The management of corneal graft rejection.

John Clifford Hill

M.B. B.S., D.O., F.R.C.S., F.R.C.Ophth.

A thesis submitted to the Faculty of Medicine, University of Cape Town, for the Degree of Doctor of Medicine.

University of Cape Town

The University has the right to reproduce this thesis in whole or in part. Copyright is held by the author.

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

University of Cape Town

This thesis is dedicated to my wife, Rhian, who encouraged me to continue and who tolerated me while I completed this work; also to my children who were undoubtedly neglected in the final stages of completion.

It is frustrating that for many blind people, the only thing that stands between them and vision is a small piece of tissue (the cornea) that cannot be rendered, or will not remain, clear.

In 1985 it was estimated that there were 28 million blind people in the world and that the majority had lost their sight from corneal disease [1].

Dieffenbach[2], one of Berlin's great surgeons of the time wrote in 1831 that the idea of keratoplasty was "... certainly one of the most audacious fantasies, and it would be the highest reward of surgery if this operation succeeded".

Table of contents

		Page No.
Acknowledgements.		7
Declaration.		8
Index to Figures.		9
Index to Tables.		13
Abbreviations used in this thesis.		15
Papers supporting this thesis:		
	Papers presented at scientific meetings.	17
	Papers published	19
Section One: Introduction.		
1.1	An historical review of corneal grafting.	22
1.2	An outline of the clinical data used in this thesis.	25
Section Two: Corneal Graft Rejection.		
2.1	An overview of transplantation immunology.	27
2.2	Cornea transplantation immunology.	35
2.3	Corneal graft rejection.	45
2.4	High-risk keratoplasty.	51
2.5	Treatment of graft rejection.	59
Section Three: The Role Of Tissue Typing In Preventing Corneal Graft Rejection.		
3.1	Introduction.	64
3.2	Results from other centres.	66
3.2	Clinical studies	76

		Page No.
Section Four: An Animal Model For High-risk Keratoplasty.		
4.1	Establishing the model and investigations into the effects of treatment regimens on graft survival.	99
4.2	Investigations into the effects of tissue mismatch on graft survival using mixed lymphocyte cultures.	112
4.3	The effect of preoperative UV-B irradiation of donor corneas on graft survival.	126
Section Five: The Role Of Cyclosporine In Preventing Corneal Graft Rejection.		
5.1	Introduction.	134
5.2	The use of topical cyclosporine in high-risk keratoplasty.	143
5.3	Preliminary report on the use of systemic cyclosporine.	150
5.4	The use of systemic cyclosporine in high-risk keratoplasty: a prospective trial.	160
5.5	Systemic cyclosporine in high-risk keratoplasty: short versus long term therapy.	173
5.6	Systemic cyclosporine in high risk keratoplasty: long term results.	186
5.7	Conclusion.	203

Page No.

Section Six: The Use Of Intravenous Corticosteroid Pulse Therapy In Corneal Graft Rejection.

6.1 Introduction. 206

6.2 Preliminary report on the use of pulse therapy in corneal graft rejection. 208

6.3 Oral versus pulse therapy in corneal graft rejection. 216

6.4 Single versus double pulse therapy. 228

6.5 Conclusion. 245

Section Seven: Conclusions. 247

References. 250

Acknowledgements.

I wish to acknowledge the help and advice of my friend and colleague Dr. Richard Maske who was my co-researcher on many of the research projects reported in this thesis.

My wife, Dr. Rhian Grötte, was my sounding board for many of the projects and problems encountered along the way.

Professor Tony Murray offered me encouragement since I first tentatively embarked on this thesis.

Jan Korrubel played a key role in making me "computer literate".

I bear a great debt to the following people who were my co-workers on research projects included in this thesis: Dr Richard Maske, Junaid Sarvan, Professor Callie Els, Dr Andrew Ivey, Sue Horak, Dr Peter Watson, and Dr Sedick Isaacs.

Statistical help and advice was given by Dr Sedick Isaacs and Dr Carl Lombard.

Many of my colleagues in the Department of Ophthalmology, Groote Schuur Hospital, have helped in the management of my corneal graft patients. They are too numerous to mention individually but their help is greatly appreciated.

Many of the research projects in this thesis were supported by financial grants from the South African Medical Research Council and from the University of Cape Town.

The animal work was approved by the Ethical Committee and Animal Users Committee of the University of Cape Town.

I, John Clifford Hill, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been , is being, or is to be submitted for another degree in this or any other university.

I empower the University of Cape Town to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signed by candidate

Date: 25/8/93

Index To Figures.

		Page No.
Fig. 1	Assessment of corneal vascularization.	54
Fig. 2.	ABO compatibility study: survival curves showing graft failure from rejection as the end-point.	81
Fig. 3.	ABO compatibility study: survival curves showing first graft rejection episode as the end-point.	82
Fig. 4.	ABO blood groups study: survival curves of group O versus groups A, B, and AB, showing graft failure from rejection as the end-point.	83
Fig. 5.	ABO blood groups study: survival curves of group O versus groups A, B, and AB, showing first graft rejection episode as the end-point.	84
Fig. 6.	ABO blood groups study: survival curves of groups O, A, B, and AB showing graft failure from rejection as the end-point.	85
Fig. 7.	ABO blood groups study: survival curves of groups O, A, B, and AB showing first graft rejection episode as the end-point.	86
Fig. 8.	HLA-DR antigen matching study: survival curves of grafts with 0 versus 1 and 2 antigen mismatches, showing graