twice for 5 minutes in a bath of PBS at room temperature. Nonspecific antibody binding sites were blocked by incubating the slides for 10 minutes with 10% nonimmune goat serum in PBS. The solution was blotted off gently and the sections were incubated with the first antibody (sera diluted 1:5 in PBS) overnight in a moist chamber at 4°C. Sections were washed 3 times for 10 minutes each in fresh baths of PBS, excess fluid was wiped off, and the biotinylated goat anti-human (or rabbit) IgG (H+L) second antibody (Zymed) was added at a dilution of 1:100 in PBS for 10 minutes at room temperature. The streptavidin-peroxidase conjugate (Zymed) was added for 5 minutes at room temperature. The slides were washed twice for 5 minutes each in PBS, and the *H_2O_2-AEC substrate-chromogen* mixture was added for 15 minutes at room temperature. Slides were washed twice for 5 minutes each in distilled water, counterstained with haematoxylin for 3 minutes, rinsed again in tap water and then mounted in GVA solution and examined using a Nikon Optiphot microscope with camera attachment (FX-35A).

MONOCLONAL ANTIBODIES (CHAPTER 4)

7.A.19 Cell Culture

Cells were cultured at 37°C in a humidified air incubator in 95% air:5% CO_2.

1. The *mouse myeloma cell line Sp2/0-Ag-14* was cultured in RPMI 1640 supplemented with 20 mM HEPES, 0.2% bicarbonate, 15% heat-inactivated FCS (Flow), azaguanine (15 μg/ml) and antibiotics (100 U/ml penicillin; 100 μg/ml streptomycin). Two days before cell fusion, the cells were seeded at a concentration of 2 X 10^5cells/ml.

2. The *hybridoma cell lines* were routinely seeded at 2 X 10^5 cells/ml in 100 mm diameter dishes (Falcon) and cultured in HT medium with 15% FCS. They were subcultured twice weekly using Versene (section 7.C).

3. *Girardi heart cells* (ATCC: CCL-27) were cultured in Minimum Essential Medium (MEM) supplemented with 5% FCS, 20 mM HEPES buffer and antibiotics (penicillin 100 U/ml; streptomycin 100 μg/ml). The cells were subcultured twice weekly by detaching them with 0.05% trypsin in PBS containing 10 mM EDTA. They were split at ratios between 1:7 and 1:10.

4. A *primary culture of human heart (endothelial-like) cells* was prepared by the method of Kasten (1973) using human heart tissue obtained at surgery from a child with a congenital heart defect (section 7.A.15). The cells were maintained in Minimum Essential Medium supplemented with 10% FCS, 20 mM HEPES and antibiotics (penicillin 100 U/ml; streptomycin 100 μg/ml).

7.A.20 EAC Rosettes: B cell purification

Lymphocytes were separated by centrifugation at 800g for 15 minutes through Ficoll-Isopaque according to standard procedures, and the cell count adjusted to 15 X 10^6 cells per ml.

Fresh sheep red blood cells (SRBC) were washed three times (1200 rpm) in ice-cold 0.15 M PBS (pH 7.4), adjusted to 5% (v/v) in 10 mls of PBS and centrifuged at 2500 rpm for 10 minutes at 4°C. 2 ml of a 4% sterile solution of AET (pH 9) was added to the supernatant and gently mixed. The cells were incubated at 37°C for 15 minutes, washed again as above (1500 rpm) with a final wash in 10 ml of RPMI containing 20 mM HEPES, 20 mM bicarbonate, 10% FCS, pH 7.3. 500 μl of these packed cells were diluted in 10 ml of the same RPMI buffer (without FCS) to give a 5% AET-SRBC suspension. Equal volumes of 5% AET-SRBC and separated lymphocytes were mixed, centrifuged at 250g for 5 minutes and left at room temperature for 2 hours. The

suspension was centrifuged through Ficoll-Isopaque at 400g for 40 minutes at 20°C, the supernatant was removed and the nonrosetted B cells at the interface were washed twice in RPMI and resuspended in *GKN buffer* at a concentration of 2 X 10^6 cells/ml, for hybridoma cell fusion.

7.A.21 Monoclonal Antibody Cell Fusions

Equal concentrations (2 X 10^6 cells/ml) of non-rosetted PBL and SP2 mouse myeloma cells were mixed in *GKN buffer* (Dulbecco's PBS, 2 g/l glucose, 10 mg/l phenol red) and warmed at 37°C for 5 minutes. The mixture was centrifuged at 650 rpm for 5 minutes, the supernatant removed and the pellet gently resuspended. The cells were fused by adding 1.5 ml of 50% polyethylene glycol (PEG) mol.wt. 4000 (Merck) in RPMI 1640 for 2 minutes, and then gradually diluting them with 10 volumes of *GKN buffer* over a period of 5 minutes. The volume was made up to 45 ml with *GKN buffer* and the suspension centrifuged at 650 rpm for 3 minutes. The pellet was diluted to a concentration of 1.6 X 10^6 cells/ml in *H.A.T medium* (10^{-4} M hypoxanthine, 4 X 10^{-7} M aminopterin, 1.6 X 10^{-5} M thymidine, 3 X 10^{-6} glycine, 10^{-3} M sodium pyruvate) containing 20% FCS and 20% SP2 conditioned medium (about 25 ml). The cell suspension was seeded at 0.5 ml/well into 24-well plates (Costar) and an additional 0.5 ml/well of *H.A.T medium* added, giving a final concentration of 8 X 10^5 cells/ml in each well. The plates were incubated at 37°C, and after 7 days 0.5 ml of the medium was replaced with fresh *HAT medium*. After a further 3 days, cultures were replenished with 0.5 ml of *HT medium* containing 20% FCS. Supernatants were then screened for antibody production.

7.A.22 Hybridoma Screening against Heart and S.pyogenes

Screening generally commenced 10-14 days after cell fusion, and was repeated every 2 days till day 20. Hybridoma supernatants were assayed for antibody against whole group A streptococci by ELISA (section 7.A.3) and against paraffin-embedded human heart sections by an immunoperoxidase second antibody assay (section 7.A.23). Positive clones were subcloned within 2 days at dilutions of 10-and 3 cells per well for the first subcloning, and at 3- and 0.5 cells per well subsequently. Culture wells were screened for antibody 11-14 days after each subcloning. Selected clones were expanded in *HT medium* at seeding densities of 2 X 10^5 cells/ml, and assayed for antibody 4 days later. Confirmed positive clones were injected intraperitoneally (10^7 cells) into pristane-primed nude mice to produce ascites, which was usually extracted about 3 weeks later.

7.A.23 Immunoperoxidase Sandwich Assay

Paraffin-embedded sections of human heart (section 7.A.16.1) were dewaxed and hydrated, and nonspecific antibody binding sites were blocked by the addition of 20% goat serum in PBS pH 7.4 for 40 minutes at room

temperature. The blocking solution was blotted off, and sections were incubated with neat hybridoma culture fluid for 40 minutes in a moist chamber at room temperature. After washing in PBS for 10 minutes, sections were incubated with a peroxidase conjugated Fab'_2 goat anti-human IgG, IgA, IgM antibody (Cappel) (dilution 1:50) for 40 minutes at room temperature. After washing as before, the *aminoethylcarbizole AEC/H_2O_2 substrate* was added for 30 minutes. The slides were rinsed in PBS, counterstained in Mayer's haematoxylin and mounted.

7.A.24 Production of Ascites

Hybridomas failed to grow in BALB/c mice and were therefore grown in nude mice. Mice were primed for development of ascites by injecting 0.5 ml of pristane (2,6,10,14-tetramethyldecanoic acid) (Sigma) into them intraperitoneally at least 2 weeks prior to introducing the hybridomas (10^7 cells in 0.5 ml PBS). 2-3 weeks later, ascites was extracted (after killing the mice by cervical dislocation), centrifuged to remove cells, aliquoted and stored at -80°C.

7.A.25 Cryopreservation of Hybridoma Cells

Hybridoma clones were preserved either within the plate by removing the supernatants, adding 200 µl/well of 10% DMSO in *HT medium* containing 15% FCS (at 4°C), and then storing the sealed plate at -80°C; or by storing individual clones in the same DMSO solution at densities of 1-5 X 10^6 cells/ml.

7.A.26 Karyotyping of Hybridomas

Hybridoma cultures in logarithmic phase of growth were arrested in mitosis by treatment with 0.2 µg/ml of colcemid (Gibco) for 45 minutes at 37°C. The cells were pelleted at 1500 rpm for 5 minutes and lysed in hypotonic solution (0.075 M KCl) for 20 minutes at room temperature. After centrifugation, cells were washed 4 times in cold fresh methanol:glacial acetic acid (3:1), suspended in 0.5 ml of this fixative and dropped from 40 cm onto cold glass slides. Chromosomes were trypsin-Giemsa banded by immersing slides in 0.05% trypsin for about 30 seconds. The slides were washed twice in PBS, air-dried, stained in 2% Giemsa pH 6.8 for 7 minutes, and mounted in DPX (BDH).

7.A.27 Immunoprecipitation of Hybridoma Immunoglobulin

Immunoprecipitations of hybridoma cell lysates and culture supernatants were performed according to a method adapted from Choi (1977). Approximately 5 X 10^6 hybridoma cells in logarithmic phase of growth were labelled with 5 µCi of ^{14}C-leucine for 24 hours. Cells were harvested by scraping and lysed in 1 ml of 1% Nonidet P-40 in 50 mM Tris, 25 mM KCl, 5 mM $MgCl_2$ pH 7.6. The cell lysate and supernatant were preprecipitated first for 1 hour at 37°C then

overnight at 4°C, using anti-chicken ovalbumen serum and a suboptimal concentration of chicken ovalbumen (Behring). Specific precipitations were performed for 3 hours at room temperature with the addition to the supernatant and lysate of optimal concentrations of Fab'$_2$ goat anti-human IgG (H+L) (Cappel) as well as total human gamma globulin (1:50 dilution). The radiolabelled precipitates were washed once with 0.5% Nonidet P-40 followed by three washes with PBS, dried overnight at 37°C and electrophoresed on 10 % SDS polyacrylamide gels. The gels were dried and exposed to X-ray film (Kodak XR-Xomat5). (As a control, PBL from a normal individual were stimulated with pokeweed mitogen in culture for 6 days prior to being similarly immunoprecipitated).

7.A.28 Indirect Immunofluorescence: Cell Surface Antigens

7.A.28.1 Cell surface immunoglobulin

Hybridoma cells were washed in PBS and resuspended in 200 μl at a density of 5 X 10^6 cells/ml in 4 separate Falcon tubes. Fab'$_2$ rabbit anti-human immunoglobulin IgG, IgM and IgA class specific antibodies (Cappel) were added to the separate tubes, the suspensions were mixed, and incubated for 30 minutes at 4°C. The cells were centrifuged twice in 2 ml of cold PBS at 400g, and the pellet suspended in 100 μl of PBS. 20 μl of a 1:20 dilution of goat anti-rabbit Fab'$_2$ FITC-conjugated antibody (Cappel) was added to each tube, and the suspension was incubated for 30 minutes at 4°C. Cells were washed twice as before, mounted in 30% sterile glycerol in PBS and examined under an Olympus BH2 microscope with epifluorescent illumination.

7.A.28.2 CD5$^+$ Antigen

Hybridomas were tested for CD5$^+$ antigen essentially by the same method (above) using a murine anti-CD5 monoclonal antibody (Dako:M705) and a FITC-conjugated goat anti-mouse IgG (γ) second antibody (Cappel).

7.A.29 Tissue Absorptions

Protein extracts of kidney and liver tissue for use as absorbents in determining antibody specificity, were prepared using CHAPS detergent by the same procedure as for the heart extract (section 7.A.11). Tissues were obtained at autopsy (less than 8 hours post mortem) and stored in liquid nitrogen until processed. The absorbent was made by mixing equal concentrations of the two extracts, centrifuging the mixture in a microfuge for 5 minutes, and washing the pellet three times in Tris-maleate buffer pH 7.25. The pellet was suspended in PBS in the same volume as the original mixture, aliquoted and lyophilized.

To remove nonspecific antibody binding (to heart), 10 μl of freeze-dried liver-kidney absorbent was added to 200 μl of serum diluted 1:10 in PBS pH 7.4. (Rabbit antisera were diluted 1:1000 for absorptions). The absorption mixtures were gently rotated on a Coulter Roller at 4°C overnight, and then centrifuged in a microfuge for 10 minutes to remove the absorbent with bound nonspecific antibody.

7.A.30 Bacterial Antigen Sonicates

Bacterial cultures in logarithmic phase of growth were washed twice in 0.01 M Tris-HCl pH 7.4 by centrifugation for 10 minutes at 10 000g. The density of the cultures was adjusted to a turbidity of E_{470} 5.0. Bacteria were sonicated on ice at 18 microns (six 1-minute bursts with 30 second intervals) and unbroken cells were removed by centrifugation at 4000 rpm for 10 minutes. The supernatant was centrifuged at 48 000g for 45 minutes and the pellet consisting of bacterial cell walls was adjusted to a turbidity of E_{470} 5.0 in 0.01 M Tris-HCl pH 7.4, and stored at -80°C.

7.A.31 Bacterial Detergent Extracts: *S.pyogenes* and *E.coli*

Bacterial cultures in logarithmic phase of growth were washed 3 times in sterile normal saline and adjusted to a turbidity of $OD_{660} = 600$ in a volume of 3 ml saline (equivalent to about 2.5 X 10^8 *S.pyogenes* cells and 3.6 X 10^8 *E.coli* cells). Suspensions were sonicated on ice at 20 microns (three 1-minute bursts with 30 second intervals), and then incubated with 5 mM CHAPS for 30 minutes at 4°C. The cell debris was removed by centrifuging in a microfuge for 5 minutes, and the supernatants were lyophilized.

7.B λGT11 CDNA LIBRARIES: CONSTRUCTION AND IMMUNOSCREENING (CHAPTER 5)

7.B.1 Preparation of RNA and Synthesis of cDNA

All RNA was handled in an RNAse-free environment as described in detail by Blumberg (1987).

7.B.1.1 RNA Extraction

RNA was extracted by the method of Chomczynski and Sacchi (1987). Heart tissue was snap-frozen in liquid nitrogen within half-a-minute of excision at surgery. Isolations were made either from accumulated tissue derived from atrial appendage of non-rheumatic hearts, or from a heart removed at transplant surgery.

5 g of frozen tissue was crushed, and ground to a powder under liquid nitrogen and homogenized in 50 ml of *RNA extraction solution D* (4 M guanidinium isothiocyanate; 25 mM sodium citrate pH 7; 0.5% sarcosyl, 0.1M ß-mercaptoethanol) using an Ultra Turrex homogenizer. The following solutions were then added sequentially, with thorough mixing after each addition: 5 ml of 2 M sodium acetate, 50 ml of water-saturated phenol and 10 ml of chloroform-isoamyl alcohol mixture (49:1). The final suspension was shaken vigorously and cooled on ice for 15 minutes, followed by centrifugation at 10 000g for 20 minutes. The aqueous phase was removed, mixed with 50 ml of isopropanol, and placed at -20°C for at least 1 hour to precipitate the RNA. Following centrifugation at 10 000g for 20 minutes, the resulting RNA pellet was dissolved in 15 ml of *solution D* and precipitated with 1 volume of isopropanol. After centrifugation for 10 minutes at 4°C, the RNA pellet was washed twice in 70% ethanol, vacuum dried and dissolved in 50 µl of 0.1% SDS at 65°C for 10 minutes. It was then either used immediately for poly $(A)^+$ RNA isolation, or stored in 70% ethanol at -80°C. The RNA was checked by fractionation on a formaldehyde 1% agarose gel (section 7.B.5.1), stained with ethidium bromide, and its integrity ascertained by the intactness of the 18S and 28S ribosomal RNA.

7.B.1.2 Poly $(A)^+$ RNA Isolation

Great care was taken to prevent degradation of poly $(A)^+$ RNA by RNAses, and to prevent loss of RNA through sticking to glass and plastic surfaces. Procedures recommended by Blumberg (1987) for creating an RNAse-free environment were followed. All glassware used was pretreated by soaking for 24 hours in 0.1% diethylpyrocarbonate (DEPC), followed by autoclaving, and siliconization with 2% dimethylsilane in CCl_4 for 6 hours. It was then baked at

140°C for 3 hours, treated with 0.01% DEPC followed again by baking and autoclaving.

The oligo-dT cellulose affinity column was prepared in a pasteur pipette, and plugged with silanized glass wool before addition of 0.5 g of oligo-dT cellulose (Collaborative Research) slurry in distilled water. The column was then washed with 10 ml of 0.1 M NaOH, and equilibrated with 10 ml of 0.01 M Tris pH 7.5, 0.5 M NaCl.

The RNA sample was centrifuged for 10 minutes to remove insoluble precipitates before adjusting its concentration to about 20 OD_{260} units/ml in equilibration buffer. The flow rate of the column was 20-30 ml/hr, and the RNA eluate was re-applied twice. Poly $(A)^-$ RNA was removed by washing 20 ml of equilibration buffer through the column. The poly $(A)^+$ RNA was then eluted from the column using a a low salt buffer (0.01 M Tris pH 7.5). Fractions of 1 ml were collected until there were no more traces of nucleic acids (A260=0). Fractions containing eluted RNA were pooled and precipitated with 1/10 volume of ammonium acetate pH 5.5, and 2.5 volumes of ethanol at -20°C. The poly $(A)^+$ RNA was stored as a precipitate until needed, whereupon it was washed twice in 70% ethanol, dried and dissolved in sterile distilled water for immediate use.

7.B.1.3 Synthesis of cDNA

cDNA was synthesized by three different methods referred to as: (1) the S1 nuclease method; (2) RNAse H method; (3) random hexanucleotide priming method. Reagents from various suppliers were used for the first two methods. The *Amersham cDNA Synthesis System Plus* was used for cDNA synthesis by the random hexanucleotide primers method (and also by the RNase H method). Several variations of each cDNA synthesis method were tried. The S1 nuclease method used in *Library A* (see below) was adapted from Maniatis et al. (1982) and Efstratiadis et al. (1978); the RNAse H method used in *Library B* was based on a protocol recommended by Promega Biotec (Madison, USA); and the RNAse H and random priming methods used in *Library A* followed the instructions of the *Amersham cDNA Synthesis System Plus*.

(Avian reverse transcriptase supplied by Seikagaku, Promega or Amersham was used).

7.B.1.3.1 S1 Nuclease Method

Poly $(A)^+$RNA was dissolved in sterile water at 1 mg/ml, and 2 μl transferred to an RNAse-free, siliconized microfuge tube. 0.5 μl of 100 mM methylmercuric hydroxide was added, and the tube heated at 70°C for 5 minutes followed by quick cooling on ice. To a second sterile microfuge tube, 20 μCi of ^{32}P-dCTP

(>400 Ci/mmol) was added and the following reagents were added at room temperature, in order:

dATP	10 mM	5 µl
dGTP	10 mM	5 µl
dTTP	10 mM	5 µl
dCTP	1 mM	1 µl
5 X RT buffer		20 µl
DTT	200 mM	5 µl
RNAsin	50 units	2 µl
Oligo (dT) primer	1 mg/ml	1 µl
Poly $(A)^+$RNA	1 mg/ml	2 µl
Sterile H_2O		50 µl
Reverse Transcriptase	40 units	4 µl
TOTAL VOLUME		100 µl

The mixture was incubated at 41°C for 1 hour, then heated at 100°C for 5 minutes, cooled on ice and the denatured proteins pelleted by centrifugation for 5 minutes. The supernatant was transferred to a microfuge tube containing 20 µCi ^{32}P-dCTP (dried down), and the following added:

(supernatant)		x µl
10 X Klenow buffer		20 µl
dATP	10 mM	5 µl
dGTP	10 mM	5 µl
dTTP	10 mM	5 µl
dCTP	10 mM	5 µl
sterile H_2O		x µl
DTT	200 mM	2 µl
Klenow polymerase 1	20 units	4 µl
TOTAL VOLUME		200 µl

The reaction was incubated at 18-20°C for 20 hours, extracted with chloroform and unincorporated nucleotides removed by chromatography through Sephadex G50. Fractions, checked by Cerenkov counting, showed two elution peaks the first of which (usually fractions 5-9) contained the cDNA. These were pooled, lyophilized, and then reconstituted in 89 µl of sterile water. 10 µl of 10 X S1 nuclease buffer and 1 unit (1 µl) of S1 nuclease enzyme was added, and the reaction incubated at 37°C for 20 minutes. The mixture was again extracted with chloroform, fractionated on Sephadex G50 and fractions containing double-stranded cDNA were pooled. (Calculation of cDNA reaction yields was done as described below).

7.B.1.3.2 RNAse H Method

Synthesis of cDNA by this method, was done either using the *Amersham cDNA Synthesis System Plus* according to instructions, or (more efficiently) following the protocol below, using mainly Promega Biotec reagents.

2 μg of Poly (A)$^+$RNA and 1 μg of oligo(dT) primer were added to an RNAse-free microfuge tube, made up to 10 μl with sterile water and then heated at 70°C for 5 minutes. The reaction was cooled to room temperature, briefly microfuged, and the following reagents added at room temperature to the annealed primer-template, in order:

(RNA-oligo(dT) in water	1 μg/2 μg RNA	10 μl)
10 X RT Buffer	(see below)	2.5 μl
DTT	100 mM	2.5 μl
Na pyrophosphate	40 mM	2.5 μl
dNTP mix	10 mM	2.5 μl
RNAsin	20 units	1 μl
Reverse transcriptase	30 units	3 μl
Sterile H_2O		1 μl
TOTAL VOLUME		25 μl

Reagents were mixed and 5 μl removed to a separate tube containing 2-5 μCi (>400 Ci/mmol) of ^{32}P-dCTP, for measuring first strand synthesis by TCA precipitation (see below). Both tubes were incubated at 42°C for 1 hour and then placed on ice. To the 20 μl tube, add the following, in order:

(First strand reaction		20 μl)
10 X 2nd strand buffer	(see below)	10 μl
DTT	100 mM	3 μl
NAD	1 mM	10 μl
RNAse H	0.8 units	x μl
DNA polymerase 1	25 units	x μl
DNA ligase	1 unit	x μl
^{32}P-dCTP	2-5 μCi	x μl
Sterile water		up to 100 μl
TOTAL VOLUME		100 μl

Reagents were mixed gently, and incubated at 15°C for 2 hours. The reaction was stopped by heating at 70°C for 10 minutes, microfuged, cooled on ice and then 4 units of T4 DNA polymerase (2 units/μg RNA) were added and the tube incubated at 37°C for 10 minutes. The reaction was stopped by addition of 10 μl of 0.2 M EDTA and placed on ice. 5 μl was removed for measuring second strand synthesis, and the remainder was extracted with phenol:chloroform and precipitated by addition of half a volume of 7.5 M ammonium acetate and 3 volumes of ice cold ethanol, followed immediately by centrifugation for 15 minutes at room temperature. The supernatant was washed with 500 μl of cold 70% ethanol, dried and reconstituted in 25 μl of *TE buffer*.

10 X First strand buffer: 500 mM Tris-HCl, pH 8.3; 750 mM KCl; 100 mM $MgCl_2$; 5 mM spermidine
10 X Second strand buffer: 500 mM Tris-HCl, pH 7.6; 1 M KCl; 50 mM $MgCl_2$; 100 mM ammonium sulphate; 500 μg/ml nuclease-free BSA

7.B.1.3.3 Random Hexanucleotide Priming Method

The *Amersham cDNA Synthesis System Plus* was used, according to instructions. Random hexanucleotide primers were used instead of an oligo(dT) primer in first strand synthesis; *E.coli* RNAse H was used in second strand synthesis; and *E.coli* T4 DNA polymerase was used to blunt-end the double-stranded cDNA.

7.B.1.3.4 Calculation of cDNA Reaction Yields

To measure cDNA reaction yields of first and second strand synthesis, radioactivity incorporated into each strand was estimated by TCA precipitation. Samples of 1 or 2 μl (or dilutions thereof, if the total cpm is too high) were spotted onto glass fibre filters, dried, and then counted by Cerenkov counting. Filters were washed six times for 5 minutes per wash in 0.5 M Na_2HPO_4, followed by two 1-minute washes in water and two 1-minute washes in 96% ethanol. The filters were dried and recounted. Percentage incorporation for each strand synthesized, was then calculated as:

(cpm on post-wash filter/cpm on pre-wash filter) X 100%

7.B.2 Cloning of cDNA into λgt11 Expression Vector

λgt11 dephosphorylated arms and other components for cloning, were purchased from Promega Biotec, Amersham or Stratagene.

7.B.2.1 T4 DNA Polymerase Blunt End Repair of cDNA

The double stranded (d/s) cDNA synthesis reaction was heat inactivated and blunt-ended using T4 DNA polymerase. In some cases, cDNA was extracted with phenol:chloroform and precipitated prior to blunt-ending. Under these conditions, additional dNTPs (5 mM each) and *T4 polymerase buffer* (18 mM $(NH_4)_2$ SO_4, 6.6 M $MgCl_2$, 10 mM ß-mercaptoethanol, 66 mM Tris-HCl pH 8.3), or *Klenow polymerase buffer* (10 mM $MgCl_2$, 50 mM NaCl, 10 mM Tris-HCl pH 7.5) were added, and the reaction performed in a total volume of 50 μl. Two units of T4 DNA polymerase per μg of original mRNA template were used, and the mixture was incubated for 15 minutes at 37°C. The reaction was terminated by addition of 1 μl of 0.5 M EDTA pH 8.0. The cDNA was extracted with phenol/chloroform, precipitated by addition of ammonium acetate and ethanol, and fractionated on a Sephadex G-50 column. cDNA derived from the S1 nuclease method was efficiently blunt-ended using mungbean nuclease.

7.B.2.2 Methylation of Internal EcoR I Sites

Up to 1 μg of cDNA was incubated at 37°C with 20 units of EcoR I methylase (Promega Biotec or Amersham) in the presence of 10 mM S-

adenosylmethionine, 10 mg/ml nuclease-free BSA, *methylation buffer* (0.1 M Tris-HCl pH 8.0, 10 mM EDTA) and sterile distilled water to a total volume of 125 μl. After 1 hour, the reaction was stopped by heating at 70°C for 10 minutes.

7.B.2.3 Ligation of EcoR I Phosphorylated Linkers to cDNA

This reaction was done using a vast molar excess (>100-fold) of phosphorylated linkers to ensure that cDNA ends became ligated to a linker and not to each other. 800-1000 ng of linkers (Amersham, Promega or New England Biolabs) were added to a mixture containing 20 μl (about 100-200 ng) of methylated blunt-ended cDNA, 1 mM ATP and 3 μl of *ligase buffer* (0.05 M Tris-HCl pH 7.6, 10 mM $MgCl_2$, 10 mM DTT, 50 μg/ml nuclease-free BSA) and water to a final volume of 30 μl. After brief mixing, 1 μl (100 Weiss units) of T4 DNA ligase was added, and the mixture was incubated at 15°C for 16-20 hours. The ligase was inactivated by heating at 70°C for 10 minutes. (The efficacy of the ligation reaction was checked on a 1% agarose gel).

7.B.2.4 EcoR I Digestion of Linkered cDNA

The linkered cDNA was digested with 100 units of concentrated EcoR I enzyme in a total reaction volume of 100 μl at 37°C for 6 hours. The reaction was terminated by heating at 70°C for 10 minutes.

7.B.2.5 Separation of Excess Linker Molecules

To remove excess EcoR I linkers and eliminate cDNA molecules smaller than 500 bp in length, the digested cDNA was fractionated by chromatography through a 15 cm Sepharose CL-4B column. The column was equilibrated with 4 volumes of *STE buffer* (100 mM NaCl, 10 mM Tris-HCl pH 8, 1 mM EDTA) before applying the cDNA in a 100 μl volume. 100 μl aliquots were collected in *STE buffer* and analysed by Cerenkov counting. The two or three fractions with the highest dpm were pooled and precipitated with sodium acetate/ethanol at -20°C overnight. Typically fractions 3 and 4 were pooled and, to exclude unwanted free linkers, no fractions after No.5 were used. Aliquots of each fraction were also checked on thin 1% agarose gels against end-labelled DNA marker fragments.

cDNA was recovered by centrifuging for 30 minutes at 4°C, and the dried pellet dissolved in *TE buffer* at a final concentration of 25-50 ng/μl.

7.B.2.6 Ligation of EcoR I-Ended cDNA into λgt11

Ligation of linkered cDNA into dephosphorylated λgt11 arms included the following control reactions: (1) whole λgt11 vector DNA, to monitor the efficiency of the packaging reactions and the colour selection; (2) a model control EcoR I insert and the λgt11 dephosphorylated arms, to monitor the

efficiency of the ligation reaction of DNA into λgt11; (3) control DNA that had been subjected to the same reactions as the cDNA (T4 DNA polymerase blunt-ending; linker addition, etc.), to check the performance of the entire cloning procedure.

To determine the optimal cloning efficiency for each cDNA population (cDNA synthesized by RNAse H, random hexanucleotide primers or S1 nuclease methods), usually three ligation reactions were performed containing different amounts of cDNA in the range 25-100 ng, and a constant amount of λgt11 dephosphorylated arms. The following table shows a typical set of ligation reactions. (*Ligation buffer*: 50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 50 μg/ml nuclease-free BSA).

TABLE 7.2.6 : Ligation Reactions of cDNA into λgt11

INSERT DNA	TUBE 1	TUBE 2 100 ng EcoR I cut ligation control DNA	TUBE 3 50 ng linkered blunt ended cloning control DNA	TUBE 4 25 ng linkered cDNA	TUBE 5 50 ng linkered cDNA	TUBE 6 100 ng linkered cDNA
Whole λgt 11 DNA	500 ng (5μl)	-	-	-	-	-
Lgt 11 arms	-	1μg (2μl)	1μg (2μl)	1μg (2μl)	1μg (2μl)	1μg (2μl)
10 x ligase Buffer	1μl	1μl	1μl	1μl	1μl	1μl
10 mM ATP	1μl	1μl	1μl	1μl	1μl	1μl
T4 DNA ligase 100 units/μl	0.1μl	0.1μl	0.1μl	0.1μl	0.1μl	0.1μl
Water	to 10μl	to 10μl	to 10μl	to 10μl	to 10μl	to 10μl

Tubes were incubated at 15°C for 15-20 hours and the ligation mix was packaged *in vitro* as described below.

7.B.2.7 Packaging of λgt11 Recombinants

Packaging extracts purchased from Amersham, Promega or Stratagene were used in various reactions. Of the three (tested on the same cDNA population), the Stratagene extract "Gigapack Plus" was found to give the highest packaging efficiency.

Packaging reactions were performed according to the suppliers' instructions. Immediately after thawing, packaging extracts and DNA were mixed and incubated at 20°C for 2 hours. 0.5 ml of phage dilution buffer (*SM Buffer*) and 10 µl of chloroform were added, the phage stock was gently mixed and stored at 4°C. (For libraries that were found to be efficient, half of this phage stock was stored in 7% DMSO at -70°C).

Controls for packaging reactions included, whole λgt11 and dephosphorylated λgt11 arms, as well as the ligated DNA cloning control (see ligation reaction table above).

In trial packaging experiments, the efficiency of the packaging system was tested using the supplied lambda c1857 Sam7 DNA together with bacterial indicator strains (LE392 for Promega "Packagene" and VCS 257 for Stratagene "Gigapack Plus").

7.B.2.8 Plating and Titration of λgt11 Libraries

To calculate the phage titre, an initial 1:100 dilution of phage in *SM buffer* representing 10^2 pfu/ml of the original 0.5 ml phage stock, was made by adding 30 µl of this stock to 270 µl of SM buffer. This was designated the 10^2 dilution. Similar dilutions were made up to dilution 10^7, in duplicate.

A single colony of Y1090 cells was inoculated into *L-broth* containing 0.4% maltose and ampicillin (50 µg/ml) and grown at 37°C with good aeration until an OD_{600} between 0.7 and 0.9 was reached. 100 µl of these cells were then dispensed into round-bottom 10 ml Falcon tubes, and 100 µl of each phage dilution added to these tubes. Mixtures were incubated at 37°C for 15 minutes, whereafter they were transferred to a 45°C heating block and 3 ml of top agar (kept ready at 45-50°C) was quickly added. Immediately, 20 µl of 100 mM IPTG and 20 µl of 40 mg/ml of X-gal (BCIG) was added to each tube, the contents were mixed and poured onto dry L-agar plates containing ampicillin. Plates were left to set for 15 minutes and then incubated at 43°C for 3.5 hours followed by overnight incubation at 37°C.

The number of plaques on plates containing fewer than 1000 pfu were counted and average titres were determined from the duplicate titration series. The phage titre/ml was calculated by multiplying the total average number of plaques by the dilution number (eg 250 plaques on the 10^5 plate = 2.5×10^7 pfu/ml). Both the total titre/ml (blue and clear plaques) and the titre of recombinants per ml (clear plaques) were calculated.

7.B.2.9 Evaluation of Libraries

For each library, the cloning efficiency per µg of cDNA was calculated. In addition, the size range of the cDNA inserts of each library was analysed on 1-

2% agarose gels, by extracting the DNA from 10-12 randomly selected recombinant clones (section 7.B.2.10.1) and digesting it with either Mlu I, or with Kpn I and Sac I. Libraries with the highest number of recombinant clones and the largest average cDNA insert range, were selected.

The libraries screened in this study were not amplified, so as to avert the possibility of losing (or reducing the abundance) of poorly growing potentially relevant recombinants. However, to guard against loss of libraries through contamination (or other circumstances), amplified stocks were made by plating out the entire library at high density on several plates (as for immunoscreening but at higher density), adding 5 ml of *SM Buffer* to each plate and harvesting the phage as for making plate lysates (section 7.B.2.10.2).

7.B.2.10 Lambda DNA Extraction

For isolation of phage DNA, high titre phage lysates were prepared on a small scale using either plate or liquid lysates. The most effective method of DNA isolation was however found to be that described by Ausubel et al. (1987). This method is described below.

7.B.2.10.1 DNA Extraction from Liquid Lysates

7.B.2.10.1.1 Liquid Lysate

An overnight culture of *E.coli* Y1090 were grown in L-broth containing 0.4% maltose and ampicillin (50 μg/ml). A single plaque from a plated λgt11 cDNA library was cored out using a sterile pasteur pipette, and transferred to 0.4 ml of *SM Buffer* for elution of phage at 4°C for 2 hours. 100 μl of eluted phage was added to 100 μl of a 10 mM $MgCl_2$/10 mM $CaCl_2$ solution and incubated for 15 minutes at 37°C. The mixture was then transferred to 50 ml of *NZC broth* (section 7.C) and shaken vigorously for 6-8 hours until lysis occurred. Immediately after lysis, a few drops of chloroform were added and the cell debris removed by centrifugation at 10 000 rpm for 10 minutes at 4°C in a Beckman JA-20 rotor. The supernatant was immediately transferred to a sterile 50 ml Falcon tube and stored at 4°C.

7.B.2.10.1.2 DNA Extraction

To 40 ml of liquid phage lysate, 8 μl of 5 mg/ml of DNAse and 20 μl of 10 mg/ml RNAase was added. The lysate was incubated for 1 hour at 37°C and the phage pelleted by centrifugation at 27 000 rpm in an SW-27 rotor for 90 minutes. The supernatant was decanted and the remaining liquid wiped from around the phage pellet. Phage were resuspended in 200 μl of 0.05 M Tris-HCl pH 8, transferred to a microfuge tube and vigorously shaken in an equal volume of phenol for 20 minutes. After centrifugation for 2 minutes, the aqueous layer was re-extracted with phenol for 20 minutes, followed by two chloroform extractions. The DNA was precipitated by addition of 1/10 volume of 3 M

sodium acetate (pH 4.8) and 2 volumes of ethanol, and left at room temperature for 1 hour. The DNA was then centrifuged for 10 minutes, washed twice in 70% ethanol, dried under vacuum and resuspended in 30 µl *TE buffer*.

7.B.2.10.2 Plate Lysates

Plaques were cored out from the agar plate, using a sterile pasteur pipette, deposited in 1 ml of SM buffer containing 20 µl of chloroform and incubated at 4°C for at least 2 hours. A range of volumes of this stock (50-300 µl) was adsorbed to 100 µl of Y1090 cells and the cells were plated out according to the standard protocol (section 7.B.3). 5 ml of *SM buffer* was then added to each plate and they were shaken gently at 4°C for 3-4 hours. Lysates were poured into sterile tubes, a few drops of chloroform added, and centrifuged at 5000 rpm for 5 minutes at 4°C to remove cellular debris. The supernatant was stored in sterile glass vials with 20 µl chloroform at 4°C, or used for DNA extraction.

7.B.3 Immunoscreening of λgt11 Libraries

7.B.3.1 Antibody Screening Procedure

A single colony of *E.coli* Y1090 cells was grown in *LB medium* containing 0.4% maltose and 50 µg/ml ampicillin at 37°C with good aeration until the OD_{550} was between 0.7 and 0.9. 300 µl of these cells were added to 300 µl of appropriately diluted phage in *SM buffer* in a 10 ml Falcon tube. Phage were adsorbed to the cells for 20 minutes at 37°C, whereupon 7.5 ml of top agar (kept at 45-50°C) was added to the tube, and the culture poured onto 150 mm agar plates. Plates were left to set on a horizontal surface at room temperature for 20 minutes, then transferred to 43°C for 3.5 hours. They were then overlaid with dry IPTG-saturated (10 mM) nitrocellulose filters (Schleicher and Schuell), the position of the filters on the plate was marked with a needle, and the plates incubated overnight at 37°C.

The following day, filters were removed, rinsed briefly in *TBS* (pH 7.9) and blocked for 1 hour at room temperature using 2% BSA, 1% goat serum, 0.05% Tween 20 in *TBS* pH 7.5. The filters were rinsed, test antibody diluted 1:100 in 15 ml *TBS* pH 7.9, was added, and incubated at 4°C for 6 hours. Filters were washed three times for 5 minutes each, and affinity-purified goat anti-human IgG (H+L) antibody conjugate (Promega) was added at a dilution of 1:10000 in *TBS*, and incubated for 1 hour at 4°C. Filters were washed, and transferred to room temperature for the addition of the *AP substrate solution*. This was prepared (per 5 ml of solution) by adding 33 µl of NBT substrate (50 mg/ml in 70% dimethylformamide) to 5 ml of *AP buffer* (100 mM Tris-HCl, pH 9.5; 100 mM NaCl, 5 mM $MgCl_2$), mixing and then adding 16.5 µl of (50 mg/ml X-gal in 100% dimethylformamide). The solution was used within 1 hour, and

added to the filters with gentle shaking for 3-5 hours or longer. The reaction was terminated by the addition of *AP stop-buffer* (20 mM Tris-HCl, pH 8.0; 5 mM EDTA).

A positive control consisting of a λgt11 chicken ovalbumen clone together with an affinity purified rabbit anti-chicken ovalbumen antiserum was used (Clontech Laboratories).

7.B.3.2 Primary Antibody Dilution and Treatment

The optimal dilution of test antibody for immunoscreening was determined by reacting different dilutions of patient sera against non-recombinant λgt11 phage to determine the lowest background against which positive clones might be detected. In addition, the λgt11 chicken ovalbumen clone, diluted $1:10^{-4}$ in non-recombinant phage, was used together with rabbit anti- chicken ovalbumen serum to establish an optimal signal:noise ratio for screening.

7.B.3.3 Removal of *E.coli* Cross-reactivity

Some batches of sera had high levels of anti-*E.coli* antibodies. These antibodies were removed either by: (1) incubating sera with different concentrations of *E.coli* lysate (1:20, 1:50, 1:100, 1:200) for 30 minutes at room temperature with gentle mixing, before using them in immunoscreening; or (2) by "pseudoscreening", in which non-recombinant λgt11 phage were plated at 10^5 phage per 150 mm dish, transferred to filters which were blocked for nonspecific binding, and then used as a solid-phase absorbents to remove anti-*E.coli* antibodies, by adding test sera for 6 hours with gentle shaking at 4°C.

7.B.3.4 Affinity Purification of Antibody on Western Blots

Certain sera were affinity purified against heart antigen before screening the libraries. An entire nitrocellulose filter from a Western blot of separated human heart extract (sections 7.A.9 and 7.A.11) was used as a solid phase absorbent. Sera were incubated with this filter for 2 hours at room temperature. Bound antibody was eluted using glycine-HCl pH 2.5 for 2 minutes, and the eluate was immediately neutralized in 750 µl (per 5 ml eluate) of 2 M Tris-HCl pH 10.

7.B.3.5 Purification of Positive Clones

Positive plaques detected by alkaline phosphatase second antibody screening, were easily located against the fainter background of negative plaques (see Fig. 5.5). (Needle and ink marks were also used for correct alignment). The exact positive plaque was cored out using a sterile pasteur pipette and transferred to 500 µl of *SM buffer* in a microfuge tube. A few drops of chloroform were added and the tube incubated overnight at 4°C. Eluted phage were plated at low density (less than 200 pfu), rescreened and the whole process repeated

until all the plaques were positive. Stocks of highly-purified phage were stored at 4°C (and at -70°C in 7% DMSO).

7.B.4 Analysis of positive clones

7.B.4.1 Analysis of Fusion Proteins

7.B.4.1.1 Isolation of Recombinant Lysogens

(For establishment of lysogeny by this procedure it was found essential to omit yeast extract from all media used).

A single colony of Y1089 cells was inoculated into 10 ml of *L-broth* (without yeast extract) containing 0.4% maltose and 50 μg/ml ampicillin and shaken overnight at 37°C. 1 ml of the overnight culture was then inoculated into 50 ml of prewarmed *L-broth* (as above), and incubated with shaking at 37°C for 2-3 hours until the OD_{600} reached 0.5 (2.5 X 10^8 cells/ml). The culture was then supplemented with 10 mM $MgCl_2$ and dispensed into 100 μl aliquots and kept on ice. Each 100 μl aliquot was infected with 10-50 μl of a phage dilution containing about 10^8 pfu, so as to achieve a multiplicity of infection (m.o.i) of 5-10. The culture was incubated at 32°C for 20 minutes, and the infected cells were diluted for plating at a density of about 200 per 90 mm plate, and incubated overnight at 32°C. From each such plate, 25 colonies were picked using sterile cocktail sticks, and each colony was spotted onto duplicate L-agar plates containing ampicillin. One plate was incubated at 32°C and the other at 43°C. Lysogens were identified by their ability to grow at 32°C but not at 43°C.

7.B.4.1.2 Fusion Proteins

50 ml of *L-broth* pH 7.5 was inoculated with a single colony of a specific Y1089 recombinant lysogen, and incubated at 32°C with vigorous shaking until the culture had grown to an OD_{600} of 0.6 (about 5 hours). The temperature of the culture was then rapidly raised to 45°C, by the addition of 50 ml of *L-broth* prewarmed to 70°C, followed by immediate immersion of the flask in a 45°C waterbath with vigorous shaking for 20 minutes. 5 mM IPTG was then rapidly added and the flask was incubated for about 45-60 minutes at 38°C. The longest incubation time without lysis occurring was determined for each recombinant lysogen, and found to be usually less than 1 hour. At this optimal time, lysogenized cells were rapidly harvested in a JA-10 rotor at 5000 rpm for 5 minutes at 30-37°C. Pelleted cells were rapidly resuspended in 1/20 the original culture volume in *TEP Buffer* (100 mM Tris-HCl pH7.4, 10 mM EDTA, 1 mM PMSF), and immediately frozen in liquid nitrogen and stored at -70°C. To lyse the induced lysogens, they were thawed at 37°C, refrozen in liquid

nitrogen, thawed again and sonicated (3 X 10 s) to reduce viscosity. The sonicated extract was centrifuged at 10 000 rpm for 10 minutes to remove cell debris. The proteins in the supernatant were precipitated with 3 volumes of saturated ammonium sulphate at 4°C for at least 60 minutes. It was stored as a slurry or else centrifuged at 10 000g for 20 minutes, the supernatant discarded and the pellet redissolved in *TEP buffer* at approximately 10 mg/ml of protein. It was then either analysed as a crude extract by immunoblotting against sera, or further processed for purification of the fusion protein on anti-ß-galactosidase immunoaffinity columns (see Results).

7.B.4.2 Analysis of DNA Positive Inserts

7.B.4.2.1 Subcloning of λgt11 Inserts into pUC19

7.B.4.2.1.1 In-gel Ligation of Insert into pUC19

Subcloning of inserts from λgt11 into pUC19 was done by in-gel ligation according to the method Struhl (1985). Recombinant λgt11 DNA was digested with EcoR I, and then subjected to electrophoresis in 0.7% low melting agarose (SeaPlaque) using *TAE Buffer*. The released insert was excised from the gel in the smallest possible volume (20-40 µl) using a sterile scalpel blade and transferred to a sterile microfuge tube. The gel slice was then heated at 70°C for 15 minutes. 7 µl of this insert DNA was combined with 2 µl of EcoRI-cut pUC19 at an insert:vector concentration ratio of 5:1, and incubated at 37°C for ≥ 3 minutes before addition of 11 µl of ice-cold ligation mixture containing 2 X *ligase buffer*, ATP and T4 DNA ligase. The reaction was quickly mixed and placed on ice. Mixtures were incubated at 15°C overnight (or up to 48 hours). For transfection, gel ligation mixtures were remelted at 73°C for 5-10 minutes, and 5 µl of ligation mixture was used to transfect 200 µl of competent *E.coli* cells.

7.B.4.2.1.2 Plasmid DNA Isolation

7.B.4.2.1.3 Small Scale (Rapid) Preparation

10 ml of sterile *L-broth* was inoculated with *E.coli* and shaken overnight at 37°C. Before DNA extraction, glycerol stocks were made by mixing equal volumes (500 µl) of overnight culture and *glycerol stock buffer* (65% glycerol/0.1 M $MgSO_4$/25 mM Tris pH 8).

Remaining cells were pelleted at 4000 g for 10 minutes, and resuspended in 200 µl of 25 mM Tris pH 8.0, 10 mM EDTA, 50 mM glucose for 5 minutes at room temperature. To lyse the cells and denature the DNA, 400 µl of 0.2 M NaOH, 1% SDS was added for 5 minutes on ice. Genomic DNA was precipitated by addition of 300 µl of 3 M potassium acetate pH 4.8, and

removed by centrifugation at room temperature for 5 minutes. Plasmid DNA, contained in the supernatant, was precipitated by adding 3/4 volume of isopropanol and incubating at -20°C for 30 minutes. The DNA was pelleted, washed twice with 70% ethanol, vacuum dried and dissolved in *TE buffer*.

7.B.4.2.1.4 Large Scale Preparation

A 100 ml overnight culture of transfected *E.coli* was grown in *L-broth* containing 50 μg/ml ampicillin was prepared. To amplify plasmid DNA, chloramphenicol was added to a final concentration of 200 μg/ml. After a further 5 hours of shaking at 37°C, the bacteria were harvested by centrifugation at 4000g for 10 minutes at 4°C. The pelleted cells were resuspended in 50 mM Tris-HCl pH 8.0 containing 25% sucrose (*Clear Lysate Buffer A*) and then incubated on ice for 5 minutes before addition of lysozyme to a final concentration of 2 mg/ml. After a further 5 minutes on ice, 1.5 ml of 250 mM EDTA, pH 8.0, was added. After another 5 minutes, 50 mM Tris-HCl pH 8.0, 6.25 mM EDTA, 0.5% Triton X-100 (*Clear Lysate Buffer B*) was added, and the suspension left on ice for 20 minutes with occasional gentle swirling. Bacterial debris was removed by centrifugation at 20 000 rpm in a Beckman JA-20 rotor for 40 minutes at 4°C. CsCl was added at 1 g per ml of supernatant, and to each 10 ml of this mixture was added, 0.8 ml of 10 mg/ml ethidium bromide . The samples were centrifuged in sealed polyallomer tubes for 36 hours at 49 000 rpm in a Beckman Ti-60 rotor at 18°C. The band containing plasmid DNA was visualised under UV light, and removed. To remove ethidium bromide, the DNA was extracted 4 to 5 times with an equal volume of water-saturated 1-butanol, until the pink colour disappeared. The aqueous phase was extensively dialysed against 0.1 X *TE* at 4°C, and DNA in the dialysate precipitated at -20°C, washed with 70% ethanol, dried and dissolved in sterile water or *TE buffer*.

7.B.4.2.1.5 Preparation of Competent *E.coli* Cells

Competent *E.coli* DK-1 cells were prepared by inoculating 3 ml of an overnight culture into 300 ml of L-broth in a 1-litre flask. The cells were grown at 37°C with vigorous shaking for about 1-2 hours until the OD_{650} was between 0.2-0.4. The cells were then pelleted at 4000g for 5 min at 4°C, and gently resuspended in 40 ml of an ice-cold sterile solution of 60 mM $CaCl_2$, 10 mM PIPES, pH 7.2. The suspension was incubated on ice for 30 minutes, and the cells again centrifuged at 4000g for 5 minutes at 4°C. The pellet was gently resuspended in 4 ml of 60 mM $CaCl_2$, 10 mM PIPES pH 7.2, and 15% glycerol. 200 μl aliquots were rapidly frozen in a dry ice/ethanol bath and immediately stored at -70°C until needed.

7.B.4.2.1.6 Plasmid DNA Transfection of *E.Coli* Cells

An aliquot of frozen competent cells was thawed on ice for 30 minutes. Between 5 and 100 ng of DNA (in *TE buffer* or ligation mixture) was added to 100 µl of cells in a round-bottom Falcon tube, and incubated on ice for 30 minutes. The cells were heat-shocked for 3 minutes in a 43°C waterbath with gentle agitation. 1 ml of *L-broth* was added to the cells which were then incubated at 37°C for 1 hour. Transfected cells (50-250 µl) were plated onto agar plates containing ampicillin (50 µg/ml), and incubated at 37°C overnight. Circularised (uncut) and cut pUC19 were always included as transfection controls.

7.B.4.2.2 Sequencing of DNA

Positive DNA inserts subcloned from λgt11 into pUC19 were sequenced using the modified T7 DNA polymerase, *Sequenase* (United States Biochemical). The pUC19 double stranded DNA (1-2 µg) was denatured at 37°C for 30 minutes in 0.2 N NaOH. This reaction was neutralised by addition of 2 µl of *TE* and 7 µl of 1 N HCl. The primer (0.5 pg in 1 µl) was added and the reaction incubated at 37°C for 30 minutes, whereupon the template- primer DNA was precipitated in 50 µl of ethanol overnight at -20°C. The DNA was pelleted by centrifugation, washed in 70% ethanol, dried and dissolved in 8 µl of water and 2 µl of *5 X sequenase buffer* (200 mM Tris-HCl, 100 mM $MgCl_2$, 250 mM NaCl, pH 7.5). To the annealed DNA mixture, the following were then added in a final volume of 15.5 µl and left for 5 minutes at room temperature: 6.25 mM fresh DTT, 0.2 µM dNTPs (dATP, dGTP, dTTP), 10 µCi ^{32}P-dCTP and 2 units of *Sequenase* enzyme. The reaction was then terminated by adding 3.5 µl of the labelled DNA mixture to each of four *termination mixes* (section 7.C) containing one of the four dideoxynucleotides. The termination reaction proceded for 5 minutes at 37°C, and was then inactivated by addition of 4 µl of *sequenase stop buffer* (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and heating at 75°C for 2 minutes. Reactions were placed on ice, and 3 µl aliquots of each were loaded onto a 1 meter vertical polyacrylamide gel and electrophoresed at 2400 V for 6-10 hours. The gels were dried and exposed to X-ray film for 24 hours.

7.B.5 General Nucleic Acid Techniques

7.B.5.1 Formaldehyde Agarose Gel Electrophoresis of RNA

RNA was fractionated on 1% agarose gels containing 2.2 M formaldehyde as described by Maniatis et al. (1982). Agarose was boiled in *RNA Running Buffer* (40 mM MOPS, 10 mM sodium acetate, 10 mM EDTA, pH 7.0) and then cooled with stirring to 60°C before the addition of formaldehyde. Horizontal gels were poured and run at 150 mA for 2-5 hours.

7.B.5.2 Agarose Gel Electrophoresis of DNA

DNA was heated at 65°C for 5 minutes prior to fractionation on horizontal agarose gels (0.5-3% depending on size of fragments to be resolved). Large gels were run for 4-6 hours at 150 mA constant current in *TAE Buffer* (4 mM Tris, 5 mM sodium acetate, 1 mM EDTA, pH 7.8). Minigels were run for 45 minutes at 50 mA in *TBE Buffer* (90 mM Tris-Borate, 90 mM boric acid, 2 mM EDTA, pH 8.3). Gels were stained in 0.5 μg/ml ethidium bromide and visualised under UV light.

7.C BUFFERS AND SOLUTIONS

The composition of some commonly used buffers and solutions used are presented below. More specialised buffers are described in the text of sections 7.A and 7.B.

ABTS/H_2O_2 ELISA substrate solution
Dissolve 40 mM ABTS (Sigma) in distilled water
Add 100μl ABTS to 10 ml fresh citric acid pH 4
Add 100μl of 30% H_2O_2 immediately before use.

AEC Immunocytochemistry substrate solution
1 mg AEC dissolved in 0.25ml N-N-dimethylformamide.
Add 3.5 ml of acetate buffer pH 5.2
Add 38μl of 3% H_2O_2
Filter before use.
Stable for only 2-3 hours.

Alkaline phosphatase (AP) buffer
100mM Tris-HCl pH 9.5
100mM NaCl
5mM $MgCl_2$

Alkaline phosphatase (AP) stop buffer
20mM Tris-HCl pH 8.0
5mM EDTA

Alkaline phosphatase (AP) substrate solution
33μl of 50mg/ml NBT in 70% dimethylformamide in 5ml *AP buffer*
Add 16.5μl of 50mg/ml X-gal in 100% dimethylformamide

Amido-black stain
0.3% Amido-black
10% Ethanol
7% Acetic acid
The nitrocellulose filter was stained in this solution for 5 minutes then destained in hot 2% acetic acid

Ampicillin plates
Autoclaved solution was allowed to cool to about 55°C before adding 2.5mls of 10mg/ml ampicillin (final concentration of 50μg/ml):

5g Tryptone	
2.5g Yeast extract	per 500mls water
2.5g NaCl	
7.5g Bacto-agar	

40ml of the solution was added per 100mm diameter plate.

ddA termination mix
80μM dGTP
80μM dATP
80μM dTTP
80μM dCTP
8μM ddATP
50mM NaCl

ddC termination mix
80μM dGTP
80μM dATP
80μM dTTP
80μM dCTP
8μM ddCTP
50mM NaCl

ddG termination mix
80μM dGTP
80μM dATP
80μM dTTP
80μM dCTP
8μM ddGTP
50mM NaCl

ddT termination mix
80μM dGTP
80μM dATP
80μM dTTP
80μM dCTP
8μM ddTGTP
50mM NaCl

Dignam buffer A
10mM HEPES, pH 7.9
1.5mM $MgCl_2$
10mM KCl
0.5mM DTT (add fresh)

Dignam buffer C
20mM HEPES, pH 7.9 at 4°C
0.42M NaCl
1.5mM $MgCl_2$
0.2mM EDTA
25% v/v glycerol
0.5mM DTT (add fresh)
0.5mM PMSF (add fresh)

Dignam buffer D
20mM HEPES, pH 7.9
0.1M KCl
0.2mM EDTA
20% v/v glycerol
0.5mM DTT (add fresh)
0.5mM PMSF (add fresh)

DNA stop buffer
0.05% bromophenol blue
0.5% SDS
1mM EDTA
50% glycerol

ELISA blocking buffer
1% BSA
1% goat serum
100mM glycine
in TBS, pH 7.5

ELISA coating buffer
15mM Na_2CO_3
35mM $NaHCO_3$
0.1g thymol
pH 9.5
Stored at 4°C, used within 2 weeks

ELISA PBS-Tween wash buffer
0.01% Tween 20
in PBS pH 7.4

GKN buffer for mAb (per liter of distilled water)
8.0g NaCl
0.4g KCl
3.56g $Na_2HPO_4 12H_2O$
2.0g Glucose
0.01g Phenol Red
Autoclave to sterilise

Glycerol stock buffer for plasmids
65% glycerol
0.1M $MgSO_4$
25mM Tris-HCl, pH 8.0

H.A.T medium for mAb Selection (100 x)
To make 25ml -
3.75ml FCS
5.00ml SP2 conditioned medium
0.25ml HAT
0.25ml Antibiotics (penicillin 100 U/ml; streptomycin 100 μg/ml)
16.5ml RPMI-H-B

Heart extract homogenization buffer
20mM Tris-HCl
150mM NaCl
1mM $CaCl_2$
1mM PMSF
pH 8.0

Heart extract solubilization buffer
20mM Tris-maleate
150mM NaCl
1mM $CaCl_2$
1mM PMSF
1mM thymol
pH to 7.25, then add 5mM CHAPS detergent.

HT/FCS/SP2 mAb conditioned medium
15ml FCS (Flow or Gibco Myoclone)
20ml SP2 24hr conditioned medium
1 ml HT 100 x
1 ml Antibiotics
66ml RPMI-HEPES-bicarbonate

Immunoblotting colour reaction solution
60mg 4-chloro-1-naphthol
20ml methanol
100ml TBS
12.5 μl 30% H_2O_2

Immunoblotting transfer buffer
0.05 M Tris
0.3 M Glycine
20% v/v Methanol

Klenow polymerase buffer (10 x)
100mM Tris-HCl, pH 7.5
100mM $MgCl_2$
500mM NaCl

L-broth
For 1 liter:
10g tryptone
5g yeast extract
5g NaCl
Set pH 7.5 using 1N NaOH

LB medium (per litre)
10g tryptone
5g yeast extract
5g NaCl
1 ml 1N NaOH

Ligase buffer (10 x)
500 mM Tris-HCl
70mM $MgCl_2$
100mM DTT
500μg/ml nuclease-free BSA

Methylation buffer
0.1M Tris-HCl, pH 8.0
10mM EDTA

NZC broth
10g NZ Amine A
5g NaCl
2g $MgCl_2.6H_2O$
Autoclave 30 min
Made to 0.1% in Casamino Acids
Made to 1 litre

PBS
7.36mM KCl
1.5mM KH_2PO_4
137mM NaCl
8.1mM $Na_2HPO_4.7H_2O$
pH 7.0

Plasmid mini-prep solution 1
25mM Tris-HCl, pH 8.0
10mM EDTA
50mM glucose

Plasmid mini-prep solution 2
0.2N NaOH
1% SDS

Plasmid mini-prep solution 3
3M potassium acetate, pH 4.8

Plasmid clear lysate buffer A
50mM Tris-HCl, pH 8.0
25% sucrose

Plasmid clear lysate buffer B
50mM Tris-HCl, pH 8.0
6.25mM EDTA
0.5% Triton X-100

Reverse transcriptase buffer (for S1 cDNA) (5 x)
250mM Tris/HCl pH 8.3
500mM KCl
30mM $MgCl_2$

Reverse transcriptase buffer (for RNAse H cDNA) (5 x)
100mM Tris-HCl, pH 8.3
10mM $MgCl_2$
140mM KCl

RNA Extraction Solution D
4 M guanidinium thiocyanate
25mM sodium citrate, pH 7.0
0.5% sarcosyl
0.1M ß-mercaptoethanol

RNA gel running buffer (5 x)
0.2M Morpholinopropansulfonic acid (MOPS)
50mM Na acetate
50mM EDTA
pH 7.0

SDS-PAGE running buffer
0.025M Tris-HCl
1.5% glycine
0.1% SDS
pH 8.3

SDS-PAGE sample buffer
48mM Tris-HCl
2.5% SDS
20% glycerol
0.25% bromophenol blue
5% ß-mercaptoethanol

Sequenase buffer (5 x)
200mM Tris-HCl
100mM $MgCl_2$
250mM NaCl
pH 7.5

Sequenase stop buffer
95% Formamide
20mM EDTA
0.05% Bromophenol blue
0.05% Xylene cyanol

S1 nuclease buffer (10 x)
0.3M Na acetate, pH 4.5
3M NaCl
100mM $ZnCl_2$

SM lambda diluent buffer
10mM Tris-HCl, pH 7.5
10mM $MgCl_2$

STE buffer
10mM Tris-HCl, pH 8.0
100mM NaCl
1mM EDTA

T4 DNA polymerase buffer (5 x)
0.09M $(NH_4)_2SO_4$
0.33M Tris-HCl, pH 8.3
0.33M $MgCl_2$
0.05M ß-mercaptoethanol

TAE agarose electrophoresis buffer (10 x)
40 mM Tris
50 mM Na acetate
10 mM EDTA
pH 7.8 with glacial acetic acid

TBE agarose electrophoresis buffer (10 x)
0.90M Tris
0.90M boric acid
25mM EDTA
pH 8.3

TE buffer (10 x)
100mM Tris-HCl
10mM EDTA
pH 8.0

TEP fusion protein buffer
100mM Tris-HCl, pH 7.4
10mM EDTA
1mM PMSF

Tris buffered saline + Tween 20 (TBST)
10mM Tris-HCl pH 8.0
150mM NaCl
0.05% Tween 20

Tris buffered saline (TBS)
25mM Tris
0.9% w/v NaCl
pH 7.5

Versene Stock Buffer (5 x) (per litre)

Na_2EDTA $(2H_2O)$	1300mg
KCL	1000mg
NaCl	40g
Na_2HPO_4	4.29g

pH 7.3

APPENDIX A

STATISTICAL TESTS AND UNIVARIATE DESCRIPTIVE STATISTICS

See next 12 pages for tables of univariate descriptive statistics for each group of subjects.

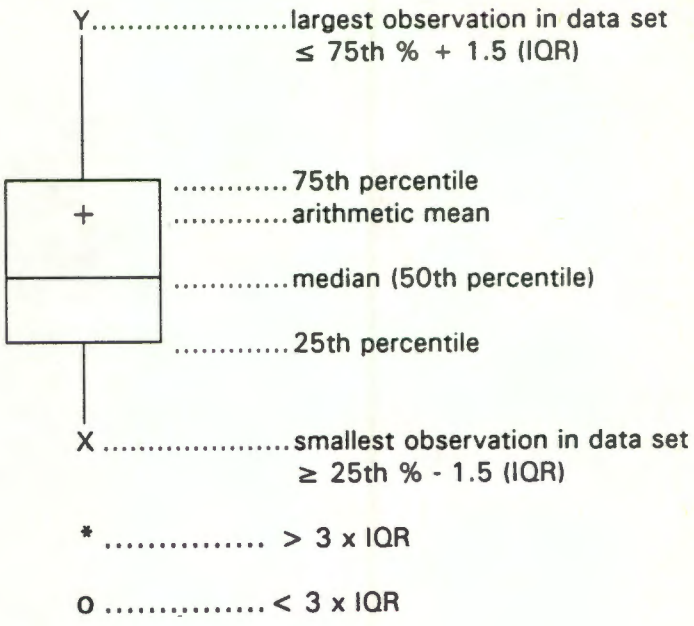


Diagram of Box Plot

The median was used to describe the average value of the variables. Where the median is used, the descriptive measure of dispersion used is the interquartile range or the distance between the 25th (Q1) and the 75th (Q3) percentile. The standard deviation is a measure of the spread of observations around the arithmetic mean. The skewness of the frequency distribution of the variable can be gleaned from the relative position of the mean and the median, together with the associated measures of dispersion.

Statistical Analyses

Mann-Whitney-U Test:
This test was used to test the hypothesis that there is no difference in the medians of the two groups compared. This test was used instead of the t-test, since the number of observations in the groups compared were generally not equal, and the underlying distributional assumptions of the t-test were not met.

Spearman Rank Correlation Coefficient:
The Spearman Rank Correlation Coefficient gives a measure of linear association between variables, without requiring that the data are normally distributed. The significance of the value of the correlation coefficient depends on the size of the sample used to calculate it. The results from the hypothesis test are greatly influenced by the sample size of each of the two variables, and caution must therefore exercised in interpreting results.

Fisher's Exact Probability Test:
This test is used when two independent sample variables are small in size and are in two mutually exclusive classifications. The test determines whether the two groups differ in the proportion in which they fall into the two classifications. The test does not depend on underlying distributional assumptions, and the level of significance (p-value) is therefore exact.

APPENDIX A

Univariate descriptive statistics for each group of subjects

VARIABLE	DESCRIPTIVE STATISTIC	CHILDREN				ADULTS		
		CONT-C	ARF	AGN	CRF-C	CONT-A	CRF-A	IHD
AGE	Number in group (N)	19	17	19	19	10	15	20
	Mean (arithmetic)	10.2	9.1	7.1	11.7	34.5	38.2	56.0
	Std. deviation	2.7	3.6	3.6	3.2	5.6	9.7	8.8
	Max	14	14	13	16	45	53	70
	Q3 (75th percentile)	12	12	11	14	38	46	62
	Median	10	10	6	12	35.5	39	57
	Q1 (25th percentile)	9	5.5	4	10	30.25	31	50
	Min.	3	3	2	6	26	20	38
	Q3-Q1	3	6.5	7	4	7.75	15	12
dnase B	Number in group (N)	19	14	19	19	10	15	18
	Mean (arithmetic)	1385.3	1451.4	736.8	426.8	1921.0	225.3	226.7
	Std. deviation	744.8	819.4	675.2	344.6	167.4	103.2	171.8
	Max	2560	2560	2560	1230	640	480	640
	Q3 (75th percentile)	1920	2080	1280	640	200	320	320
	Median	1280	1280	400	240	180	200	200
	Q1 (25th percentile)	640	640	320	200	80	200	110
	Min.	80	160	200	80	80	60	60
	Q3-Q1	1280	1440	960	440	120	120	210
IgG	Number in group (N)	20	18	18	19	10	15	20
	Mean (arithmetic)	16.6	22.1	20	19.1	13.1	22.1	15.2
	Std. deviation	3.8	6.0	8.2	4.7	2.0	6.0	4.0
	Max	24.6	30.3	35.0	32.4	15.8	33.8	7.6
	Q3 (75th percentile)	19	26.5	23.6	20.8	15.1	26.3	5.2
	Median	17	22.5	18.2	18.2	13.1	22.4	3.9
	Q1 (25th percentile)	14.3	20.1	15.1	16.4	11.5	19.5	2.4
	Min.	7.8	5.7	2.2	9.8	9.4	10.8	1.2
	Q3-Q1	4.7	6.4	8.5	4.4	3.6	6.8	2.8

APPENDIX A

Univariate descriptive statistics for each group of subjects

VARIABLE	DESCRIPTIVE STATISTIC	CHILDREN				ADULTS		
		CONT-C	ARF	AGN	CRF-C	CONT-A	CRF-A	IHD
IgA	Number in group (N)	20	18	18	19	10	15	20
	Mean (arithmetic)	2.1	3.2	1.8	3.5	2.4	4.8	4.0
	Std. deviation	0.8	1.7	0.9	1.3	0.9	2.6	1.8
	Max	4.1	6.8	3.3	6.7	4.0	11.4	7.6
	Q3 (75th percentile)	2.6	4.5	2.6	3.9	3.0	5.7	5.2
	Median	2.0	3.0	1.6	3.7	2.3	4.2	3.9
	Q1 (25th percentile)	1.5	2.1	1.1	2.4	1.8	3.3	2.4
	Min.	1.0	0.9	0.2	1.6	1.1	1.7	1.2
	Q3-Q1	1.1	2.4	1.5	1.5	1.2	2.4	2.8
IgM	Number in group (N)	20	18	18	19	10	15	20
	Mean (arithmetic)	2.3	2.8	2.4	2.9	2.1	2.6	1.9
	Std. deviation	1.0	1.4	1.5	1.4	1.1	1.4	1.1
	Max	5.0	5.6	7.1	5.2	4.6	5.2	5.0
	Q3 (75th percentile)	2.7	3.8	2.9	4.3	2.9	2.9	2.4
	Median	2.3	2.7	2.0	2.4	1.8	2.3	1.6
	Q1 (25th percentile)	1.7	1.6	1.6	1.6	1.2	1.6	1.2
	Min.	0.4	0.5	0.5	1.1	1.0	1	0.5
	Q3-Q1	1.0	2.2	1.3	2.7	1.7	1.3	1.2
Protein M1	Number in group (N)	17	15	16	19	10	15	19
	Mean (arithmetic)	0.380	0.512	0.389	0.248	0.154	0.346	0.203
	Std. deviation	0.203	0.353	0.201	0.176	0.065	0.162	0.108
	Max	0.765	1.192	0.781	0.717	0.274	0.637	0.510
	Q3 (75th percentile)	0.498	0.816	0.565	0.330	0.212	0.468	0.274
	Median	0.372	0.437	0.324	0.222	0.126	0.385	0.176
	Q1 (25th percentile)	0.288	0.195	0.212	0.124	0.108	0.218	0.130
	Min.	0.035	0.114	0.035	0.059	0.072	0.067	0.066
	Q3-Q1	0.21	0.702	0.353	0.206	0.104	0.25	0.144

APPENDIX A

Univariate descriptive statistics for each group of subjects

VARIABLE	DESCRIPTIVE STATISTIC	CHILDREN				ADULTS		
		CONT-C	ARF	AGN	CRF-C	CONT-A	CRF-A	IHD
Protein M3	Number in group (N)	17	15	16	19	10	15	19
	Mean (arithmetic)	0.266	0.231	0.280	0.160	0.090	0.317	0.132
	Std. deviation	0.189	0.199	0.140	0.102	0.033	0.151	0.058
	Max	0.829	0.203	0.497	0.444	0.145	0.596	0.227
	Q3 (75th percentile)	0.298	0.288	0.373	0.203	0.109	0.425	0.181
	Median	0.266	0.196	0.318	0.143	0.084	0.325	0.135
	Q1 (25th percentile)	0.179	0.087	0.161	0.103	0.068	0.214	0.087
	Min.	0.028	0.013	0.020	0.045	0.040	0.070	0.026
	Q3-Q1	0.119	0.201	0.212	0.100	0.041	0.211	0.094
Protein M5	Number in group (N)	17	15	16	19	10	15	19
	Mean (arithmetic)	0.139	0.215	0.159	0.112	0.074	0.242	0.136
	Std. deviation	0.081	0.208	0.095	0.073	0.026	0.121	0.125
	Max	0.345	0.807	0.411	0.331	0.115	0.396	0.550
	Q3 (75th percentile)	0.187	0.298	0.213	0.152	0.098	0.326	0.141
	Median	0.124	0.141	0.135	0.073	0.078	0.298	0.101
	Q1 (25th percentile)	0.104	0.081	0.102	0.06	0.051	0.117	0.079
	Min.	0.013	0.020	0.001	0.035	0.040	0.034	0.025
	Q3-Q1	0.083	0.217	0.111	0.092	0.047	0.209	0.062
Protein M6	Number in group (N)	17	15	16	19	10	15	19
	Mean (arithmetic)	0.222	0.390	0.270	0.230	0.143	0.242	0.189
	Std. deviation	0.102	0.270	0.167	0.219	0.053	0.121	0.096
	Max	0.464	0.934	0.650	1.055	0.252	0.396	0.470
	Q3 (75th percentile)	0.291	0.620	0.353	0.275	0.181	0.326	0.228
	Median	0.197	0.337	0.223	0.167	0.133	0.298	0.191
	Q1 (25th percentile)	0.173	0.172	0.155	0.101	0.097	0.117	0.119
	Min.	0.064	0.077	0.004	0.071	0.087	0.034	0.055
	Q3-Q1	0.119	0.448	0.198	0.143	0.085	0.209	0.109

APPENDIX A

Univariate descriptive statistics for each group of subjects

VARIABLE	DESCRIPTIVE STATISTIC	CHILDREN				ADULTS		
		CONT-C	ARF	AGN	CRF-C	CONT-A	CRF-A	IHD
Protein M12	Number in group (N)	17	15	16	19	10	15	19
	Mean (arithmetic)	0.272	0.366	0.325	0.179	0.091	0.342	0.168
	Std. deviation	0.138	0.323	0.167	0.136	0.044	0.161	0.126
	Max	0.588	1.029	0.582	0.509	0.163	0.532	0.605
	Q3 (75th percentile)	0.366	0.456	0.474	0.241	0.142	0.504	0.183
	Median	0.259	0.281	0.292	0.135	0.078	0.370	0.150
	Q1 (25th percentile)	0.210	0.130	0.201	0.098	0.060	0.215	0.110
	Min.	0.042	0.021	0.003	0.031	0.036	0.062	0.010
	Q3-Q1	0.156	0.326	0.273	0.143	0.083	0.289	0.073
Protein M18	Number in group (N)	17	15	16	19	10	15	19
	Mean (arithmetic)	0.314	0.379	0.320	0.198	0.125	0.368	0.190
	Std. deviation	0.150	0.256	0.159	0.127	0.040	0.164	0.085
	Max	0.630	0.939	0.610	0.545	0.191	0.585	0.320
	Q3 (75th percentile)	0.383	0.468	0.446	0.276	0.156	0.497	0.248
	Median	0.333	0.350	0.276	0.175	0.121	0.346	0.196
	Q1 (25th percentile)	0.244	0.175	0.226	0.115	0.091	0.296	0.122
	Min.	0.061	0.069	0.007	0.059	0.077	0.074	0.039
	Q3-Q1	0.139	0.293	0.220	0.161	0.066	0.201	0.126
Protein M19	Number in group (N)	17	15	16	19	10	15	19
	Mean (arithmetic)	0.238	0.329	0.266	0.198	0.125	0.265	0.149
	Std. deviation	0.158	0.291	0.164	0.127	0.040	0.117	0.080
	Max	0.666	0.990	0.658	0.545	0.191	0.402	0.333
	Q3 (75th percentile)	0.284	0.414	0.340	0.276	0.156	0.393	0.185
	Median	0.230	0.211	0.269	0.175	0.121	0.289	0.135
	Q1 (25th percentile)	0.154	0.114	0.152	0.115	0.091	0.145	0.083
	Min.	0.020	0.020	0.003	0.059	0.077	0.059	0.013
	Q3-Q1	0.130	0.300	0.188	0.161	0.070	0.248	0.102

APPENDIX A

Univariate descriptive statistics for each group of subjects

VARIABLE	DESCRIPTIVE STATISTIC	CHILDREN				ADULTS		
		CONT-C	ARF	AGN	CRF-C	CONT-A	CRF-A	IHD
Protein M24	Number in group (N)	17	15	16	19	10	15	19
	Mean (arithmetic)	0.067	0.146	0.104	0.103	0.155	0.143	0.087
	Std. deviation	0.033	0.157	0.052	0.095	0.329	0.084	0.039
	Max	0.125	0.589	0.222	0.444	1.087	0.330	0.159
	Q3 (75th percentile)	0.091	0.153	0.133	0.123	0.106	0.181	0.110
	Median	0.063	0.133	0.097	0.087	0.038	0.134	0.094
	Q1 (25th percentile)	0.039	0.032	0.071	0.037	0.029	0.085	0.057
	Min.	0.018	0.008	0.007	0.024	0.019	0.002	0.011
	Q3-Q1	0.052	0.121	0.062	0.086	0.077	0.096	0.053
Heart 1:100	Number in group (N)	20	18	15	19	10	15	20
	Mean (arithmetic)	54.2	130.8	78.5	122.9	99.0	192.2	113.8
	Std. deviation	56.6	78.0	99.3	41.6	28.5	62.2	80.0
	Max	218.5	259.5	311.5	207.0	148.0	303.5	321.0
	Q3 (75th percentile)	85.6	184.9	153.0	151.5	120.8	227.0	167.0
	Median	37.8	133.3	48.5	116.0	95.0	203.0	88.3
	Q1 (25th percentile)	16.1	62.8	9.5	97.0	73.9	166.5	61.8
	Min.	-3.5	-13.5	-21.5	36.5	55.5	56.0	10.0
	Q3-Q1	69.5	122.0	143.5	54.5	46.9	60.5	105.3
Heart 1:1000	Number in group (N)	20	18	15	19	10	15	20
	Mean (arithmetic)	17.7	30.4	20.3	43.5	43.7	61.5	32.5
	Std. deviation	18.8	24.4	25.5	30.3	27.1	33.8	26.2
	Max	53.5	78.5	77.0	112.0	85.5	124.5	95.0
	Q3 (75th percentile)	30.5	45.1	34.5	66.5	69.4	93.5	54.1
	Median	12.8	27.0	26.0	31.5	43.2	49.5	29.8
	Q1 (25th percentile)	1.5	9.9	-1.5	20.5	19.8	34.5	7.1
	Min.	-6.0	-8.0	-17.0	4.5	4.0	17.0	-1.0
	Q3-Q1	29.0	35.3	36	46.0	49.6	59.0	47

APPENDIX A

Univariate descriptive statistics for each group of subjects

VARIABLE	DESCRIPTIVE STATISTIC	CHILDREN				ADULTS		
		CONT-C	ARF	AGN	CRF-C	CONT-A	CRF-A	IHD
Heart 1:10000	Number in group (N)	20	18	15	19	10	15	20
	Mean (arithmetic)	8.2	9.4	8.5	24.5	22.4	33.9	13.2
	Std. deviation	10.0	24.4	16.6	29.7	32.9	44.1	21.8
	Max	35.0	75.5	37.5	76.0	66.0	152.0	58.0
	Q3 (75th percentile)	13.5	13.6	22.5	52.5	49.0	49.5	31.9
	Median	7.5	4.8	9.5	9.0	29.8	18.0	12.0
	Q1 (25th percentile)	3.1	-2.5	-8.0	1.0	-10.0	1.0	-6.9
	Min.	-7.5	-17.5	-16.0	-13.0	-24.0	-3.5	-27.5
	Q3-Q1	10.4	16.1	30.5	51.5	59.0	48.5	38.8
Myosin 1:100	Number in group (N)	20	16	15	18	10	15	20
	Mean (arithmetic)	162.8	167.7	142.4	128.7	88.2	177.2	106.3
	Std. deviation	112.5	129.2	132.3	65.2	48.4	81.3	78.8
	Max	453.5	469.0	384.0	366.0	161.5	380.0	327.5
	Q3 (75th percentile)	227.9	227.8	247.0	146.5	133.4	204.0	127.4
	Median	131.0	135.3	88.5	114.8	82.3	159.0	96.0
	Q1 (25th percentile)	102.0	87,9	30.5	92.4	40.5	136.0	47.3
	Min.	18.0	-3.0	-20.0	71.5	31.0	77.5	14.5
	Q3-Q1	125.9	139.9	216.5	54.1	92.9	68.0	80.1
Myosin 1:1000	Number in group (N)	20	16	15	18	10	15	20
	Mean (arithmetic)	50.1	27.0	16.9	27.1	37.4	30.3	25.3
	Std. deviation	112.0	42.7	30.1	23.2	26.3	27.2	30.0
	Max	514.0	130.5	75.5	61.5	68.0	72.5	93.0
	Q3 (75th percentile)	42.5	55.4	26.5	52.6	58.9	43.5	45.9
	Median	26.8	11.5	16.0	21.5	44.8	31.0	21.2
	Q1 (25th percentile)	8.5	-1.9	2.0	11.1	14.3	23.5	-0.8
	Min.	-10.5	-25.0	-30.5	-16.5	-6.5	-49.0	-18.5
	Q3-Q1	34.0	57.3	24.5	41.5	44.6	20.0	46.6

APPENDIX A

Univariate descriptive statistics for each group of subjects

VARIABLE	DESCRIPTIVE STATISTIC	CHILDREN				ADULTS		
		CONT-C	ARF	AGN	CRF-C	CONT-A	CRF-A	IHD
Myosin 1:10000	Number in group (N)	20	16	15	18	10	15	20
	Mean (arithmetic)	2.6	-3.9	6.6	9.4	12.4	10.5	10.0
	Std. deviation	26.3	21.3	26.0	19.0	26.2	18.4	24.8
	Max	79.5	36.5	58.5	40.5	60.0	39.0	66.5
	Q3 (75th percentile)	16.6	8.5	28.5	28.9	32.2	26.5	31.8
	Median	2.3	-2.3	-1.5	6.0	10.3	16.0	1.3
	Q1 (25th percentile)	-15.0	-21.5	-8.0	-6.0	-2.0	-4.5	-9.1
	Min.	-39.0	-46.0	-30.5	-21.5	-34.5	-28.5	-25.5
	Q3-Q1	31.6	30.0	36.5	34.9	34.3	31.0	40.9
Collagen II 1:100	Number in group (N)	ND	15	18	19	10	15	19
	Mean (arithmetic)		0.015	0.005	0.004	0.02	0.001	0.106
	Std. deviation		0.020	0.009	0.008	0.02	0.003	0.308
	Max		0.055	0.036	0.028	0.056	0.012	1.001
	Q3 (75th percentile)		0.036	0.007	0.009	0.0454	0	0.01
	Median		0.003	0.0005	0	0.012	0	0
	Q1 (25th percentile)		0	0	0	0	0	0
	Min.		0	0	0	0	0	0
	Q3-Q1		0.036	0.007	0.009	0.045	0	0.01
Actin 1:10	Number in group (N)	15	16	16	19	9	13	19
	Mean (arithmetic)	1.636	0.202	0.084	0.195	0.117	28.098	0.105
	Std. deviation	5.634	0.072	0.693	0.060	0.05	100.776	0.037
	Max	22.0	0.336	0.352	0.292	0.187	363.5	0.163
	Q3 (75th percentile)	0.272	0.262	0.313	0.240	0.185	0.208	0.147
	Median	0.205	0.203	0.267	0.209	0.096	0.160	0.097
	Q1 (25th percentile)	0.095	0.135	0.184	0.138	0.083	0.111	0.071
	Min.	0.058	0.099	-2.5	0.072	0.049	0.050	0.036
	Q3-Q1	0.177	0.128	0.129	0.102	0.102	0.098	0.076

APPENDIX A

Univariate descriptive statistics for each group of subjects

VARIABLE	DESCRIPTIVE STATISTIC	CHILDREN				ADULTS		
		CONT-C	ARF	AGN	CRF-C	CONT-A	CRF-A	IHD
Actin 1:100	Number in group (N)	14	16	16	19	9	12	19
	Mean (arithmetic)	0.068	0.118	2.540	0.083	0.049	0.077	0.037
	Std. deviation	0.046	0.056	9.563	0.053	0.027	0.040	0.023
	Max	0.182	0.265	38.4	0.191	0.093	0.144	0.100
	Q3 (75th percentile)	0.091	0.134	0.242	0.111	0.067	0.100	0.048
	Median	0.068	0.119	0.151	0.072	0.052	0.085	0.036
	Q1 (25th percentile)	0.032	0.075	0.087	0.033	0.025	0.046	0.023
	Min.	0.007	0.033	0.027	0.018	0.008	0.004	0.005
	Q3-Q1	0.059	0.058	0.155	0.078	0.043	0.055	0.025
Actin 1:1000	Number in group (N)	14	15	16	19	9	13	19
	Mean (arithmetic)	0.006	0.037	2.60	0.023	0.007	0.208	0.004
	Std. deviation	0.015	0.037	10.240	0.037	0.009	0.689	0.008
	Max	0.048	0.126	41.000	0.115	0.019	2.500	0.033
	Q3 (75th percentile)	0	0.063	0.076	0.044	0.016	0.030	0.005
	Median	0	0.031	0.032	0.000	0.000	0.023	0.001
	Q1 (25th percentile)	0	0.000	0.013	0.000	0.000	0.001	0.000
	Min.	0	0.000	0.000	0.000	0.000	0.000	0.000
	Q3-Q1	-	0.063	0.063	0.044	0.016	0.029	0.005
Phosphorylase B 1:100	Number in group (N)	20	17	14	19	10	15	20
	Mean (arithmetic)	114.3	209.7	81.5	203.1	188.7	294.2	207.3
	Std. deviation	72.0	159.0	114.0	67.3	60.4	98.6	132.9
	Max	303.0	426.0	331.5	356.5	288.0	450.2	588.5
	Q3 (75th percentile)	145.6	338.5	116.4	223.5	245.8	359.5	307.6
	Median	106.8	214.5	53.3	195.0	180.5	318.0	185.0
	Q1 (25th percentile)	72.4	57.8	1.1	160.5	135.9	222.0	97.1
	Min.	24.5	-41.5	-44.5	81.5	93.5	65.0	11.5
	Q3-Q1	73.3	280.7	115.3	63	110.0	137.5	210.5

APPENDIX A

Univariate descriptive statistics for each group of subjects

VARIABLE	DESCRIPTIVE STATISTIC	CHILDREN				ADULTS		
		CONT-C	ARF	AGN	CRF-C	CONT-A	CRF-A	IHD
Phosphorylase B 1:1000	Number in group (N)	20	17	14	19	10	15	20
	Mean (arithmetic)	16.9	51.5	6.0	40.0	41.8	87.7	46.7
	Std. deviation	27.0	56.8	32.6	27.8	29.3	39.2	33.6
	Max	103.5	171.5	70.0	114.0	89.0	191.5	125.0
	Q3 (75th percentile)	26.4	85.8	27.0	56.0	66.9	97.0	72.9
	Median	12.0	52.5	-3.25	42.0	40.8	80.5	42.3
	Q1 (25th percentile)	1.1	5.0	-19.25	10.5	22.8	59.0	20.0
	Min.	-23.0	-38.0	-33.5	3.5	-12.0	44.5	-15.5
	Q3-Q1	25.3	80.75	46.3	45.5	44.1	38.0	52.9
Phosphorylase B 1:10000	Number in group (N)	20	17	14	19	10	15	20
	Mean (arithmetic)	4.5	9.8	1.2	8.5	16.6	24.4	13.9
	Std. deviation	20.5	32.4	22.8	25.8	32.8	25.3	26.0
	Max	75.5	66.0	47.5	67.5	74.5	78.5	77.5
	Q3 (75th percentile)	10.5	37.3	14.9	33.5	47.6	33.5	19.5
	Median	4.0	3.5	-3.3	-1.5	6.8	17.0	9.75
	Q1 (25th percentile)	-5.6	-13.75	-15.5	-10.5	-3.8	10.5	-3.75
	Min.	-27.0	-50.5	-34.0	-22.0	-35.0	-9.0	-26.0
	Q3-Q1	16.0	51.0	30.4	44.0	51.5	23	23.3
Streptococci 1:100	Number in group (N)	20	17	15	19	10	15	20
	Mean (arithmetic)	376.3	369.1	381.4	323.5	312.5	335.6	312.6
	Std. deviation	71.9	62.7	69.4	129.4	51.0	137.2	67.0
	Max	525.5	466.0	506.0	530.5	403.5	554.5	401.5
	Q3 (75th percentile)	439.4	413.8	443.5	394.6	354.9	406.5	347.5
	Median	378.3	374.0	354.0	318.5	310.3	359.5	332.8
	Q1 (25th percentile)	301.4	322.5	324.5	269.5	272.9	289.0	265.2
	Min.	280.0	232.0	291.5	-19.5	236.5	17.0	165.0
	Q3-Q1	138.0	91.3	119.0	125.0	82.0	117.5	82.3

APPENDIX A

Univariate descriptive statistics for each group of subjects

VARIABLE	DESCRIPTIVE STATISTIC	CHILDREN				ADULTS		
		CONT-C	ARF	AGN	CRF-C	CONT-A	CRF-A	IHD
Streptococci 1:1000	Number in group (N)	20	17	15	19	10	15	20
	Mean (arithmetic)	229.3	239.2	224.3	154.9	139.2	198.1	137.1
	Std. deviation	94.2	92.2	79.1	127.2	55.4	113.0	82.5
	Max	389.5	368.0	336.5	379.0	207.0	385.5	291.0
	Q3 (75th percentile)	310.6	318.0	276.5	259.5	188.8	269.5	201.5
	Median	236.8	250.0	257.5	154.5	153.0	202.0	163.8
	Q1 (25th percentile)	161.0	175.0	150.5	78.5	70.0	146.0	47.5
	Min.	50.0	49.0	82.0	-172.5	64.5	-111.0	22.5
	Q3-Q1	150.0	143.0	126.0	181.0	118.8	123.5	154.0
Streptococci 1:10000	Number in group (N)	20	17	15	19	10	15	20
	Mean (arithmetic)	56.0	74.0	66.6	20.7	31.8	28.4	24.3
	Std. deviation	60.0	118.6	44.7	86.4	34.5	102.5	34.7
	Max	161.0	179.5	142.5	131.5	106.0	184.5	114.0
	Q3 (75th percentile)	99.4	96.5	112.5	58.0	43.4	53.5	39.8
	Median	51.8	69.0	59.0	34.5	25.0	45.0	15.3
	Q1 (25th percentile)	26.8	42.5	31.5	6.5	16.0	24.0	1.1
	Min.	-69.0	-7.0	8.5	-297.0	-15.0	-311.0	-15.0
	Q3-Q1	72.6	54.0	81.0	51.5	127.0	29.5	38.6
ASO	Number in group (N)	19	14	19	19	10	15	17
	Mean (arithmetic)	351.6	588.6	469.5	263.2	184	236	531.8
	Std. deviation	165.8	297.0	291.8	114.9	71.1	112.7	382.6
	Max	640	1280	1280	480	320	480	1920
	Q3 (75th percentile)	320	720	640	320	240	240	560
	Median	320	480	400	240	160	240	480
	Q1 (25th percentile)	240	320	200	160	120	160	320
	Min.	120	240	200	120	120	60	160
	Q3-Q1	80	400	440	160	120	80	240

APPENDIX A

Univariate descriptive statistics for each group of subjects

VARIABLE	DESCRIPTIVE STATISTIC	CHILDREN				ADULTS		
		CONT-C	ARF	AGN	CRF-C	CONT-A	CRF-A	IHD
IgG	Number in group (N)	19	18	3	17	7	14	19
	Mean (arithmetic)	17	8.7	7	10.3	16.3	7.1	9.2
	Std. deviation	11.8	5.3	3	7.7	9.4	6.5	7.4
	Max	39	20	10	29	30	20	32
	Q3 (75th percentile)	29	11.75	10	16	21	11.25	13
	Median	15	8	7	7	18	5	8
	Q1 (25th percentile)	6	6.25	4	5	10	3	4
	Min.	0	0	4	0	0	0	2
	Q3-Q1	23	5.5	6	11	11	8.25	9
	Range	0-39	0-20	4-6	0-29	0-30	0-20	2-30
IgM	Number in group (N)	19	18	3	17	7	14	19
	Mean (arithmetic)	14	10.6	4.3	10.4	12.3	5.5	12.1
	Std. deviation	12.2	7.4	2.5	7.0	9.1	6.3	8.5
	Max	42	30	7	30	30	22	40
	Q3 (75th percentile)	19	11.75	7	14	16	7.5	15
	Median	10	8.5	4	9	10	3.5	11
	Q1 (25th percentile)	5	6.25	2	5.5	6	2	6
	Min.	2	4	2	2	2	0	2
	Q3-Q1	14	5.5	5	8.5	10	5.5.	9
	Range	2-40	4-26	2-5	2-28	2-28	0-22	2-38

APPENDIX B

CLINICAL AND DEMOGRAPHIC PARTICULARS OF ARF PATIENTS

Patient No	Age	Sex	Race	Area	Card	Arth	Chorea	Eryth	Nodules	ASO	DNASE B	ESR	IgG	IgA	IgM
51	9	M	Col	?	x	x				400	?	134	25.0	6.4	4.5
53	4	F	Col	U	x		x			1600	1280	70	26.4	1.3	1.7
56	13	M	Col	U	x	x				320	160	120	13.1	2.5	1.4
57	6	F	Col	U	x					400	640	150	22.4	2.2	3.9
58	10	F	Col	U	x	x				200	1280	85	22.0	2.5	3.8
61	5	M	Col	R	x					400	320	70	28.6	1.0	3.7
62	12	F	Col	R	x					1600	1280	134	22.7	3.5	5.6
63	10	F	Col	U	x	x				200	1280	60	21.9	2.9	3.0
64	3	M	Col	R	x					400	?	51	16.6	1.6	2.2
65	12	M	Col	R	x				x	200	1920	65	22.5	6.8	4.4
66	7	M	Bl	R	x					200	?	40	25.3	3.0	3.1
68	4	F	Col	R	x					1200	2560	90	27.3	2.6	3.7
70	13	M	Col	U	x	x				200	2560	110	26.7	4.9	2.4
300	11	F	Bl	U	x	x				400	1920	77	21.1	3.1	2.4
313	9	M	Col	U	x					400	2560	134	22.4	3.0	1.0

Key: M = Male, F = Female, Col = Coloured, Bl = Black, U = Urban, R = Rural, Arth = Arthritis, Eryth = Erythema marginatum, Card = Carditis

APPENDIX C

ADMISSION CRITERIA FOR PATIENTS

ACUTE RHEUMATIC FEVER (ARF)

i) must fulfill the Jones Criteria (as revised by the American Heart Association 1984),
ii) must have carditis according to usual clinical criteria,
iii) must have been given no medication except penicillin or antifailure treatment,
iv) must have informed parental consent,
v) must have had no heart operations,
vi) can have an initial attack of ARF or have acute on chronic rheumatic fever,
v) symptoms present for less than 14 days.

CHRONIC RHEUMATIC HEART DISEASE (CRF-a, CRF-c)

i) must be regular attenders at the clinic,
ii) must have had no heart operations,
iii) must have had no episodes of ARF within the previous 12 months,
iv) must have no signs of ARF at the time of entry,
v) must be on no drugs other than penicillin or antifailure therapy,
vi) must have been on no steroids during the previous 12 months,
vii) must have been documented to have had regular penicillin prophylaxis
viii) must have clinically and echocardiographically documented rheumatic valvular disease,
ix) must have informed parental consent (i.e. children),
x) must be older than 5 years.

ACUTE GOMERULONEPHRITIS (AGN)

i) the symptoms must have been present for less than 7 days, and they must have been in-patients for less than 48 hours,
ii) the features must be of classic AGN, i.e. oliguria, dark urine, fluid overload, haematuria, and hypertension,
iii) the haematuria must be on dipstix at least 3+,
iv) there must be at most 2+ proteinuria on dipstix,
v) serum albumin must be greater than 30 g/L, and cholesterol less than 6 mmol/L,
vi) they must have received no medication other than penicillin, diuretics or anti-hypertensives,
vii) there must be no organic heart disease,
viii) there must be no history of rheumatic fever.

ISCHAEMIC HEART DISEASE (IHD)

Subjects in this group were drawn from patients undergoing cardiac catheterization and coronary angiography for ischaemic heart disease. None of them had any history or clinical evidence of rheumatic heart disease.

NORMAL CONTROLS (CONT-a, CONT-c)

i) must have no immunological disease,
ii) must have no illness which might affect immune system,
iii) must have had no history of acute rheumatic fever,
iv) must have a clinically normal heart,
v) must be on no medication,
vi) must be older than 5 years,
vii) must have informed parental consent (i.e. children).

REFERENCES

Abou-Zeid, C., E. Filley, J. Steele, and G.A.W. Rook. (1987)
A simple new method for using antigens separated by polyacrylamide gel electrohoresis to stimulate lymphocytes in vitro by converting lines cut from western blots into antigen-bearing particles. J. Immunol. Methods **98**:5-10.

Adam, S.A., T. Nakagawa, M.S. Swanson, T.K. Woodruff, and G. Dreyfuss. (1986)
mRNA polyadenylate-binding protein: gene isolation and sequencing and identification of a ribonucleoprotein consensus sequence. Mol. Cell. Biol. **6**:2932-2943.

Adelstein, R.S., M.A. Conti, G.S. Johnson, I. Pastan, and T.D. Pollard. (1972)
Isolation and characterization of myosin from cloned mouse fibroblasts. Proc. Natl. Acad. Sci. USA **69**:3693-3697.

Aitchison, E.J., P.A. Lambert and I.D. Farrell. (1986)
Antigenic composition of an endocarditis-associated isolate of *Streptococcus faecalis* and identification of its glycoprotein antigens by ligand blotting with lectins. J. Med. Microbiol. **21**:161-167.

Alderuccio, F., A.J. Barnett, J.H. Campbell, J.S. Pedersen and B.H. Toh. (1986)
Scl-95/100: doublet of endothelial marker autoantigens in progressive systemic sclerosis. Clin. Exp. Immunol. **64**:94-100.

Alvarez, F.L., N. Neu, N.R. Rose, S.W. Craig, and K.W. Beisel. (1987)
Heart-specific autoantibodies induced by Coxsackievirus B3: Identification of heart autoantigens. Clin. Immunol. Immunopathol. **43**:129-139.

American Heart Association: Committee Report. (1984)
Jones criteria (revised) for guidance in the diagnosis of rheumatic fever. Circulation **69**:204A-208A.

Anderson, P.J. (1985)
The recovery of nitrocellulose-bound protein. Anal. Biochem. **148**:105-110.

Anderson, S. (1981)
Shotgun DNA sequencing using cloned DNase I-generated fragments. Nucleic Acids Res. **9**:3015-3027.

Andriopoulos, N.A., J. Mestecky, E.J. Miller, and E.L. Bradley. (1976)
Antibodies to native and denatured collagen in sera of patients with rheumatoid arthritis. Arthritis Rheum. **19**:613-617.

Andrzejewski, C. Jr., J. Rauch, E. Lafer, B.D. Stollar, and R.S. Schwartz. (1980)
Antigen binding diversity and idiotypic cross-reactions among hybridoma autoantibodies to DNA. J. Immunol. **126**:226-231.

Anhalt, G.J., R.S. Labib, J.J. Voorhees, et al. (1982)
Induction of pemphigus in neonatal mice by passive transfer of IgG from patients with the disease. N. Engl. J. Med. **306**:1189-1196.

Appleton, R.S., B.C. Victorica, D. Tamer, and E.M. Ayoub. (1985)
Specificity of persistence of antibody to the streptococcal group A carbohydrate in rheumatic valvular heart disease. J. Lab. Clin. Med. **105**:114-119.

Asherson, R.A., and E.N. Harris. (1986)
Anticardiolipin antibodies:clinical associations. Postgrad. Med. J. **62**:1081-1087.

Atassi, M.Z. (1975)
Antigenic structure of myoglobin: the complete immunochemical anatomy of a protein and conclusions relating to antigenic structures of proteins. Immunochemistry **12**:423-438.

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. (Eds.) (1987)
Current protocols in molecular biology. John Wiley and Sons, New York.

Avrameas, S. (1986)
Natural autoreactive B cells and autoantibodies: The "know thyself" of the immune system. Ann. Inst. Pasteur/Immunol **137** D:150-156.

Avrameas, S., G. Dighiero, P. Lymberi, et al. (1983)
Studies on natural antibodies and autoantibodies. Ann. Immunol. **134D**:103-113.

Avrameas, S., B. Guilbert, and G. Dighiero. (1981)
Natural antibodies against tubulin, actin, myoglobulin, thyroglobulin, fetuin, albumin and transferrin are present in normal human sera and monoclonal immunoglobulins from multiple myeloma and Waldenstrm's macroglobulinemia may express similar antibody specificities. Ann. Immunol. (Inst. Pasteur), **132C**:231-236.

Ayakawa, G.Y., A.S. Bleiweis, P.J. Crowley, and M.W. Cunningham. (1988)
Heart cross-reactive antigens of mutans streptococci share epitopes with group A streptococci and myosin. J. Immunol. **140**:253-257.

Ayakawa, G.Y., J.L. Siegel, P.J. Crowley, and A.S. Bleiweis. (1985)
Immunochemistry of the *Streptococcus mutans BHT* cell membrane: Detection of determinants cross-reactive with human heart tissue. Infect. Immun. **48**:280-286.

Ayoub, E.M. (1984)
The search for host determinants of susceptibility to rheumatic fever: the missing link. Circulation **69**:197-201.

Ayoub, E.M., and B.A. Dudding. (1970)
Streptococcal Group A carbohydrate antibody in rheumatic and non rheumatic bacterial endocarditis. J. Lab. Clin. Med. **76**:322-332.

Baccala, R., T.V. Quang, M. Gilbert, T. Ternynck, and S. Avrameas. (1989)
Two murine natural polyreactive autoantibodies are encoded by nonmutated germ-line genes. Proc. Natl. Acad. Sci. USA **86**:4624-4628.

Bach, J.F. (1986)
The original nature of autoantibodies. 13th Forum in Immunology (discussion Pg 178). Ann. Inst. Pasteur/Immunol. **137D**:149-182.

Bach, J.F. (1989)
Pathogenic Autoantibodies. In: B Lymphocytes: Function and Regulation. P. Del Guercio, J.M. Cruse, eds. Contrib Microbiol Immunol. Basel, Karger, 1989, vol 11, p.263-288.

Barbacid, M., D. Bolognesi, and S.A. Aaronson. (1980)
Humans have antibodies capable of recognizing oncoviral glycoproteins: Demonstration that these antibodies are formed in response to cellular modification of glycoproteins rather than as consequence of exposure to virus. Proc. Natl. Acad. Sci. U.S.A. 77:1617-1621.

Batteiger, B., W.J. Newhall, and R.B. Jones. (1982)
The use of Tween 20 as a blocking agent in the immunological detection of proteins transferred to nitrocellulose membranes. J. Immunol. Methods **55**:297-307.

Beachey, E.H., and J.M. Seyer. (1986)
Protective and nonprotective epitopes of chemically synthesized peptides of the amino terminal region of type 6 streptococcal M protein. J. Immunol. **136**:2287-2292.

Beachey, E.H., J.M. Seyer, J.B. Dale, and D.L. Hasty. (1983)
Repeating covalent structure and protective immunogenicity of native and synthetic polypeptide fragments of type 24 streptococcal M protein: Mapping of protective and nonprotective epitopes with monoclonal antibodies. J. Biol. Chem. **258**:13250-13257.

Beachey, E.H., J.M. Seyer, J.B. Dale, W.A. Simpson, and A.H. Kang. (1981)
Type-specific protective immunity evoked by synthetic peptide of *Streptococcus pyogenes* M protein. Nature (Lond). **292**:457-459.

Beachey, E.H., J.M. Seyer and A.H. Kang. (1978)
Repeating covalent structure of streptococcal M Protein. Proc. Natl. Acad. Sci. USA **75**:3163-3167.

Beachey, E.H., J.M. Seyer and A.H. Kang. (1980)
Primary structure of protective antigens of type 24 streptococcal M protein. J. Biol. Chem. **255**:6284-6289.

Beachey, E.H., J.M. Seyer and A.H. Kang. (1980)
Studies on the primary structure of streptococcal M protein antigens. In: Streptococcal Diseases and the Immune Responses. J.B. Zabriskie and S.E. Read, eds. Academic Press Inc., New York, p.149-160.

Beachey, E.H., G.H. Stollerman, E.Y. Chiang, T.M. Chiang, J.M. Seyer, and A.H. Kang. (1977)
Purification and properties of M protein extracted from Group A streptococci with pepsin: covalent structure of the amino terminal region of type 24 M antigen. J. Exp. Med. **145**:1469-1483.

Beachey, E.H., A. Tartar, J.M. Seyer, and L. Chedid. (1984)
Epitope-specific protective immunogenicity of chemically synthesized 13-, 18- and 23-residue peptide fragments of streptococcal M protein. Proc. Natl. Sci. USA **81**:2203-2207.

Beisiegel, U. (1986)
Protein blotting. Electrophoresis **7**:1-18.

Bell, J.A., T.H. Pennington, and D.T. Petrie. (1987)
Western blot analysis of staphylococcal antibodies present in human sera during health and disease. J. Med. Microbiol. **23**:95-99.

Benatar, A., D.W. Beatty, and D.G. Human. (1988)
Immunological abnormalities in children with acute rheumatic carditis and acute post-streptococcal glomerulonephritis. Int. J. Cardiol. **21**:51-58.

Benson, S.A., and R.K. Taylor. (1984)
A rapid small-scale procedure for isolation of phage lambda DNA. Biotechniques **2**:126-127.

Berzofsky, J.A., and A.N. Schechter. (1981)
The concepts of crossreactivity and specificity in immunology. Molec. Immunol. **18**:751-763.

Berzofsky, J.A., S.L. Epstein, and I.J. Berkower. (1989)
Antigen-antibody interactions and monoclonal antibodies. In: Fundamental Immunology, second edition. W.E. Paul, ed. Raven Press Ltd., New York, p.315-356.

Besredka, M. (1901)
Les antihemolysines naturelles. Ann. Inst. Pasteur. **15**:785-807.

Bevan, M.J. (1977)
In a radiation chimera, host H-2 antigens determine immune responsiveness of donor cytotoxic cells. Nature **269**:417-419.

Binz, H., and H. Wigzell. (1976)
Specific transplantation tolerance induced by autoimmunization against the individual's own naturally occurring idiotypic, antigen-binding receptors. J. Exp. Med. **144**:1438-1457.

Bisno, A.L. (1979)
Nonsuppurative poststreptococcal sequelae: Rheumatic fever and glomerulonephritis. In: Principles and Practice of Infectious Diseases, 2nd edition. G.L. Mandell, R.G. Douglas, and J.E. Bennett, eds. John Wiley & Sons, New York, p. 1133-1142.

Bisno, A.L. (1980)
The concept of rheumatogenic and nonrheumatogenic group A streptococci. In: Streptococcal Diseases and the Immune response. S. E. Read and J.B. Zabriskie, eds. Academic Press, New York, p.789-803.

Bisno, A.L. (1985)
The rise and fall of rheumatic fever. J.A.M.A. **254**:538-541.

Bisno, A.L., X. Berrios, F. Quesney, D.M. Monroe Jr., J.B. Dale, and E.H. Beachey. (1982)
Type-specific antibodies to structurally defined fragments of streptococcal M proteins in patients with acute rheumatic fever. Infect. Immun. **38**:573-579.

Bluestone, R., L.S. Goldberg, R.M. Katz et al. (1970)
Juvenile rheumatoid arthritis: A serologic survey of 200 consecutive patients. J. Pediatr. **77**:98-102.

Blumberg, D.D. (1987)
Creating a ribonuclease-free environment. Methods Enzymol. **152**:20-24.

Bogard, W.C., E. Hornberger, and P.C. Kung. (1985)
Production and characterisation of human monoclonal antibodies against Gram negative bacteria. In: Human Hybridomas and Monoclonal Antibodies. E.G. Engleman, S.K.H. Foung, J. Larrick and A. Raubitschek, eds. Plenum, New York, p.95.

Boissier, M-C., X-Z. Feng, A. Carlioz, R. Roudier, and C. Fournier. (1987)
Experimental autoimmune arthritis in mice. I. Homologous type II collagen is responsible for self-perpetuating chronic polyarthritis. Ann. Rheum. Dis. **46**:691-700.

Boonpucknavig, S., R. Udomsangpetch, and B. Pongpanich. (1984)
Immunological studies on acute rheumatic fever and rheumatic heart disease. J. Clin. Lab. Immunol. **13**:133-136.

Bottazzo, G-F., A. Florin-Christensen, A. Fairfax, G. Swana, D. Doniach, and U. Groeschel-Stewart. (1976)
Classification of smooth muscle autoantibodies detected by immunofluorescence. J. Clin. Path. **29**:403-410.

Bourguignon, L.Y.W., M.L. Nagpal, and Y. C. Hsing. (1981)
Phosphorylation of myosin light chain during capping of mouse T-lymphoma cells. J. Cell. Biol. **91**:889-894.

Boyden, S.V. (1963)
Natural antibodies and the immune response. Adv. Immunol. 5:1-28.

Bretherton, L., C. Brown, J.S. Pederson, B.H. Toh, F.N. Clarcke, I.R. Mackay, and I.D. Gust. (1983)
ELISA assay for IgG autoantibody to G-actin: comparison of chronic active hepatitis and acute viral hepatitis. Clin. Exp. Immunol. 51:611-616.

Brown, P., M. Coker-Vann, K. Pomeroy, M. Franko, D.M. Asher, C.J. Gibbs Jr., and D. Carleton Gajdusek. (1986)
Diagnosis of Creutzfeldt-Jakob disease by western blot identification of marker protein in human brain tissue. New Eng. J. Med. 314:547-551.

Burastero, S.E., P. Casali, R.L. Wilder, and A.L. Notkins. (1988)
Monoreactive high affinity and polyreactive low affinity rheumatoid factors are produced by CD5+ B cells from patients with rheumatoid arthritis. J. Exp. Med. 168:1979-1992.

Burnet, F.M. (1959)
The clonal selection theory of acquired immunity. Cambridge University Press, London.

Burnett, K.G., J.P. Leung, and J. Martinis. (1985)
Human monoclonal antibodies to defined antigens: Towards clinical applications. In: Human Hybridomas and Monoclonal Antibodies. E.G. Engleman, S.K.H. Foung, J. Larrick and A. Raubitschek, eds. Plenum, New York, p.113.

Burnie, J., and I. Clark. (1989)
Diagnosing endocarditis with the cloned 112 kDa antigen of *Enterococcus faecalis*. J. Immunol. Methods 123:217-225.

Burnie, J., and R.C. Matthews. (1987)
Immunoblot analysis: a new method for fingerprinting hospital pathogens. J. Immunol. Methods 100:41-46.

Cairns, L., F.S. Rosen, and Y. Borel. (1986)
Mice naturally tolerant to C5 have T cells that suppress the response to this antigen. Eur. J. Immunol. 16:1277-1282.

Cameron, D.J., and B.F. Erlanger. (1977)
Evidence for multispecificity of antibody molecules. Nature 268:763-765.

Carroll, P., D. Stafford, R.S. Schwartz, and B.D. Stollar. (1985)
Murine monoclonal anti-DNA autoantibodies bind to endogenous bacteria. J. Immunol. 135:1086-1090.

Carson, D.A., P.P. Chen, R.I. Fox et al. (1987)
Rheumatoid factors and immune networks. Annu. Rev. Immunol. 5:109-126.

Casali, P., S.E. Burastero, M. Nakamura, G. Inghirami, and A.L. Notkins. (1987)
Human lymphocytes making rheumatoid factor and antibody to ssDNA belong to Leu-1+ B-cell subset. Science 236:77-81.

Casali, P., and A.L. Notkins. (1989)
CD5+ B lymphocytes, polyreactive antibodies and the human B-cell repertoire. Immunology Today 10:364-368.

Casali, P., and A.L. Notkins. (1989)
Probing the human B-cell repertoire with EBV: Polyreactive antibodies and CD5+ B lymphocytes. Annu. Rev. Immunol. 7:513-535.

Casali, P., B.S. Prabhakar, and A.L. Norkins. (1988)
Characterization of multi-reactive autoantibodies and identification of Leu-1+B lymphocytes as cells making antibodies binding multiple self and exogenous molecules. Int. Rev. Immunol. 3:17-45.

Cassidy, J.T., A. Burt, R. Petty et al. (1969)
Selective IgA deficiency in connective tissue diseases. New Engl. J. Med. 280:275-279.

Chambers, J.C, and J.D. Keene. (1985)
Isolation and analysis of cDNA clones expressing human lupus La antigen. Proc. Natl. Acad. Sci. U.S.A. 82:2115-2119.

Chambers, J.C., D. Kenan, B.J. Martin, and J.D. Keene. (1988)
Genomic structure and amino acid sequence domains of the human La autoantigen. J. Biol. Chem. 263:18043-18051.

Cheadle, W.B. (1889)
Harveian lectures on the various manifestations of the rheumatic state as exemplified in childhood and early life. Lancet I 821-832.

Chen, J-H., D.H. Kono, Z. Yong, M.S. Park, M.B.A. Oldstone, and D.T.Y. Yu. (1987)
A *Yersinia pseudotuberculosis* protein which cross-reacts with HLA-B27. J. Immunol. 139:3003-3011.

Chiles, T.C., T.W. O'Brien, and M.S. Kilberg. (1987)
Production of monospecific antibodies to a low-abundance hepatic membrane protein using nitrocellulose immobilized protein as antigen. Anal. Biochem. 163:136-142.

Choi, Y.S. (1977)
Serological precipitation method for studying biosynthesis and secretion of immunoglobulins by human peripheral blood lymphocytes. J. Immunol. Methods 14:37-40.

Chomczynski, P., and N. Sacchi. (1987)
Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156-159.

Clague, R.B., M.J. Shaw, and P.J.L. Holt. (1981)
Incidence and correlation between serum IgG and IgM antibodies to native type II collagen in patients with chronic inflammatory arthritis. Ann. Rheum. Dis. 40:6-10.

Clough, J.D., and R. Valenzuela. (1980)
Relationship of renal histopathology in SLE nephritis to immunoglobulin class of anti-DNA. Am. J. Med. 68:80-85.

Cohen, I.R. (1984)
Autoimmunity: Physiologic and Pernicious. In: Adv. In Int. Med. Stollerman, ed. p.147-165.

Cohen, I.R. (1989)
An electic summary of the symposium on autoimmunity. Immunology Today 10:394-396.

Cohen, I.R., and A. Cooke. (1986)
Natural autoantibodies might prevent autoimmune disease. Immunology Today 7:363-364.

Colaco, C.B., and D.K. Male. (1985)
Antiphospholipid antibodies in syphilis and a thrombotic subset of SLE: Distinct profiles of epitope specificity. Clin. Exp. Immunol. 59:449-456.

Congeni, B., C. Rizzo, J. Congeni, and V.V. Sreenivasan. (1987)
Outbreak of acute rheumatic fever in northeast Ohio. J. Pediatr. 111:176-179.

Cossio, P., C. Diez, E. Szarfman et al. (1974)
Demonstration of a serum gamma globulin factor which reacts with endocardium and vascular structures. Circulation 49:13-21.

Cote, R.J., and A.N. Houghton. (1985)
The generation of human monoclonal antibodies and their use in the analysis of the humoral immune response to cancer. In: Human Hybridomas and Monoclonal Antibodies. E.G. Engleman, S.K.H. Foung, J. Larrick and A. Raubitschek, eds. Plenum, New York, p.189.

Cote, R.J., D.M. Morrissey, A.N. Houghton, E.J. Beattie, Jr, H.F. Oettgen, and L.J. Old. (1983)
Generation of human monoclonal antibodies reactive with cellular antigens. Proc. Natl. Acad. Sci. USA 80:2026-2030.

Cote, R.J., D.M. Morrissey, A.N. Houghton, T.M. Thomson, M.E. Daly, H.F. Oettgen, and L.J. Old. (1986)
Specificity analysis of human monoclonal antibodies reactive with cell surface and intracellular antigens. Proc. Natl. Acad. Sci. USA 83:2959-2963.

Crawford, L., K. Leppard, D. Lane, and E. Harlow. (1982)
Cellular proteins reactive with monoclonal antibodies directed against simian virus 40 T-antigen. J. Virol. 42:612-620.

Croce, C.M., M. Shander, J. Martinis, L. Cicurel, G.G. D'Ancona, and H. Koprowski. (1980b)
Preferential retention of human chromosome 14 in mouse x human B cell hybrids. Eur. J. Immunol. 10:486-488.

Cummins, P., and M.L. Auckland. (1984)
Variable myosin light chain expression in tetralogy of Fallot. Journal of Molecular and Cellular Cardiology 16:66.

Cummins, P., B. McGurk, and W.A. Littler. (1981)
Radioimmunoassay of human cardiac tropomyosin in acute myocardial infarction. Clinical Science 60:251-259.

Cunningham, A.L., I.R. Mackay, I.H. Frazer, C. Brown, J.S. Pedersen, B.-H. Toh, B.D. Tait, and F.M. Clarke. (1985)
Antibody to G-actin in different categories of alcoholic liver disease: quantification by an ELISA and significance for alcoholic cirrhosis. Clin. Immunol. Immunopathol. 34:158-164.

Cunningham, M.W. N.K. Hall, K.K. Krisher, and A.M. Spanier. (1986)
A study of anti-group A streptococcal monoclonal antibodies cross-reactive with myosin. J. Imunol. 136:293-298.

Cunningham, M.W., K. Krisher, and D.C. Graves. (1984)
Murine monoclonal antibodies reactive with human heart and group A streptococcal membrane antigens. Infect. Immun. **46**:34-41.

Cunningham, M.W., J.M. McCormack, P.G. Fenderson, M-K. Ho, E.H. Beachey, and J.B. Dale. (1989)
Human and murine antibodies cross-reactive with streptococcal M protein and myosin recognize the sequence GLN-LYS-SER-LYS-GLN in M protein. J. Immunol. **143**:2677-2683.

Cunningham, M.W., J.M. McCormack, L.R. Talaber, J.B. Harley, E.M. Ayoub, R.S. Muneer, L.T. Chun, and D.V. Reddy. (1988)
Human monoclonal antibodies reactive with antigens of the group A streptococcus and human heart. J. Immunol. **141**:2760-2766.

Cunningham, M.W., and S.M. Russell. (1983)
Study of heart-reactive antibody in antisera and hybridoma culture fluids against group A streptococci. Infect. and Immun. **42**:531-537.

Cunningham, M.W., and R.A. Swerlick. (1986)
Polyspecificity of anti-streptococcal murine monoclonal antibodies and their implications in autoimmunity. J. Exp. Med. **164**:998-1012.

Cunningham-Rundles, C., and M.K.L. Cheung. (1985)
Cross-reacting idiotypes in human sera. Immunology Today **6**:14-16.

Czarnocka, B., J. Ruf, M. Ferrand, P. Carayon, and S. Lissitzky. (1985)
Purification of the human thyroid peroxidase and its identification as the microsomal antigen involved in autoimmune thyroid diseases. FEBS Lett **190**:147.

Dale, J.B., and E.H. Beachey. (1982a)
Protective antigenic determinant of streptococcal M protein shared with sarcolemmal membrane protein of human heart. J. Exp. Med. **156**:1165-1176.

Dale, J.B., and E.H. Beachey. (1982b)
Protective antibody against a peptide fragment of type 5 streptococcal M protein cross-reacts with human heart tissue. Trans. Assoc. Am. Physicians **95**:286-291.

Dale, J.B., and E.H. Beachey. (1985a)
Multiple heart-crossreactive epitopes of streptococcal M proteins. J. Exp. Med. **161**:113-122.

Dale, J.B., and E.H. Beachey. (1985b)
Epitopes of streptococcal M proteins shared with cardiac myosin. J. Exp. Med. **162**:583-591.

Dale, J.B., and E.H. Beachey. (1986a)
Localization of protective epitopes of the amino terminus of type 5 streptococcal M protein. J. Exp. Med. **163**:1191-1202.

Dale, J.B., and E.H. Beachey. (1986b)
Sequence of myosin-crossreactive epitopes of streptococcal M protein. J. Exp. Med. **164**:1785-1790.

Dale, J.B., and E.H. Beachey. (1987)
Human cytotoxic T lymphocytes evoked by group A streptococcal M proteins. J. Exp. Med. **166**:1825-1835.

Dale, J.B., I. Ofek, and E.H. Beachey. (1980)
Heterogeneity of type-specific and cross-reactive antigenic determinants within a single M protein of group A streptococci. J. Exp. Med. **151**:1026-1038.

Dale, J.B., J.M. Seyer, and E.H. Beachey. (1983)
Type-specific immunogenicity of a chemically synthesized peptide fragment of type 5 streptococcal M protein. J. Exp. Med. **158**:1727-1732.

Dale, J.B., W.A. Simpson, I. Ofek, and E.H. Beachey. (1981)
Blastogenic responses of human lymphocytes to structurally defined polypeptide fragments of streptococcal M protein. J. Immunol. **126**:1499-1505.

Dales, S., R.S. Fujinami, and M.B.A. Oldstone. (1983)
Serologic relatedness between Thy-1.2 and actin revealed by monoclonal antibody. J. Immunol. **131**:1332-1338.

Damian, R.T. (1987)
Molecular mimicry revisited. Parasitology Today **3**:263-266.

Das, S.K., J.T. Cassidy, and R.E. Petty. (1972)
Antibodies against heart muscle and nuclear constituents in cardiomyopathy. Am. Heart J. **83**:159-166.

DeLozanne, A., M. Lewis, J.A. Spudich, and L.A. Leinwand. (1985)
Cloning and characterization of a nonmuscle myosin heavy chain cDNA. Proc. Natl. Acad. Sci. USA **82**:6807-6810.

Denis, K.A., R. Wall, and A. Saxon. (1983)
Human-human B cell hybridomas from in vitro stimulated lymphocytes of patients with common variable immunodeficiency. J. Immunol. **131**:2273-2278.

Dennert, G. (1971)
The mechanism of antibody-induced stimulation and inhibition of the immune response. J. Immunol. **106**:951-955.

De Scheerder, I., J. Vanderkerckhove, G. De Schrijver, M. Hoste, D. Clement, R. Wieme, and R. Pannier. (1985)
Detection of anti-contractile antibodies after cardiac surgery using ELISA assay. Clin. Exp. Immunol. **60**:403-406.

Devereux, J., P. Haeberli, and O. Smithies. (1984)
A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Research **12(1)**:387-395.

Devereux, J. (1989)
The GCG Sequence Analysis Software Package, Version 6.0, Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wisconsin, USA, 53705.

Diamond, A.G., G.W. Butcher, and J.C. Howard. (1983)
Localized conformational changes induced in a Class 1 major histocompatibility antigen by the binding of moncoclonal antibodies. J. Immunol. **132**:1169-1175.

Dighiero, G., B. Guilbert, and S. Avrameas. (1982)
Naturally occurring antibodies against nine common antigens in humans sera. II. High incidence of monoclonal Ig exhibiting antibody activity against actin and tubulin and sharing antibody specificities with natural antibodies. J. Immunol. **128**:2788-2792.

Dighiero, G., B. Guilbert, J-P. Fermand, P. Lymberi, F. Danon, and S. Avrameas. (1983)
Thirty-six human monoclonal immunoglobulins with antibody activity against cytoskeleton proteins, thyroglobulin, and native DNA: Immunologic studies and clinical correlations. Blood **62**:264-270.

Dighiero, G., P. Lymberi, D. Holmberg, I. Lundquist, A. Coutinho, and S. Avrameas. (1985)
High frequency of natural autoantibodies in normal newborn mice. J. Immunol. **134**:765-771.

Dighiero, G., P. Lymberi, J.C. Mazie, S. Rouyre, G.S. Butler-Browne, R.G. Whalen, and S. Avrameas. (1983)
Murine hybridomas secreting natural monoclonal antibodies reacting with self antigens. J. Immunol. **131**:2267-2272.

Dignam, J.D., R.M. Lebovitz, and R.G. Roeder. (1983)
Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nuc. Acids Res. **11**:1475-1489.

DiSciascio, G., and A. Taranta. (1980)
Rheumatic fever in children. Am. Heart J. **99**:635-658.

Doyle, G., D. Everhart, C. Mallett, G. Ayakawa, and A.S. Bleiweis. (1986)
Demonstration of shared antigenic determinants between *Streptococcus mutans BHT* cell membrane, human heart tissue and myosin using monoclonal antibodies to *S. mutans*. J. Gen. Microbiol. **132**:2885-2892.

Dresser, D.W. (1978)
Most IgM-producing cells in the mouse secrete autoantibodies (rheumatoid factor). Nature **274**:480-483.

Dressler, W. (1959)
The post-myocardial - infarction syndrome. Am. Arch. Int. Med. **103**:28-42.

Dropcho, E.J., Y-T. Chen, J.B. Posner, and L.J. Old. (1987)
Cloning of a brain protein identified by autoantibodies from a patient with paraneoplastic cerebellar degeneration. Proc. Natl. Acad. Sci. USA **84**:4552-4556.

Dübois, D.B., and R.D. Rossen. (1983)
Use of human antibodies to identify antigens in cultured human tumor cells: Detection of discrete antigen molecules using electroblotting and enzyme-linked antibody probes. J. Immunol. Methods **63**:7-24.

Dübois, P., M. Dardenne, T. Fandeur, O. Mercereau-Puijalon, D. Mattei, B. Müller-Hill, T. Blisnick, and L. Pereira da Silva. (1988)
Structure and function of a thymic peptide is mimicked by *Plasmodium falciparum* peptides. Ann. Inst. Pasteur/Immunol. **139**:557-567.

Duggan, D.B., and A. Schattner. (1986)
Unusual manifestations of monoclonal gammopathies. Autoimmune and idiopathic syndromes. Am. J. Med. **81**:864-870.

Dulbecco, R., M. Unger, M. Bologna, H. Battifora, P. Syka, and S. Okada. (1981)
Cross-reactivity between Thy-1 and a component of intermediate filaments demonstrated using a monoclonal antibody. Nature (London) **292**:772-774.

Dziarski, R. (1987)
Natural autoantibodies might prevent autoimmune disease. Immunology Today **8**:132.

Dziarski, R. (1988)
Autoimmunity: polyclonal activation or antigen induction? Immunology Today **9**:340-342.

Effros, R.B., C.M. Hulette, R. Ettenger, L.C. Dillard, E. Zeller, R. Duong, and R.L. Walford. (1986)
A human-human hybridoma secreting anti-HLA class II antibody. J. Immunol. **137**:1599-1603.

Efstratiadis, F., F.C. Kafotos, A.M. Maxam, and T. Maniatis. (1978)
Enzymatic in vitro synthesis of globin genes. Cell **7**:279-288.

Ehrenfeld, E.N., I. Gery, and A.M. Davies. (1961)
Specific antibodies in heart disease. Lancet I:1138-1141.

Ehrlich, P., and J. Morgenroth. (1901)
Ueber Haemolysine. Berl. Klin. Wschr. **21**:569-574.

Eichmann, K. (1978)
Expression and function of idiotypes on lymphocytes. Adv. Immunol. **26**:195-254.

Eichmann, K., and K. Rajewsky. (1975)
Induction of T and B cell immunity by anti-idiotypic antibody. Eur. J Immunol. **5**:661-666.

Eisenbarth, G.S., A. Linnenbach, R. Jackson, R. Scearce, and C.M. Croce. (1982)
Human hybridomas secreting anti-islet autoantibodies. Nature **300**:264-267.

Elkon, K.B., E. Bonfa, J. Li-Chu, and A.P. Parnassa. (1987)
Characterization of autoantigen and autoantibodies by immunoblotting. Electrophoresis **8**:445.

Ellis, J., L.S. Ozaki, R.W. Gwadz, A.H. Cochrane, V. Nussenzweig, R.S. Nussenzweig, and G.N. Godson. (1983)
Cloning and expression in *E.coli* of the malarial sporozoite surface antigen gene from *Plasmodium knowlsei*. Nature **302**:536-538.

Engleman, E.G., S.K.H. Foung, J. Larrick, and A. Raubitschek. (Eds.) (1985)
Human hybridomas and monoclonal antibodies. Plenum Press, New York.

Engvall, E., and P. Perlman. (1972)
Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. J. Immunol. **109**:129-135.

Fagraeus, A., C. Orvell, R. Norberg, and E. Norrby. (1983)
Monoclonal antibodies to epitopes shared by actin and vimentin obtained by paramyxovirus immunization. Exp. Cell Res. **145**:425-432.

Fairfax, A.J., and U. Groschel-Stewart. (1977)
Myosin autoantibodies detected by immunofluorescence. Clin. Exp. Immunol. **28**:27-34.

Falk, J.A., J.L. Fleischman, J.B. Zabriskie and R.E. Falk. (1973)
A study of HLA antigen phenotype in rheumatic fever and rheumatic heart disease patients. Tissue Antigens **3**:173-178.

Fallon, J.R., and V.T. Nachmias. (1980)
Localization of cytoplasmic and skeletal myosins in developing muscle cells by double-label immunofluorescence. J. Cell Biol. **87**:237-247.

Fasler, S., F. Skvaril, and H.U. Lutz. (1988)
Electrophoretic properties of human IgG and its subclasses on sodium dodecyl-sulfate-polyacrylamide gel electrophoresis and immunoblots. Anal. Biochem. 174:593-600.

Feinstein, A.R., and M. Spagnuolo. (1962)
The clinical patterns of acute rheumatic fever: A reappraisal. Medicine **41**:279-305.

Fellows, G., N. Gittoes, D.G.I. Scott, J.S. Coppock, A. Wainwright, M.Goodall, and B.M. Turner. (1988)
Individual variation in the isotype profile of anti-histone autoantibodies in systemic lupus erythematosus. Clin. Exp. Immunol. **72**:440-445.

Fenderson, P.G., V. A. Fischetti, and M.W. Cunningham. (1989)
Tropomyosin shares immunologic epitopes with group A streptococcal M proteins. J. Immunol. **142**:2475-2481.

Fischetti, V.A., K.F. Jones, B.N. Manjula, and J.R. Scott. (1983)
Streptococcal M6 protein expressed in *Escherichia coli*: localization, purification and comparison with streptococcal derived M protein. J. Exp. Med. **159**:1083-1095.

Fischetti, V.A., D.A.D. Parry, B.L. Trus, S.K. Hollingshead, J.R. Scott, and B.N. Manjula. (1988)
Conformational characteristics of the complete sequence of group A streptococcal M6 protein. Struct. Funct. Genet. **3**:60-69.

Fischetti, V.A., and B.N. Manjula. (1982)
Biologic and immunologic implications of the structural relationship between streptococcal M protein and Mammalian tropomyosin. Semin. Infect. Dis. 4:411-418.

Fisher, D.Z., N. Chaudhary, and G. Blobel. (1986)
cDNA sequencing of nuclear lamins A and C reveals primary and secondary structural homology to intermediate filament proteins. Proc. Natl. Acad. Sci. USA **83**:6450-6454.

Franke, W.W., E. Schmid, C. Grund, and B. Geiger. (1982)
Intermediate filament proteins in nonfilamentous structures: Transient disintegration and inclusion of subunit proteins in granular aggregates. Cell **30**:103-113.

Friedman, I., A. Laufer, N. Ron, and A.M. Davies. (1971)
Experimental myocarditis: in vitro and in vivo studies of lymphocytes sensitized to heart extracts and group A streptococci. Immunology. **20**:225-232.

Fujinami, R.S., and M.B.A. Oldstone. (1985)
Amino acid homology between the encephalitogenic site of myelin basic protein and virus: Mechanism for autoimmunity. Science **230**:1043-1045.

Fujinami, R.S., M.B.A. Oldstone, Z. Wroblewska, M.E. Frankel, and H. Koprowski. (1983)
Molecular mimicry in virus infection: Crossreaction of measles virus phosphoprotein or of herpes simplex virus protein with human intermediate filaments. Proc. Natl. Acad. Sci. USA **80**:2346-2350.

Gabbiani, G., G.B. Ryan, J-P. Lamelin, P. Vassalli, G. Majno, C.A. Bouvier, A. Cruchaud, and E.F. Lüscher. (1973)
Human smooth muscle autoantibody. Its identification as anti-actin antibody and a study of its binding to "non-muscular" cells. Amer. J. Pathol. **72**:473-488.

Gadd, S.J., and L.K. Ashman. (1983)
Binding of mouse monoclonal antibodies to human leukaemic cells via the Fc receptor: a possible source of `false positive' reactions in specificity screening. Clin. Exp. Immunol. **54**:811-818.

Gaffar, S.A., C.D. Surh, and M.D. Glassy. (1986b)
Variations in the secretion of monoclonal antibodies by human-human hybridomas. Hybridoma **5**:93-105.

Gambke, B., and N.A. Rubinstein. (1984)
A monoclonal antibody to the embryonic myosin heavy chain of rat skeletal muscle. J. Biol. Chem. **259**:12092-12100.

Gershoni J.M., and G.E. Palade. (1983)
Protein blotting: Principles and applications. Anal. Biochem. **131**:1-15.

Getzoff, E.D., J.A. Tainer, R.A. Lerner, and H.M. Geysen. (1988)
The chemistry and mechanism of antibody binding to protein antigens. Adv. Immunol. **43**:1-98.

Ghosh, S., and A.M. Campbell. (1986)
Multispecific monoclonal antibodies. Immunology Today **7**:217-222.

Gibofsky, A., and J.B. Zabriskie. (1988)
Acute rheumatic fever: Clinical and immunopathologic aspects.
In Infections in the Rheumatic Diseases. L. Espinoza, D. Goldenberg, F. Arnett, and G. Alarcon, eds. Grune & Stratton, Inc., p.367-373.

Glynn, L.E., and E.J. Holborrow. (1961)
Relation between blood groups, secretor status and susceptibility to rheumatic fever. Arthritis Rheum. **4**:203-207.

Goding, J.W. (1980)
Antibody production by hybridomas. J. Immunol. Methods. **39**:285-308.

Golding, B., G. Inghirami, E. Peters, T. Hoffman, J. Balow, and G. Tsokos. (1987)
In vitro generated human monoclonal TNP-specific B cell lines: evidence that human and murine anti-TNP monoclonal antibodies cross-react with *E.coli* beta-galactosidase. J. Immunol. **139**:4061-4066.

Goldstein, I., B. Halpern, and L. Robert. (1967)
Immunological relationship between streptococcus A polysaccharide and the structural glycoproteins of heart valve. Nature (London) **213**:44-47.

Gooderham, K. (1985)
Protein Blotting. In: Techniques in Molecular Biology. Walker and Gaastra, eds. Groom and Helm, p.49-61.

Goroncy-Bermes, P., J.B. Dale, E.H. Beachey, and W. Opferkuch. (1987)
Monoclonal antibody to human renal glomeruli cross-reacts with streptococcal M protein. Infect. Immun. **55**:2416-2419.

Gosslau, B., and H-J. Barrach. (1979)
Enzyme-linked immunosorbent microassay for quantification of specific antibodies to collagen type I, II, III. J. Immunol. Methods **29**:71-77.

Gottlieb, A.B., B. Lifshitz, S.M. Fu, L. Staiano-Coico, C.Y. Wang, and D.M. Carter. (1986)
Expression of HLA-DR molecules by keratinocytes, and presence of langerhans cells in the dermal infiltrate of active psoriatic plaques. J. Exp. Med. **164**:1013-1028.

Gowrishankar, R., and S.C. Agarwal. (1980)
Leucocyte migration inhibition with human heart valve glycoproteins and group A streptococcal ribonucleic acid proteins in rheumatic heart disease and post-streptococcal glomerulonephritis. Clin. Exp. Immunol. **39**:519-525.

Grabar, P. (1983)
Autoantibodies and the physiological role of immunoglobulins. Immunol. Today 4:337-340.

Gray, E.D., L.W. Wannamaker, E.M. Ayoub, A. El Kholy, and Z.H. Abdin. (1981)
Cellular immune responses to extracellular streptococcal products in rheumatic heart disease. J. Clin. Invest. **68**:665-671.

Gripenberg, M., and P. Kurki. (1986)
Demonstration of human autoantibodies by quantitative enzyme immunoassays. J. Immunol. Methods **92**:145-159.

Guilbert, B., G. Dighiero, and S. Avrameas. (1982)
Naturally occurring antibodies against nine common antigens in human sera. I. Detection, isolation, and characterization. J. Immunol. **128**:2779-2787.

Haber, E. (1982)
Immunological probes in cardiovascular disease. Br. Heart J. **47**:1-10.

Habets, W.J.A. (1986)
Immunobiology of anti-nuclear antibodies in rheumatic diseases. PhD Thesis.

Habets, W.J.A., J.H.M. Berden, S.O. Hoch, and W.J. van Venrooij. (1985)
Further characterization and subcellular localization of Sm and UI ribonucleoprotein antigens. Eur. J. Immunol. **15**:992-997.

Habets, W.J.A., D.J. De Rooij, M.H. Salden, A.P. Verhagen, C.A. Van Eekelen, L.B. Van de Putte, and W.J. Van Venrooij. (1983)
Antibodies against distinct nuclear matrix proteins are characteristic for mixed connective tissue disease. Clin. Exp. Immunol. **54**:265-276.

Habets, W.J.A., M.H. Hoet, P. Bringmann, R. Luhrmann, and W.J. van Venrooij. (1985)
Autoantibodies to ribonucleoprotein particles containing U2 small nuclear RNA. EMBO J. **4**:1545-1550.

Habets, W.J.A., P.T.G. Sillekens, M.H. Hoet, J.A. Schalken, A.J.M. Roebroek, J.A.M. Leunissen, W.J.M. van de Ven, and W.J. van Venrooij. (1987)
Analysis of a cDNA clone expressing a human autoimmune antigen: Full-length sequence of the U2 small nuclear RNA-associated B" antigen. Proc. Natl. Acad. Sci. U.S.A. **84**:2421-2425.

Hall, R., J.E. Hyde, M. Goman, D.L. Simmons, I.A. Hope, M. Mackay, J. Scaife, B. Merkli, R. Richle, and J. Stocker. (1984)
Major surface antigen gene of a human malaria parasite cloned and expressed in bacteria. Nature **311**:379-382.

Hamada, N., C. Grimm. H. Mori, and L.J. De Groot. (1985)
Identification of a thyroid microsomal antigen by Western blot and immunoprecipitation. J. Clin. Endocrinol. Metab. **61**:120-128.

Hamsten, A., R. Norberg, M. Bjorkholm, U. De Faire, and G. Holm. (1986)
Antibodies to cardiolipin in young survivors of myocardial infarction: an association with recurrent cardiovascular events. Lancet i:113-115.

Hardy, R.R., K. Hayakawa, M. Shimizu, K. Yamasaki, and T. Kishimoto. (1987)
Rheumatoid factor secretion from human Leu-1+ B-cells. Science **236**:81-83.

Harley, E.H., and P. Klemp. (1982)
A specific DNA immune complex assay, and its application in SLE. Clinica. Chimica. Acta. **122**:213-223.

Harris, D.E., L. Cairns, F.S. Rosen, and Y. Borel. (1982)
A natural model of immunologic tolerance. Tolerance to murine C5 is mediated by T cells, and antigen is required to maintain unresponsiveness. J. Exp. Med. **156**:567-584.

Haskard, D.O., and J.R. Archer. (1984)
The production of human monoclonal autoantibodies from patients with rheumatoid arthritis by the EBV-hybridoma technique. J. Immunol. Methods **74**:361-367.

Haspel, M.V., T. Onodera, B.S. Prabhakar, M. Horita, H. Suzuki, and A.L. Notkins. (1983)
Virus-induced autoimmunity: Monoclonal antibodies that react with endocrine tissues. Science **220**:304-306.

Haspel, M.V., T. Onodera, B.S. Prabhakar, P.R. McClintock, K. Essani, U.R. Ray, S. Yagihashi, and A.L. Notkins. (1983)
Multiple organ-reactive monoclonal autoantibodies. Nature (London) **304**:73-76.

Hayakawa, K., R.R. Hardy, M. Honda, et al. (1984)
Ly-1 B cells: Functionally distinct lymphocytes that secrete IgM autoantibodies. Proc. Natl. Acad. Sci. USA **81**:2494-2498.

Heidelberger, M. (1983)
Precipitating cross-reactions among pneumococcal types. Infect. Immun. **41**:1234-1244.

Henry, C., and N.K. Jerne. (1968)
Competition of 19S and 7S antigen receptors in the regulation of the primary immune response. J. Exp. Med. **128**:133-152.

Hess, E.V., C.W. Fink, A. Taranta, and M. Ziff. (1964)
Heart muscle antibodies in rheumatic fever and other diseases. J. Clin. Invest. **43**:886-893.

Heyman, B., G. Andrighetto, and H. Wigzell. (1982)
Antigen dependent IgM-mediated enhancement of the sheep erythrocyte response in mice: Evidence for induction of B cells with specificities other than that of the injected antibodies. J. Exp. Med. **155**:994-1009.

Heyman, B., M.V. Hobbs, and W.O. Weigle. (1985)
IgM-mediated enhancement of in vivo anti-sheep erythrocyte antibody responses: Isotype analysis of the enhanced responses. Cell. Immunol. **92**:134-141.

Hirayu, H., P. Seto, R.P. Magnusson, S. Filetti, and B. Rapoport. (1987)
Molecular cloning and partial characterization of a new autoimmune thyroid disease-related antigen. J. Clin. Endocrinol. Metab. **64**:578-584.

Hobbs, R.N., A-L. Clayton, and R.M. Bernstein. (1987)
Antibodies to the five histones and poly(adenosine diphosphate-ribose) in drug induced lupus: implications for pathogenesis. Ann. Rheum. Dis. **46**:408-416.

Holmberg, D., and A. Coutinho. (1985)
Natural antibodies and autoimmunity. Immunology Today **6**:356-357.

Holmdahl, R., C. Bailey, I. Enander, R. Mayer, L. Klareskog, T. Moran, and C. Bona. (1989)
Origin of the autoreactive anti-type II collagen response: II. Specificities, Antibody Isotypes and usage of V gene families of anti-type II collagen B cells. J. Immunol. **142**:1881-1886.

Hollingshead, S.K., V.A. Fischetti, and J.R. Scott. (1987)
A highly conserved region present in transcripts encoding heterologous M proteins of group A sreptococci. Infect. Immun. **55**:3237-3239.

Hosein, B., M. McCarty, and V.A. Fischetti. (1979)
Amino acid sequence and physicochemical similarities between streptococcal M protein and mammalian tropomyosin. Proc. Natl. Acad. Sci. USA **76**:3765-3768.

Hosier, D.M., J.M. Craenen, D.W. Teske, and J.J. Wheller. (1987)
Resurgence of acute rheumatic fever. Am. J. Dis. Child **141**:730-733.

Houtman, P.M., C.G.M. Kallenberg, P.C. Limburg, M.G. Huitema, M.H. van Rijswijk, and T.H. The. (1985)
Quantitation of antibodies to nucleoribonucleoprotein by ELISA: relation between antibody levels and disease activity in patients with connective tissue disease. Clin. Exp. Immunol. **62**:696-704.

Huber, S.A., and P.A. Lodge. (1984)
Coxsackievirus B-3 myocarditis in Balb/c mice: Evidence for autoimmunity to myocyte antigens. Am. J. Pathol. **116**:21-29.

Husby, G., I. van de Rijn, J.B. Zabriskie et al. (1976)
Antibodies reacting with cytoplasm of subthalamic and caudate nuclei neurons in chorea and acute rheumatic fever. J. Exp. Med. **144**:1094-1110.

Hutto, J.H., and E.M. Ayoub. (1980)
Cytotoxicity of lymphocytes from patients with rheumatic carditis to cardiac cells in vitro. In: Streptococcal Diseases and the Immune Response. S.E. Read and J.B. Zabriskie, eds. Academic Press, New York. p.733-738.

Ilyas, A.A., R.H. Quarles, M.C. Dalakas, et al. (1985)
Polyneuropathy with monoclonal gammopathy: Glycolipids are frequently antigens for IgM paraproteins. Proc. Natl. Acad. Sci. USA **82**:6697-6700.

Irie, R.F., K. Irie, and D.L. Morton. (1974)
Natural antibody in human serum to a neoantigen in human cultured cells grown in Fetal bovine serum. J. Natl. Cancer Inst. **52**:1051-1057.

Isenberg, D., and Y. Shoenfeld. (1987)
The origin and significance of anti-DNA antibodies. Immunol. Today **8**:279-282.

Ishigatsubo, Y., A.D. Steinberg, and D.M. Klinman. (1988)
Autoantibody production is associated with polyclonal B cell activation in autoimmune mice which express the lpr or gld genes. Eur. J. Immunol. **18**:1089-1094.

James, K., and G.T. Bell. (1987)
Human monoclonal antibody production: Current status and future prospects. J. Immunol. Methods **100**:5-40.

Jemmerson, R., P.R. Morrow, N.R. Klinman, and Y. Paterson. (1985)
Analysis of an evolutionarily conserved antigenic site on mammalian cytochrome c using synthetic peptides. Proc. Natl. Acad. Sci. USA. **82**:1508-1512.

Jones, K.F., S.A. Kahn, B.W. Erickson, S.K. Hollingshead, J.R. Scott, and V.A. Fischetti. (1986)
Immunochemical localization and amino acid sequences of crossreactive epitopes within the group A streptococcal M6 protein. J. Exp. Med. **164**:1226-1238.

Jones, K.F., and V.A. Fischetti. (1987)
Biological and immunochemical identity of M protein on group G streptococci with M protein on group A streptococci. Infect. Immun. **55**:502-506.

Jones, T.D. (1944)
The diagnosis of rheumatic fever. J.A.M.A. **126**:481-484.

Kabat, E.A., K.G. Nickerson, J. Liao, L. Grossbard, E.F. Osserman, E. Glickman, L. Chess, J.B. Robbins, R. Schneerson, and Y. Yang. (1986)
A human monoclonal macroglobulin with specificity for *α*(2--->8)-linked poly-n-acetyl neuraminic acid, the capsular polysaccharide of group B meningococci and *Escherichia coli K1*, which crossreacts with polynucleotides and with denatured DNA. J. Exp. Med. **164**:642-654.

Kahn, C.R. (1979)
Autoantibodies to the insulin receptor: clincal and molecular aspects. Fed. Proc. Fed. Am. Soc. Exp. Biol. **38**:2607-2609.

Kaplan, E.J., B.F. Anthony, S.S. Chapman, E.M. Ayoub, and L.W. Wannamaker. (1970)
The influence of the site of infection on the immune response to group A streptococci. J. Clin. Invest. **49**:1405-1414.

Kaplan, M.H. (1963)
Immunologic relation of streptococcal and tissue antigens. I. Properties of an antigen in certain strains of group A streptococci exhibiting an immunologic cross-reaction with human heart tissue. J. Immunol. **90**:595-606.

Kaplan, M.H. (1967)
Multiple nature of the cross-reactive relationship between antigens of group A streptococci and mammalian tissue. In: Cross-Reacting Antigens and Neoantigens. J.J. Trentin, ed. Williams & Wilkins Co., Baltimore, p.48-60.

Kaplan, M.H. (1969)
Cross-reaction of Group A streptococci and heart tissue: varying serologic specificity of cross-reactive antisera and relation to carrier-hapten specificity. Transplant. Proc. I (Suppl. 4):976-980.

Kaplan, M.H. (1980)
Streptococcal cross reactions with tissue antigens: cardiac myofibers, smooth muscle, and heart valve fibroblasts. Significance for pathogenesis in rheumatic fever. In: Streptococcal Diseases and the Immune Response. S.E. Read and J.B. Zabriskie, eds. Academic Press, New York, p.625-632.

Kaplan, M.H., R. Bolande, L. Rakita, and J. Blair. (1964)
Presence of bound immunoglobulins and complement in the myocardium in acute rheumatic fever. N. Engl. J. Med. **271**:637-645.

Kaplan, M.H., and J.D. Frengley. (1969)
Autoimmunity to the heart in cardiac disease: Current concepts of the relation of autoimmunity to rheumatic fever, postcardiotomy and postinfarction syndromes and cardiomyopathies. Am. J. Cardiol. **24**:459-473.

Kaplan, M.H., and H. Meyeserian. (1962)
An immunologic cross-reaction between group A streptococcal cells and human heart tissue. Lancet. I:706-710.

Kaplan, M.H., H. Meyeserian, and I. Kushner. (1961)
Immunologic studies of heart tissue: IV serologic reactions with human heart tissue as revealed by immunofluorescent methods: Isoimmune, Wassermann and autoimmune reactions. J. Exp. Med. **113**:17-35.

Kaplan, M.H., and K.H. Svec. (1964)
Immunologic relation of streptococcal and tissue antigens. III. Presence in human sera of streptococcal antibody cross-reactive with heart tissue. Association with streptococcal infection, rheumatic fever, and glomerulonephritis. J. Exp. Med. **119**:651-666.

Karsenti, E., B. Guilbert, M. Bornenes, and S. Avrameas. (1977)
Antibodies to tuberculin in normal non-immunized animals. Proc. Nat. Acad. Sci. **74**:3997-4001.

Karush, F. (1978)
The affinity of antibody: Range, variability, and the role of multivalence. In: Comprehensive Immunology, Vol 5, Immunoglobulins. G.W. Litman and R.A. Good, eds. Plenum, New York, p.85-116.

Kasp-Grochowska, E., D. Kingston, and L.E. Glynn. (1972)
Immunology of bovine heart valves. II. Reaction with connective tissue components. Annals of Rheum. Dis. **31**:290-297.

Kasten, F.H. (1973)
Mammalian myocardial cells in tissue culture. In: Tissue Culture, Methods and Applications. P.F. Kruse, Jr., M.K. Patterson, eds. Academic Press, chapter 10, p.72-81.

Kätkä, K., J. Eskola, K. Granfors et al. (1988)
Serum IgA deficiency and anti-IgA antibodies in pernicious anemia. Clin. Immunol. Immunopathol. **46**:55-60.

Katus, H.A., T. Yasuda, H.K. Gold, R.C. Leinbach, H.W. Strauss, C. Waksmonski, E. Haber, and B.A. Khaw. (1984)
Diagnosis of acute myocardial infarction by detection of circulating cardiac myosin light chains. Am. J. Cardiol. **54**:964-970.

Kaufman, K.D., B. Rapoport, P. Seto, G.D. Chazenbalk, and R.P. Magnusson. (1989)
Generation of recombinant, enzymatically active human thyroid peroxidase and its recognition by antibodies in the sera of patients with Hashimoto's Thyroiditis. J. Clin. Invest. **84**:394-403.

Kazyumba, G., M. Berney, G. Brighouse, et al. (1986)
Expression of the B cell repertoire and autoantibodies in human African trypanosomiasis. Clin. Exp. Immunol. **65**:10-18.

Kehoe, M.A., T.P. Poirier, E.H. Beachey, and K.N. Timmis. (1985)
Cloning and genetic analysis of serotype 5M protein determinant of group A streptococci: Evidence for multiple copies of the M5 determinant in the *Streptococcus pyogenes* genome. Infect. Immun. **48**:190-197.

Kemp, D.J., R.L. Coppel, A.F. Cowman, R.B. Saint, G.V. Brown and R.F. Anders. (1983)
Expression of *Plasmodium falciparum* blood-stage antigens in *Escherichia coli*: Detection with antibodies from immune humans. Proc. Natl. Acad. Sci. U.S.A. **80**:3787-3791.

Kennedy, W.P. (1924)
The production of spermatoxins. Quart. J. Exp. Physiol. **14**:279-283.

Khanna, A.K., D.R. Buskirk, R.C. Williams Jr, A. Gibofsky, M.K. Crow, A. Menon, M. Fotino, H.M. Reid, T. Poon-King, P. Rubinstein and J.B. Zabriskie. (1989)
Presence of a non-HLA B cell antigen in rheumatic fever patients and their families as defined by a monoclonal antibody. J. Clin. Invest. **83**:1710-1716.

Khaw, B.A., J.T. Fallon, G.A. Beller, and E. Haber. (1979)
Specificity of localization of myosin-specific antibody fragments in experimental myocardial infarction. Circulation **60**:1527-1531.

Kieber-Emmons, T., and H. Kohler. (1986)
Evolutionary origin of autoreactive determinants (autogens). Proc. Natl. Acad. Sci. U.S.A. **83**:2521-2526.

Kingston, D., and L.E. Glynn. (1976)
Antistreptococcal antibodies reacting with brain tissue. I. Immunofluorescent studies. Br. J. Exp. Pathol. **57**:114-128.

Klein, R., B. Maisch, K. Kochsiek, and P.A. Berg. (1984)
Demonstration of organ specific antibodies against heart mitochondria (anti-M7) in sera from patients with some forms of heart diseases. Clin. Exp. Immunol. **58**:283-292.

Klemp, P., R.C. Cooper, F.J. Strauss, E.R. Jordaan, J.Z. Przybojewski, and N. Nel. (1988)
Anti-cardiolipin antibodies in ischaemic heart disease. Clin. Exp. Immunol. **74**:254-257.

Klinman, D.M., Y. Ishigatsubo, and A.D. Steinberg. (1988)
Acquisition and maturation of expressed B cell repertoires in normal and autoimmune mice. J. Immunol. **141**:801-806.

Klinman, D.M., and A.D. Steinberg. (1987)
Systemic autoimmune disease arises from polyclonal B cell activation. J. Exp. Med. **165**:1755-1760.

Knudsen, K.A. (1985)
Proteins transferred to nitrocellulose for use as immunogens. Anal. Biochem. **147**:285-288.

Koffler, D., V. Agnello, and H.G. Kunkel. (1974)
Polynucleotide immune complexes in serum and glomeruli of patients with systemic lupus erythematosus. Am. J. Pathol. **74**:109-124.

Koga, K, S. Abe, H. Hashimoto, and M. Yamaguchi. (1987)
Western-blotting method for detecting antibodies against human muscle contractile proteins in myositis. J. Immunol. Methods 105:15-21.

Kotani, T., K. Umeki, S. Matsunaga, E. Kato, and S. Ohtaki. (1986)
Detection of autoantibodies to thyroid peroxidase in autoimmune thyroid diseases by micro-ELISA and immunoblotting. J. Clin. Endocrinol. Metab. 62:928-933.

Kotb, M., H.S. Courtney, J.B. Dale, and E.H. Beachey. (1989)
Cellular and biochemical responses of human T lymphocytes stimulated with streptococcal M proteins. J. Immunol. 142:966-970.

Kotb, M., J.B. Dale, and E.H. Beachey. (1987)
Specific phosphorylation of 25-kDa and 35-kDa proteins in human lymphocytes stimulated with streptococcal M protein. Fed. Proc. 46:445.

Kozbor, D., and C.M. Croce. (1985)
Fusion partners for production of human monoclonal antibodies. In: Human Hybridomas and Monoclonal Antibodies. E.G. Engleman, S.K.H. Foung, J. Larrick and A. Raubitschek, eds. Plenum, New York, p.21.

Kozbor, D., and J.C. Roder. (1983)
The production of monoclonal antibodies from human lymphocytes. Immunol. Today. 4:72-79.

Kozin, F., M. Fowler, and B.A. and S.M. Koethe. (1980)
A comparison of the sensitivities and specificities of different substrates for the fluorescent antinuclear antibody test. Am. J. Clin. Path. 74:785-790.

Krisher, K., and M.W. Cunningham. (1985)
Myosin: a link between streptococci and heart. Science 227:413-415.

Kurki, P. (1978)
Determination of anti-actin antibodies by a solid-phase immunoenzymatic assay and by indirect immunofluorescence technique. Clin. Immunol. Immunopathol. 11:328-338.

Kurki, P., T. Helve, and I. Virtanen. (1983)
Antibodies to cytoplasmic intermediate filaments in rheumatic diseases. J. Rheumatol. 10:558-562.

Kurki, P., and I. Virtanen. (1984)
The detection of human antibodies against cytoskeletal components. J. Immunol. Methods 67:209-223.

Labib, R.S., G.J. Anhalt, H.P. Patel, D.F. Mutasim, and L.A. Diaz. (1986)
Molecular heterogeneity of the bullous pemphigoid antigens as detected by immunoblotting. J. Immunol. 136:1231-1235.

Labrousse, H., F. Tron, S. Avrameas, and J.F. Bach. (1980)
Techniques: Direct evaluation of class-specific anti-DNA antibodies by an immunoenzymatic technique. J. Clin. Lab. Immunol. 3:191-195.

Lafer, E.M., J. Rauch, C. Andrzejewski Jr., D. Mudd, B. and B. Furie, R.S. Schwartz, and B.D. Stollar. (1981)
Polyspecific monoclonal lupus autoantibodies reactive with both polynucleotides and phospholipids. J. Exp. Med. 153:897-909.

Lagace, J., and B.R. Brodeur. (1985)
Parameters affecting in vitro immunisation of human lymphocytes. J. Immunol. Methods **85**:127-136.

Lamb, J.R., R.E. O'Hehir, and D.B. Young. (1988)
The use of nitrocellulose immunoblots for the analysis of antigen recognition by T lymphocytes. J. Immunol. Methods **110**:1-10.

Lampson, L.A., and C.A. Fisher. (1985)
Immunoblot specificity of monoclonal antibodies assayed against complex extracts. Anal. Biochem. **144**:55-64.

Lancefield, R.C. (1962)
Current knowledge of type-specific M antigens of group A streptococci. J. Immunol. **89**:307-313.

Lane, D.P., and W.K. Hoeffler. (1980)
SV40 large T shares an antigenic determinant with a cellular protein of molecular weight 68,000. Nature **288**:167-170.

Lane, D., and H. Koprowski. (1982)
Molecular recognition and the future of monoclonal antibodies. Nature **296**:200-202.

Larrick, J.W., K.E. Truitt, A.A. Raubitschek, G. Senyk, and J.C.N. Wang. (1983)
Characterisation of human hybridomas secreting antibody to tetanus toxin. Proc. Natl. Acad. Sci. USA **80**:6376-6380.

Lazarides, E., and K. Weber. (1974)
Actin antibody: the specific visualization of actin filaments in nonmuscle cells. Proc. Nat. Acad. Sci. USA **71**:2268-2272.

Legocki, R.P. and D.P.S. Verma. (1981)
Multiple immunoreplica technique: Screening for specific proteins with a series of different antibodies using one polyacrylamide gel. Anal. Biochem. **111**:385-392.

Lerner, M.R., and J.A. Steitz. (1979)
Antibodies to small nuclear RNAs complexed with proteins are produced by patients with systemic lupus erythematosus. Proc. Natl. Acad. Sci. USA. **76**:5495-5497.

Leuker, R.D., Z.H. Abdin, and R.C. Williams Jr. (1975)
Peripheral blood T and B lymphocytes during acute rheumatic fever. J. Clin. Invest. **55**:975-985.

Lifshitz,, R., B. Parhami, and E. Mozes. (1981)
Enhancing effect of murine anti-idiotypic serum on the proliferative response specific for poly(LTyr,LGlu)poly(DLAla)-poly(LLys)[(TG)-A-L]. Eur. J. Immunol. **11**:27-31.

Lin, W., and H. Kasamatsu. (1983)
On the electrotransfer of polypeptides from gels to nitrocellulose membranes. Anal. Biochem. **128**:302-311.

Linder, E., V-P. Lehto, and S. Stenman. (1979)
Activation of complement by cytoskeletal intermediate filaments. Nature **278**:176-178.

Lipman, D.J., and W.R. Pearson. (1985)
Rapid and sensitive protein similarity searches. Science **227**:1435-1441.

Litsey, S.E., J.A. Noonan, W.N. O'Connor, C.M. Cottrill, and B. Mitchell. (1985)
Maternal connective tissue disease and congenital heart block: Demonstration of immunoglobulin in cardiac tissue. New Engl. J. Med. **312**:98-100.

Little, J.R., and Eisen, H.N. (1969)
Specificity of the immune response to the 2,4-dinitrophenyl and 2,4,6-trinitrophenyl groups. Ligand binding and fluorescence properties of crossreacting antibodies. J. Exp. Med. **129**:247-265.

Lockshin, M.D., M.L. Druzin, S. Goei, T. Qamar, M.S. Magid, L. Jovanovic, and M4. Ferenc. (1985)
Antibody to cardiolipin as a predictor of fetal distress or death in pregnant patients with systemic lupus erythematosus. N. Engl. J. Med. **313**:152-156.

Lyampert, I.M., N.A. Borodiyuk, and G.A. Ugryumova. (1968)
The reaction of heart and other organ extracts with the sera of animals immunized with group A streptococci. Immunology **15**:845-854.

Lyampert, I.M., and T.A. Danilova. (1975)
Immunological phenomena associated with cross-reactive antigens of microorganisms and mammalian tissues. Prog. Allergy **18**:423-477.

Lyampert, I.M., O.L. Vvedenskaya, and T.A. Danilova. (1966)
Study on streptococcus group A antigens common with heart tissue elements. Immunology **11**:313-320.

Lyon, J.A., R.H Geller, J.D. Haynes, J.D. Chulay, and J.L. Weber. (1986)
Epitope map and processing scheme for the 195,000-dalton surface glycoprotein of *Plasmodium falciparum* merozoites deduced from cloned overlapping segments of the gene. Proc. Natl. Acad. Sci. USA **83**: 2989-2993.

Maddison, P.J., R.P. Skinner, P. Vlachoyiannopoulos, D.M. Brennand, and D. Hough. (1985)
Antibodies to nRNP, Sm, Ro(SSA) and La(SSB) detected by ELISA: their specificity and inter-relations in connective tissue disease sera. Clin. Exp. Immunol. **62**:337-345.

Mahana, W., B. Guilbert, and S. Avrameas. (1988)
Regulation of the humoral immune response by polyspecific natural autoantibodies. Ann. Inst. Pasteur/Immunol. **139**:349-360.

Maisch, B., P. Deeg, G. Liebau, and K. Kochsiek. (1983)
Diagnostic relevance of humoral and cytotoxic immune reactions in primary and secondary dilated cardiomyopathy. Am. J. Cardiol. **52**:1072-1078.

Maisch, B., H. Wilke, C. Marcin et al. (1984)
Adriamycin cardiotoxicity: An echo-cardiographic and immunological follow-up study. Circulation **70** (Suppl.II):149.

Male, D.K. (1986)
Idiotypes and autoimmunity. Clin. Exp. Immunol **65**:1-9.

Male, D., B. Champion, and A. Cooke. (1987)
Advanced Immunology. J.B. Lipincott. Co, Philadelphia.

Maniatis, T., E.F. Fritsch, and J. Sambrook. (1982)
Molecular Cloning: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Manjula, B.M., A.S. Acharya, T. Fairwell, and V.A. Fischetti. (1986)
Antigenic domains of the streptococcal pep M5 protein. Localization of epitopes crossreactive with type 6 M protein and identification of a hypervariable region of the M molecule. J. Exp. Med. **163**:129-138.

Manjula, B.M., and V.A. Fischetti. (1980)
Tropomyosin-like seven residue periodicity in three immunologically distinct streptococcal M proteins and its implications for the antiphagocytic property of the molecule. J. Exp. Med. **151**:695-708.

Manjula, B.M., B.L. Trus, and V.A. Fischetti. (1985)
Presence of two distinct regions in the coiled coil structure of the streptococcal Pep M 5 protein: relationship to mammalian coiled coil proteins and implications to its biological properties. Proc. Natl. Acad. Sci. USA **82**:1064-1068.

Manoussakis, M.N., A.G. Tzioufas, M.P. Silis, P.J. Pange, J. Goudevenos, and H.M. Moutsopoulos. (1987)
High prevalence of anti-cardiolipin and other autoantibodies in a healthy elderly population. Clin. Exp. Immunol. **69**:557-565.

Marchuk, D., S. McCrohon, and E. Fuchs. (1984)
Remarkable conservation of structure among intermediate filament genes. Cell **39**:491-498.

Maron, R., and I.R. Cohen. (1980)
H-2K mutation controls immune response phenotype of autoimmune thyroiditis: Critical expression of mutant gene product in both thymus and thyroid glands. J. Exp. Med. **152**:1115-1120.

Maron, R., and I.R. Cohen. (1983)
Thymic reconstitution of H-2-linked T-cell responses to thyroglobulin or insulin. Immunogenetics **17**:95-100.

Massell, B.F., D.C. Fyler, and S.B. Roy. (1958)
The clinical picture of rheumatic fever. Diagnosis, immediate prognosis course and therapeutic implications. Am. J. Cardiol. 1:436-449.

Massell, B.G., F. Amezcua, and S. Pelragonio. (1964)
Evolving picture of rheumatic fever: Data from 40 years at the House of the Good Samaritan. JAMA **188**:287.

Matsiota, P., S. Chamaret, L. Montagnier, and S. Avrameas. (1987)
Detection of natural autoantibodies in the serum of anti-HIV-positive individuals. Ann. Inst. Pasteur/Immunol. **138**:223-233.

Matsiota, P., P. Druet, P. Dosquet, B. Guilbert, and S. Avrameas. (1987)
Natural autoantibodies in systemic lupus erythematosus. Clin. Exp. Immunol. **69**:79-88.

Matter, L., J.A. Wilhelm, F. Roth, and K. Schopfer. (1986)
Abnormal humoral immune response to *Staphylococcus aureus* in patients with *Staphylococcus aureus* hyper IgE syndrome. Clin. Exp. Immunol. **66**:450-456.

McLaren, M.J., D.M. Hawkins, H.J. Koornhof, K.R. Bloom, D.M. Bramwell-Jones, E. Cohen, G.E. Gale, K. Kannrek, A.S. Lachman, J.B. Lakier, W.A. Pocock, and J.B. Barlow. (1975)
Epidemiology of rheumatic disease in black schoolchildren of Soweto, Johannesburg. Br. Med. J. **3**:474-478.

McLaughlin, J.F., P.Y. Paterson, and S.H. Embury. (1972)
Rheumatic carditis: In vitro responses of peripheral blood leukocytes to heart and streptococcal antigens. Arthritis Rheum. 15:600-608.

McLellan, T., and J.A.M. Ramshaw. (1981)
Serial electrophoretic transfers: A technique for the identification of numerous enzymes from single polyacrylamide gels. Biochem. Genetics 19:647-654.

Merrifield, R.B. (1963)
Solid phase peptide synthesis 1. The synthesis of a tetrapeptide. J. Am. Chem. Soc. 85:2149-2154.

Mierendorf, R.C., C. Percy, and R.A. Young. (1987)
Gene isolation by screening λgt11 libraries with antibodies. Methods Enzymol. 152:458-469.

Miller, L., L. Gray, E. Beachey, and M. Kehoe. (1988)
Antigenic variation among group A streptococcal M proteins. Nucleotide sequence of the serotype 5 M protein gene and its relationship with genes encoding types 6 and 24 M proteins. J. Biol. Chem. 263:5668-5673.

Morton, K.E., T.P. Gavaghan, S.A. Krilis, G.E. Daggard, D.W. Baron, J.B. Hickie, and C.N. Chesterman. (1986)
Coronary artery bypass graft failure: an autoimmune phenomenon? Lancet ii:1353-1356.

Möttönen, T., P. Hannonen, M. Oka, J. Rautiainen, I. Jokinen, H. Avrilommi, T. Palosuo, and K. Aho. (1988)
Antibodies against native type II collagen do not precede the clinical onset of rheumatoid arthritis. Arthritis Rheum. 31:776-779.

Mouw, A.R., E.H. Beachey, and V. Burdett. (1988)
Molecular evolution of streptococcal M protein: cloning and nucleotide sequence of the type 24 M protein gene and relation to other genes of *Streptococcus pyogenes*. J. Bacteriol:170:676-684.

Murray, G.C., M.M. Monteil and R.H. Persellin. (1978)
A study of HLA antigens in adults with acute rheumatic fever. Arthritis Rheum. 21:652-656.

Naparstek, Y., J. Andre-Schwartz, T. Manser, et al. (1986)
A single germline V_H gene segment of normal A/J mice encodes autoantibodies characteristic of systemic lupus erythematosus. J. Exp. Med. 164:614-626.

Netter, H.J., H.H. Guldner, C. Szostecki, H-J. Lakomek, and H. Will. (1988)
A recombinant autoantigen derived from the human (U1) small nuclear RNP-specific 68-kd protein. Arthritis Rheum. 31:616-622.

Neu, N., K.W. Beisel, M.D. Traystman, N.R. Rose, and S.W. Craig. (1987)
Autoantibodies specific for the cardiac myosin isoform are found in mice susceptible to coxsackievirus B_3-induced myocarditis. J. Immunol. 138:2488-2492.

Neu, N., S.W. Craig, N.R. Rose, F. Alvarez, and K.W. Beisel. (1987)
Coxsackievirus induced myocarditis in mice: cardiac myosin autoantibodies do not cross-react with the virus. Clin. Exp. Immunol. 69:566-574.

Nigg, E.A., G. Walter, and S.J. Singer. (1982)
On the nature of crossreactions observed with antibodies directed to defined epitopes. Proc. Natl. Acad. Sci. USA **79**:5939-5943.

Nisonoff, A., E. Margoliash, and M. Reichlin. (1967)
Anti-bodies to rabbit cytochrome c arising in rabbits. Science **155**:1273-1275.

Nowack, H., E. Hahn, and R. Timpl. (1975)
Specificity of the antibody response in inbred mice to bovine type I and type II collagen. Immunol. **29**:621-628.

Ogasawara, M., D.H. Kono, and D.T.Y Yu. (1986)
Mimicry of human histocompatibility HLA-B27 antigens by *Klebsiella pneumoniae*. Infect. Immun. **51**:901-908.

Old, L.J. (1981)
Cancer immunology: The search for specificity - G.H.A. Clowes Memorial Lecture. Cancer Res. **41**:361-375.

Oldstone, M.B.A. (1987)
Molecular mimicry and autoimmune disease. Cell. **50**:819-820.

Oldstone, M.B.A., Fujinami, R.S., and P.W. Lampert. (1980)
In: Progress in Medical Virology. J.L. Melnick, ed. Karger, Basel, Switzerland, 26:p 45-93.

Oldstone, M.B.A., and A.L. Notkins. (1986)
Molecular mimicry. In: Concepts in Viral Pathogenesis II. A.L. Notkins and M.B.A. Oldstone, eds. Springer-Verlag, New York, p. 195-202.

Olmsted, J.B. (1981)
Affinity purification of antibodies from diazotised paper blots of heterogeneous protein samples. J. Biol. Chem. **256**:11955-11957.

Olsson, L., and P. Brams. (1985)
Human-human hybridoma technology. Five years of technical improvement, and its application in cancer biology. In: Human Hybridomas and Monoclonal Antibodies. E.G. Engleman, S.K.H. Foung, J. Larrick and A. Raubitschek, eds. Plenum, New York, p. 227.

Olsson, L., and H.S. Kaplan. (1980)
Human-human hybridomas producing monoclonal antibodies of predefined antigenic specificity. Proc. Natl. Acad. Sci. USA **77**:5429-5431.

Olsson, L., H. Kronstrom, A. Cambon-de Mouzon, C. Honsik, T. Brodin, and B. Jakobsen. (1983)
Antibody producing human-human hybridomas. 1. Technical aspects. J. Immunol. Methods **61**:17-32.

Owen, M.J., J. Auger, B.H. Barber, A.J. Edwards, F.S. Walsh, and M.J. Crumpton. (1978)
Actin may be present on the lymphocyte surface. Proc. Nat. Acad. Sci. USA **75**:4484-4488.

Ozaki, L.S., D. Mattei, M. Jendoubi, P. Druihle, T. Blisnick, M. Guillotte, O. Puijalon, and L.P. Da Silva. (1986)
Plaque antibody selection: rapid immunological analysis of a large number of recombinant phage clones positive to sera raised against *Plasmodium falciparum* antigens. J. Immunol. Methods **89**:213-219.

Painter, C., M. Monastier, B. Bonin, and C.A. Bona. (1986)
Functional and molecular studies of V genes expressed in autoantibodies. Immunol. Rev. **94**:75-98.

Parekh, B.S., H.B. Mehta, M.D. West, and R.C. Montelaro. (1985)
Preparative elution of proteins from nitrocellulose membranes after separation by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Anal. Biochem. **148**:87-92.

Parham, P. (1984)
Changes in conformation with loss of alloantigenic determinants of a histocompatibility antigen (HLA-B7) induced by monoclonal antibodies. J. Immunol. **132**:2975-2983.

Parslow, J.M.M., T.A. Poulton, and F.C. Hay. (1987)
Characterization of sperm antigens reacting with human antisperm antibodies. Clin. Exp. Immunol. **69**:179-187.

Patarroyo, M.E., R. Winchester, A. Vejerano, A. Gibofsky, F. Chalem, J.B. Zabriskie, and H.G. Kunkel. (1979)
Association of B-cell alloantigen with susceptibility to rheumatic fever. Nature **278**:173-174.

Paterson, P.Y. (1980)
Rheumatic fever. In: The Biologic and Clinical Basis of Infectious Diseases, 2nd edition. W.B. Saunders Co, Philadelphia, p.209-221.

Paul, D.L. (1986)
Molecular cloning of cDNA for rat liver gap junction protein. J. Cell Biol. **103**:123-134.

Phillips, G.N., P.F. Flicker, C. Cohen, B.N. Manjula, and V.A. Fischetti. (1981)
Streptococcal M protein α-helical coiled coil structure and arrangement on the cell surface. Proc. Natl. Acad. Sci. USA **78**:4689-4693.

Pierres, M., C. Devaux, M. Dosseto, and S. Marchetto. (1981)
Clonal analysis of B- and T-cell responses to Ia antigens. I. Topology of epitope regions on I-Ak and I-Ek molecules analyzed with 35 monoclonal alloantibodies. Immunogenetics **14**:481-495.

Plater-Zyberk, C., R.N. Maini, K. Lam, T.D. Kennedy, and G. Janossy. (1985)
A rheumatoid arthritis B-cell subset expresses a phenotype similar to that in chronic lymphocytic leyukemia. Arthritis Rheum. **28**:971-976.

Playfair, J.H. (1982)
Immunity to malaria. Br. Med. Bull. **38**:153-159.

Poirier, T.P., M.A. Kehoe, J.B. Dale, K.N. Timmis, and E.H. Beachey. (1985)
Expression of protective and cardiac tissue cross-reactive epitopes of Type 5 streptococcal M protein in *Escherichia coli*. Infect. Immun. **48**:198-203.

Polla, B.S., and D. Young. (1989)
Heat shock proteins and immunity. Immunol. Today **10**:393-394.

Pollard, T.D., and R.R. Weihing. (1974)
Actin and myosin and cell movement. CRC Critical Reviews in Biochemistry **2**:1-65.

Poon-King, T., I. Mohammed, R. Cox, E.V. Potter, N.M. Simon, A.C. Siegel, and D.P. Earle. (1967)
Recurrent epidemic nephritis in South Trinidad. N. Engl. J. Med. **277**:728-733.

Portmann, L., N. Hamada, G. Heinrich, and L.J. DeGroot. (1985)
Anti-thyroid peroxidase antibody in patients with autoimmune thyroid disease: possible identity with anti-microsomal antibody. J. Clin. Endocrinol. Metab. **61**:1001-1003.

Potasman, I., F.G. Araujo, and J.S. Remington. (1986)
Toxoplasma antigens recognized by naturally occurring human antibodies. J. Clin. Microbiol. **24**:1050-1054.

Potter, E.V., M. Svartman, T. Poon-King, and D.P. Earle. (1977)
The families of patients with acute rheumatic fever or glomerulonephritis in Trinidad. Am. J. Epidemiol. **106**:130-138.

Prabhakar, B.S., J. Saegusa, T. Onodera, and A.L. Notkins. (1984)
Lymphocytes capable of making monoclonal autoantibodies that react with multiple organs are a common feature of the normal B cell repertoire. J. Immunol. **133**:2815-2817.

Query, C.C., and J.D. Keene. (1987)
A human autoimmune protein associated with U1 RNA contains a region of homology that is crossreactive with Retroviral $p30^{gag}$ antigen. Cell **51**:211-220.

Raison, R.L., K.Z. Walker, C.R.E. Halnan, D. Briscoe, and A. Basten. (1982)
Loss of secretion in mouse-human hybrids need not be due to the loss of a structural gene. J. Exp. Med. **156**:1380-1389.

Rammelkamp, C.H., F.W. Denny, and L.W. Wannamaker. (1952)
Studies on the epidemiology of rheumatic fever in the armed services. In: Rheumatic Fever. L. Thomas, ed. University of Minnesota Press, Minneapolis, p.72-89.

Rapoport, B., P. Seto, and R.P. Magnusson. (1984)
Immunoprecipitation of radiolabeled human thyroid cell proteins by serum from patients with autoimmune thyroid disease. Endocrinology **115**:2137-2144.

Rauch, J., H. Tannenbaum, B.D. Stollar, and J. Schwartz. (1984)
Monoclonal anti-cardiolipin antibodies bind to DNA. Eur. J. Immunol. **14**:529-534.

Regelmann, W.E., E.D. Gray, R. Talbot, L. Cairns, D. Martin, L.C. Miller, J.B. Zabriskie, D. Braun, and S.S. Rich. (1987)
Distribution and inheritance of "rheumatic" antigens in peripheral blood of RHD patients and their families. Xth Lancefield International Symposium on Streptococci and Streptococcal Diseases **58**:85.

Reininger, L., P. Ollier, P. Poncet, et al. (1987)
Novel V genes encode virtually identical variable regions of six murine monoclonal anti-bromelain-treated red blood cell autoantibodies. J. Immunol. **138**:316-323.

Reisfeld, R.A., and B.D. Kahan. (1970)
Biological and chemical characterization of human histocompatibility antigens. Fed. Proc. **29**:2034-2040.

Rose, N.R., H. Friedman. (Eds.) (1980)
Manual of Clinical Immunology. Second Edition. American Society for Microbiology, Washington, D.C.

Rose, N.R., A. Herskowitz, D.A. Neumann, and N. Neu. (1988)
Autoimmune myocarditis: A paradigm of post-infection autoimmune disease. Immunology Today **9**:117-120.

Rosenblatt, H.M., N. Parikh, J.E. McClure, I. Meza, S. Hwo, J. Bryan, and W.T. Shearer. (1985)
Mitogen-like monoclonal anti-actin antibodies. J. Immunol. **135**:995-1000.

Rosenthal, J., and M.D. Benson. (1980)
Diffuse fasciitis and eosinophilia with symmetric polyarthritis. Ann. Intern. Med. **92**:507-509.

Rosenthal, A., G. Czoniczer, and B.F. Massell. (1968)
Rheumatic fever under three years of age. Pediatrics **41**:612-619.

Rothbard, J.B., R. Fernandez, and G.K. Schoolnik. (1984)
Strain-specific and common epitopes of gonococcal pili. J. Exp. Med. **160**:208-221.

Rotta, J. (1983)
Pathogenesis of rheumatic fever: a review. Ann. Trop. Paediatr. **3**:1-8.

Rousset, B., F. Bernier-Valentin, C. Poncet, J. Orgiazzi, A. Madec, J.C. Monier, and R. Mornex. (1983)
Anti-tubulin antibodies in autoimmune thyroid disorders. Clin. Exp. Immunol. **52**:325-332.

Rungger-Brändle, E., and G. Gabbiani. (1983)
The role of cytoskeletal and cytocontractile elements in pathologic processes. Am. J. Pathol. **110**:361-392.

Ruppel, A., Y.E. Shi, D.X. Wei, and H.J. Diesfeld. (1987)
Sera of *Schistosoma japonicum*-infected patients cross-react with diagnostic 31/32 kD proteins of *S. mansoni*. Clin. Exp. Immunol. **69**:291-298.

Russel, H., R.R. Facklam, and R.L. Edwards. (1976)
Enzyme-linked immunosorbent assay for streptococcal M protein antibodies. J. Clin. Microbiol. **3**:501-505.

Saah, A.J., H. Farzadegan, R. Fox, P. Nishanian, C.R. Rinaldo Jr., J.P. Phair, J.L. Fahey, T-H. Lee, B.F. Polk, and the Multicenter Aids Cohort Study. (1987)
Detection of early antibodies in human immunodeficiency virus infection by enzyme-linked immunosorbent assay, Western blot, and radioimmunoprecipitation. J. Clin. Microbiol. **25**:1605-1610.

Sa Ferreira, J.A., B. Galvao-Castro, W. Macedo, and C. Castro. (1983)
Immunoglobulins and other serological parameters in Chagas' disease: evidence for increased IgA levels in the chronic digestive form. Clin. Exp. Immunol. **52**:266-270.

Sargent, S.J., E.H. Beachey, C.E. Corbett, and J.B. Dale. (1987)
Sequence of protective epitopes of streptococcal M proteins shared with cardiac sarcolemmal membranes. J. Immunol. **139**:1285-1290.

Sasaki, T., F. Endo, M. Mikami, Y. Sekiguchi, K. Tada, Y. Ono, N. Ishida, and K Yoshinaga. (1984)
Establishment of human monoclonal anti-DNA antibody producing cell lines. J. Immunol. Methods **72**:157-165.

Satoh, J., K. Essani, P.R. McClintock, and A.L. Notkins. (1984)
Human multiple organ-reactive monoclonal autoantibody recognizes growth hormone and a 35K protein. J. Clin. Invest. **74**:1526-1531.

Satoh, J., B.S. Prabhakar, M.V. Haspel, F. Ginsberg-Fellner, and A.L. Notkins. (1983)
Human monoclonal autoantibodies that react with multiple endocrine organs. New Engl. J. Med. **309**:217-220.

Sattentau, Q.J., A.G. Dalgleish, R.A. Weiss, and P.C.L. Beverley. (1986)
Epitopes of the CD4 antigen and HIV infection. Science **234**:1120-1123.

Schaller, J.G., and I. Szer. (1989)
Rheumatic Disorders. In: Immunologic Disorders in Infants and Children, 3rd edition. E.R. Stiehm, ed. W.B. Saunders Co., Philadelphia, p.542-590.

Schimpl, A., and E. Wecker. (1973)
Stimulation of IgG antibody response in vitro by T cell-replacing factor. J. Exp. Med. **137**:547--552.

Schultheiss H-P., P. Schwimmbeck, H.-D. Bolte, and M. Klingenberg. (1984)
The antigenic characteristics and the significance of the adenine nucleotide translocator as a major autoantigen to antimitochondrial antibodies in dilated cardiomyopathy. In: Advances in Myocardiology, Vol 6. N.S. Dhalla and D.C. Hearse, eds. Plenum Publishing Corporation, New York.

Schultheiss, H.P., and H.D. Bolte. (1985)
Immunological analysis of auto-antibodies against the adenine nucleotide translocator in dilated cardiomyopathy. J. Mol. Cell. Cardiol. **17**:603-617.

Schwaber, J.F., M.R. Posner, S.F. Schlossman, and H. Lazarus. (1984)
Human-human hybrids secreting pneumococcal antibodies. Hum. Immunol. **9**:137-143.

Schwartz, R.S. (1986)
Are natural autoantibodies real? Ann. Inst. Pasteur/Immunol. **137D**:156-159.

Schwartz, R.S., and S.K. Datta. (1989)
Autoimmunity and autoimmune diseases. In: Fundamental Immunology, second edition. W.E. Paul, ed. Raven Press Ltd., New York, p.819-866.

Scott, J.R., and V.A. Fischetti. (1983)
Expression of streptococcal M protein in *Escherichia coli*. Science **221**:758-760.

Seibert, F. (1928)
The chemical composition of the active principle of tuberculin. IX. The fractional heat coagulation of the protein of tuberculin. Am. Rev. Tuberc. Pulm. Dis. **17**:394-401.

Seigneurin, J.M., C. Desgranges, D. Seigneurin, J. Paire, J.C. Renversez, B. Jacquemont, and C. Micouin. (1983)
Herpes simplex virus glycoprotein D: Human monoclonal antibody produced by bone marrow cell line. Science **221**:173-175.

Seligmann, M., et al. (1986)
The origin and nature of autoantibodies. Ann. Inst. Pasteur/Immunol. **137D**:149-182.

Senitzer, D., and E.H. Freimer. (1984)
Autoimmune mechanisms in the pathogenesis of rheumatic fever. Rev. Infect. Dis. **6**:832-839.

Sercarz, E. (1987)
Ir gene regulation: Past failures to present cogent mechanisms and to delete diverting oversimplications - a commentary. J. Mol. Cell. Immunol. **3**:277.

Seyer, J.M., A.H. Kang, and E.H. Beachey. (1980)
Primary structural similarities between type 5 and 24 M proteins of *Streptococcus pyogenes*. Biochem. Biophys. Res. Commun. **92**:546-553.

Shastry, P., S. Naik, M. Joshi, and S. Kinare. (1988)
Persistence of heart reactive antibodies (HRA) in acute rheumatic fever (ARF) and rheumatic heart disease (RHD) patients. J. Clin. Lab. Immunol. **27**:87-90.

Sheshberadaran, H., and E. Norrby. (1984)
Three monoclonal antibodies against measles virus F protein cross-react with cellular stress proteins. J. Virol. **52**:995-999.

Shoenfield, Y., S.C. Hsu-Lin, J.E. Gabriels, L.E. Silberstein, B.C. Furie, B. Furie, B.D. Stollar, and R.S. Schwartz. (1982)
Production of autoantibodies by human-human hybridomas. J. Clin. Invest. **70**:205-208.

Shoenfeld, Y., and D.A. Isenberg. (1989)
The mosaic of autoimmunity. Immunology Today **10**:123-126.

Shoenfeld, Y., and R.S. Schwartz. (1984)
Immunologic and genetic factors in autoimmune diseases. N. Engl. J. Med. **311**:1019-1029.

Sikora, K., T. Alderson, J. Phillips, and J.V. Watson. (1983)
Human hybridomas from patients with malignant disease. Br. J. Cancer **47**:135-145.

Silverstein, S.C., R.M. Steinman, and Z.A. Cohn. (1977)
Endocytosis. Annu. Rev. Biochem. **46**:669-722.

Smith, D.E., and P.A. Fisher. (1984)
Identification, developmental regulation, and response to heat shock of two antigenically related forms of a major nuclear envelope protein in Drosophila embryos: Application of an improved method for affinity purification of antibodies using polypeptides immobilized on nitrocellulose blots. J. Cell Biol. **99**:20-28.

Snyder, H.W. Jr., and E. Fleissner. (1980)
Specificity of human antibodies to oncovirus glycoproteins: Recognition of antigen by natural antibodies directed against carbohydrate structures. Proc. Natl. Acad. Sci. U.S.A. **77**:1622-1626.

Souroujon, M., M.E. White-Scharff, J. Andre-Schwartz, et al. (1988)
Preferential autoantibody reactivity of the pre-immune B cell repertoire in normal mice. J. Immunol. **140**:4173-4179.

Spanier, J.G., S.J.C. Jones, and P. Cleary. (1984)
Small DNA deletions creating avirulence in *Streptococcus pyogenes*. Science **225**:935-938.

Spinola, S.M., and J.G. Cannon. (1985)
Different blocking agents cause variation in the immunologic detection of proteins transferred to nitrocellulose membranes. J. Immunol. Methods **81**:161-165.

Srinivasappa, J., J. Saegusa, B.S. Prabhakar, M.K. Gentry, M.J. Buchmeier, T.J. Wiktor, H. Koprowski, M.B.A. Oldstone, and A.L. Notkins. (1986)
Molecular mimicry: Frequency of reactivity of monoclonal antiviral antibodies with normal tissues. J. Virol. **57**:397-401.

Stall, A.M., P.A. Lalor, L.A. Herzenberg, and L.A. Herzenberg. (1986)
LY-1 B cells and autoantibodies. 13th Forum in Immunology. Ann. Inst. Pasteur/Immunol. **137D**:173-176.

Stanhope, J.M. (1975)
New Zealand trends in rheumatic fever, 1885-1971. N.Z. Med. J. **82**:297-299.

Stanley, J.R., T. Tanaka, S. Mueller, V. Klaus-Kovtun, and D. Roop. (1988)
Isolation of complementary DNA for bullous pemphigoid antigen by use of patients' autoantibodies. J. Clin. Invest. **82**:1864-1870.

Steele, E.J., and A.J. Cunningham. (1978)
High proportion of Ig-producing cells making autoantibody in normal mice. Nature **274**:483-484.

Steffan, C., and R. Timpl. (1963)
Antigenicity of collagen and its application in the serologic investigation of rheumatoid arthritis sera. Int. Arch. Allergy Appl. Immunol. **22**:333-349.

Steinert, P.M., and D.R. Roop. (1988)
Molecular and cellular biology of intermediate filaments. Ann. Rev. Biochem. **57**:593-625.

Stetler, D.A., and S.T. Jacob. (1985)
Immunization of rabbits with purified RNA polymerase I induces a distinct population of antibodies against nucleic acids as well as anti-RNA polymerase I antibodies, both characteristic of systemic lupus erythematosus. Proc. Natl. Acad. Sci. USA **82**:6797-6801.

Stollerman, G.H. (1974)
The streptococcus rheumatic fever and rheumatic heart disease. In: Cardiovascular Disease in the Tropics. A.G. Shaper, M.S.R. Hutt and Z. Fejfar, eds. British Medical Associates, London.

Stollerman, G.H. (1975)
Rheumatic Fever and Streptococcal Infection. Grune and Stratton, Inc., New York.

Stollerman, G.H. (1988)
Rheumatic and heritable connective tissue diseases of the cardiovascular system. In: Heart Disease: A Textbook of Cardiovascular Medicine, third edition. E. Braunwald, ed. W.B. Saunders, Philadelphia, p.1706-1733.

Stollerman, G.H., A.J. Lewis, I. Schultz, and A. Taranta. (1956)
Relationship of immune response to group A streptococci to the course of acute, chronic and recurrent rheumatic fever. Am. J. Med. **20**:163-169.

Stone, D., and L.B. Smillie. (1978)
The amino acid sequence of rabbit skeletal α-Tropomyosin. J. Biol. Chem. **253**:1137-1148.

Stossel, T.P. (1975)
Phagocytosis: recognition and ingestion. Semin. Hematol. **12**:83-116.

Stossel, T.P., and T.D. Pollard. (1973)
Myosin in polymorphonuclear leukocytes. J. Biol. Chem. **248**:8288-8294.

Stott, D.I. (1985)
Autoantibodies and nuclear antigens. Immunol. Today **6**:314-315.

Stott, D.I. (1989)
Immunoblotting and dot blotting. J. Immunol. Methods **119**:153-187.

Strickland, F.M., J.T. Gleason, and J. Cerny. (1987)
Reexpression of a T15 idiotope on variant immunoglobulins after the binding of another anti-idiotopic antibody. J. Immunol. **138**:3868-3872.

Struhl, K. (1985)
A rapid method for creating recombinant DNA molecules. Biotechniques 3:452-453.

Stuart, J.M., E.H. Huffstutter, A.S. Townes, and A.H. Kang. (1983)
Incidence and specificity of antibodies to types I, II, III, IV and V collagen in rheumatoid arthritis and other rheumatic diseases measured by ^{125}I-radioimmunoassay. Arthritis Rheum. **26**:832-840.

Stuart, J.M., W.C. Watson, and A.H. Kang. (1988)
Collagen autoimmunity and arthritis. FASEB Jnl. **2**:2950-2956.

Sturgess, J.M., J. Chao, and J.A.P. Turner. (1980)
Transposition of ciliary microtubules: Another cause of impaired ciliary motility. New Engl. J. Med. **303**:318-322.

Swerlick, R.A., M.W. Cunningham, and N.K. Hall. (1986)
Monoclonal antibodies cross-reactive with group A streptococci and normal and psoriatic human skin. J. Invest. Dermatol. **87**:367-371.

Syu, W-J., and L. Kahan. (1987)
Use of protein-stained immunoblots for unequivocal identification of antibody specifities. J. Immunol. Methods **103**:247-252.

Tainer, J.A., E.D. Getzoff, H.Alexander, R.A. Houghten, A.J. Olson, R.A. Lerner, and W.A. Hendrickson. (1984)
The reactivity of anti-peptide antibodies is a function of the atomic mobility of sites in a protein. Nature (London) **312**:127-134.

Talmage, D.W. (1959)
Immunological specificity: Unique combinations of selected natural globulins provide an alternative to the classical concept. Science **129**:1643-1648.

Tan, E.M. (1989a)
Interactions between autoimmunity and molecular and cell biology: Bridges between clinical and basic sciences. J. Clin. Invest. **84**:1-6.

Tan, E.M. (1989b)
Antinuclear antibodies: Diagnostic markers for autoimmune diseases and probes for cell biology. Adv. Immunol. **44**:93-151.

Taranta, A. (1976)
Rheumatic fever made difficult. A critical review of pathogenetic theories. Paediatrician **5**:74-95.

Taranta, A. (1985)
Rheumatic fever. In: Arthritis and Allied Conditions, 10th edition. D.J. McCarty, ed. Lea and Febiger, Philadelphia.

Tegtmeyer, P., M. Schwartz, J.K. Collins, and K. Rundell. (1975)
Regulation of tumor antigen synthesis by simian virus 40 gene A. J. Virol. **16**:168-178.

Teng, N.N.H., K.S. Lam, F.C. Riera, and H.S. Kaplan. (1983)
Construction and testing of mouse-human heteromyelomas for human monoclonal antibody production. Proc. Natl. Acad. Sci. USA **80**:7308-7312.

Teng, N.N.H., H.S. Kaplan, J.M. Herbert, C. Moore, H. Douglas, A. Wunderlich, and A.J. Braude. (1985)
Protection against Gram negative bacteremia and endotoxinemia with human monoclonal IgM antibodies. Proc. Natl. Acad. Sci. USA **82**:1790-1794.

Thompson, K.M., M.D. Melamed, K. Eagle, B.D. Gorick, T. Gibson, A.M. Holburn, and N.C. Hughes-Jones. (1986)
Production of human monoclonal IgG and IgM antibodies with anti-D (Rhesus) specificity using heterohybridomas. Immunology **58**:157-160.

Toh, B.H. (1979)
Smooth muscle autoantibodies and autoantigens. Clin. Exp. Immunol. **38**:621-628.

Top, F.H., L.W. Wannamaker, W.R. Maxted, and G.V. Anthony. (1967)
M antigens among group A streptococci isolated from skin lesions. J. Exp. Med. **126**:667-685.

Towbin, H., T. Staehlin, and J. Gordon. (1979)
Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350-4354.

Tron, F., L. Jacob, and J-F. Bach. (1983)
Murine monoclonal anti-DNA antibodies with an absolute specificity for DNA have a large amount of idiotypic diversity. Proc. Natl. Acad. Sci. USA **80**:6024-6027.

Tron, F., C. Le Guern, P-A. Cazenave, and J-F Bach. (1982)
Intrastrain recurrent idiotypes among anti-DNA antibodies of (NZBxNZW)F1 hybrid mice. Eur. J. Immunol. **12**:761-766.

Uhl, J., and R.C. Newton. (1988)
Quantitation of related proteins by Western blot analysis. J. Immunol. Methods **110**:79-84.

Underwood, P. (1985)
Theoretical considerations of the ability of monoclonal antibodies to detect antigenic differences between closely related variants, with particular reference to heterospecific reactions. J. Immunol. Methods **85**:295-307.

Unny, S.K., and B.L. Middlebrooks. (1983)
Streptococcal rheumatic carditis. Microbio. Rev. **47**:97-120.

Van de Rijn, I., A.S. Bleiweis, and J.B. Zabriskie. (1976)
Antigens in *Streptococcus mutans* cross reactive with human heart muscle. J. Dent. Res. Special Issue C:C59-C64.

Van de Rijn, I., and R.E. Kessler. (1980)
Growth characteristics of group A streptococci in a new chemically defined medium. Infect. Immun. **27**:444-448.

Van de Rijn, I., J.B. Zabriskie, and M. McCarty. (1977)
Group A streptococcal antigens cross reactive with myocardium. Purification of heart-reactive antibody and isolation and characterization of the streptococcal antigen. J. Exp. Med. **146**:579-599.

Van Meel, F.C.M., P.G.A. Steenbakkers, and J.C.H. Oomen. (1985)
Human and chimpanzee monoclonal antibodies. J. Immunol. Methods **80**:267-276.

Van Regenmortel, M.H.V. (1986)
Which structural features determine protein antigenicity? In: TIBS, p. 36-39.

Van Regenmortel, M.H.V. (1987)
Antigenic cross-reactivity between proteins and peptides: new insights and applications. In: TIBS, p237-240.

Van Regenmortel, M.H.V. (1989)
Structural and functional approaches to the study of protein antigenicity. Immunology Today **10**:266-271.

Veasy, L.G., S.E. Wiedmeier, G.S. Orsmond, H.D. Ruttenberg, M.M. Boucek, S.J. Roth, V.F. Tait, J.A. Thompson, J. A. Daly, E.L. Kaplan, and H.R. Hill. (1987)
Resurgence of acute rheumatic fever in the intermountain area of the United States. N. Engl. J. Med. **316**:421-427.

Viljanen, M.K., and J. Punnonen. (1989)
The effect of storage of antigen-coated polystyrene microwells on the detection of antibodies against *Borrelia burgdorferi* by enzyme immunoassay (EIA). J. Immunol. Methods **124**:137-141.

Voller, A., D. Bidwell, and A. Bartlett. (1980)
Enzyme linked immunosorbent assay. In: Manual of Clinical Immunology. N.R. Rose, and H. Friedman, eds. American Society for Microbiology, Washington, D.C. p.359-371.

Wada, K., S. Ueno, T. Hazama, S. Ogasahara, J. Kang, M. Takahashi, and S. Tarui. (1983)
Radioimmunoassay for antibodies to human skeletal muscle myosin in serum from patients with polymyositis. Clin. Exp. Immunol. **52**:297-304.

Wald, E.R., B. Dashefsky, C. Feidt, D. Chiponis, and C. Byers. (1987)
Acute rheumatic fever in western Pennsylvania and the Tristate area. Pediatrics **80**:371-374.

Wallis, R.S., S.L.M. Alde, D.V. Havlir, M.H. Amir-Tahmasseb, T.M. Daniel, and J.J. Ellner. (1989)
Identification of antigens of *Mycobacterium tuberculosis* using human monoclonal antibodies. J. Clin. Invest. **84**:214-219.

Ward, C., K. Gelsthorpe, R.W. Doughty, and C.A. Hardisty. (1976)
HLA antigens and acquired valvular heart disease. Tissue Antigens **7**:227-231.

Ward, K.H., H. Anwar, M.R.W. Brown, J. Wale, and J. Gowar. (1988)
Antibody response to outer-membrane antigens of *Pseudomonas aeruginosa* in human burn wound infection. J. Med. Microbiol. **27**:179-190.

Wason, W.M. (1973)
Regulation of the immune response with antigen specific IgM antibody: A dual role. J. Immunol. **110**:1245-1252.

Watson, W.C., M.A. Cremer, P.H. Wooley, and A.S. Townes. (1986)
Assessment of the potential pathogenicity of type II collagen autoantibodies in patients with rheumatoid arthritis. Evidence of restricted IgG_3 subclass expression and activation of complement C5 to C5a. Arthritis Rheum. **129**:1316-1321.

Weber, K., and U. Groeschel-Stewart. (1974)
Antibody to myosin: the specific visualization of myosin-containing filaments in nonmuscle cells. Proc. Nat. Acad. Sci. USA 71:4561-4564.

Weigle, W.O. (1980)
Analysis of autoimmunity through experimental models of thyroiditis and allergic encephalomyelitis. Adv. Immunol. 30:159-273.

Westerwoudt, R.J. (1985)
Improved fusion methods. IV. Technical aspects. J. Immunol. Methods 77:181-196.

Westhof, E., D. Altschuh, D. Moras, A.C. Bloomer, A. Mondragon, A. Klug, and M.H.V. Van Regenmortel. (1984)
Correlation between segmental mobility and the location of antigenic determinants in proteins. Nature 311:123-126.

Widdowson, J.P., W.R. Maxted, C.M. Notley, and A.M. Pinney. (1974)
The antibody responses in man to infection with different serotypes of group A streptococci. J. Med. Microbiol. 7:483-496.

Williams, C.L., and V.A. Lennon. (1986)
Thymic B lymphocyte clones from patients with myasthenia gravis secrete monoclonal striational autoantibodies reacting with myosin, α actinin, or actin. J. Exp. Med. 164:1043-1059.

Williams, R.C. Jr. (1985)
Molecular mimicry and rheumatic fever. Clin. Rheum. Dis. 11:573-590.

Wilson, G.B., and F.J. Dixon. (1973)
Antiglomerular basement membrane antibody induced glomerulonephritis. Kidney Int. 3:74.

Wilson, I.A., H.L. Niman, R.A. Houghten, A.R. Cherenson, M.L. Connolly, and R.A. Lerner. (1984)
The structure of an antigenic determinant in a protein. Cell 37:767-778.

Wilson, M.G., M.D. Schweitzer, and R. Lubschez. (1943)
The familial epidemiology of rheumatic fever: Genetic and epidemiologic studies. J. Pediatr. 22:468-492.

Winfield, J.B. (1983)
Cryoglobulinemia. Hum. Pathol. 14:350-354.

Winger, L., C. Winger, P. Shastry, A. Russel, and M. Longenecker. (1983)
Efficient generation in vitro, from human peripheral blood cells, of monoclonal Epstein-Barr virus transformants producing specific antibody to a variety of antigens without prior deliberate immunisation. Proc. Natl. Acad. Sci. USA 80:4484-4488.

Wolfgram, L.J., K.W. Beisel, and N.R. Rose. (1985)
Heart-specific autoantibodies following murine coxsackievirus B3 myocarditis. J. Exp. Med. 161:1112-1121.

Wolfgram, L.J., K.W. Beisel, A. Herskowitz and N.R. Rose. (1986)
Variations in the susceptibility to Coxsackie B3-induced myocarditis among different strains of mice. J. Immunol. 136:1846-1852.

Wood, J.N., L. Hudson, T.M. Jessell, and M. Yamamoto. (1982)
A monoclonal antibody defining antigenic determinants on sub-populations of mammalian neurones and *Trypanosoma cruzi* parasites. Nature (London) **296**:34-38.

Wooley, P.H., H.S. Luthra, S. Singh, A. Huse, J.M. Stuart, and C.S. David. (1984)
Passive transfer of arthritis in mice by human anti-type II collagen antibody. Mayo Clinic Proc. **59**:737-743.

Wraith, D.C., H.O. McDevitt, L. Steinman, and H. Acha-Orbea. (1989)
T cell recognition as the target for immune intervention in autoimmune disease. Cell **57**:709-715.

Wunderlich, D., Y.A. Teramoto, C. Alford, and J. Schlom. (1981)
The use of lymphocytes from axillary lymph nodes of mastectomy patients to generate human monoclonal antibodies. Eur. J. Cancer and Clin. Oncol. **17**:719-730.

Yadin, O., B. Sarov, L. Naggan, H. Slor, and Y. Shoenfeld. (1989)
Natural autoantibodies in the serum of healthy women - a five-year follow-up. Clin. Exp. Immunol. **75**:402-406.

Yewdall, V., J.S. Cameron, A.W. Nathan et al. (1983)
Systemic lupus erythematosus and IgA deficiency. J. Clin. Lab. Immunol. **10**:13-18.

Yoshinoya, S., and R.M. Pope. (1980)
Detection of immune complexes in acute rheumatic fever and their relationship to HLA-B5. J. Clin. Invest. **65**:136-145.

Yoshitake, S., Y. Yamada, E. Ishikawa, and R. Massayeff. (1979)
Conjugation of glucose oxidase from *Aspergillus niger* and rabbit antibodies using N-hydroxysuccinimide ester of N-(4-carboxycyclohexylmethyl)-maleimide. Eur. J. Biochem. **101**:395-399.

Young, D.B. and J.R. Lamb. (1986)
T-lymphocytes respond to solid phase antigen: a novel approach to the molecular analysis of cellular immunity. Immunology **59**:167.

Zabriskie, J.B. (1967)
Mimetic relationships between group A streptococci and mammalian tissues. Adv. Immunol. **7**:147-188.

Zabriskie, J.B. (1982)
Streptococcal diseases and their relationship to antigenic mimicry in the group A streptococcus. In: Clinical Aspects of Immunology, Vol II. P.J. Lachman and D.K. Peters, eds. Blackwell Scientific Publications, Oxford, p.1428-1444.

Zabriskie, J.B. (1985)
Rheumatic fever: the interplay between host, genetics, and microbe. Circulation **71**:1077-1086.

Zabriskie, J.B. (1986)
Rheumatic fever: A model for the pathological consequences of microbial-host mimicry. Clin. Exp. Rheumatol. **4**:65-73.

Zabriskie, J.B., S.E. Read, and R.J. Ellis. (1971)
Cellular and humoral studies in diseases with heart-reactive antibodies. Prog. in Immunol. 213-229.

Zabriskie, J.B., and E.H. Freimer. (1966)
An immunological relationship between the group A streptococcus and mammalian muscle. J. Exp. Med. **124**:661-672.

Zabriskie, J.B., and J.E. Friedman. (1983)
The role of heart binding antibodies in rheumatic fever. Adv. Exp. Med. Biol. **161**:457-470.

Zabriskie, J.B., and A. Gibofsky. (1986)
Genetic control of the susceptibility to infection with pathogenic bacteria. Microbiol. Immunol. **124**:1-20.

Zabriskie, J.B., K.C. Hsu, and B.C. Seegal. (1970)
Heart-reactive antibody associated with rheumatic fever: characterization and diagnostic significance. Clin. Exp. Immunol. **7**:147-159.

Zanders, E.D., and T. Lehner. (1981)
Separation and characterization of a protein antigen from cells of *Streptococcus mutans*. J. Gen. Microbiol. **122**:217-225.

Zanetti, M. (1988)
Self-immunity and the autoimmune network: A molecular perspective to ontogeny and regulation of the immune system. Ann. Inst. Pasteur/Immunol. **139**:619-631.

Zouali, M., R.S. Schwartz, and B.D. Stollar. (1988)
Origin and diversification of anti-DNA antibodies. Immunol. Rev. **105**:137-159.

Zurawski, V.R. Jr., E. Haber, and P.H. Black. (1978)
Production of antibody to tetanus toxoid by continuous human lymphoblastoid cell lines. Science **199**:1439-1441.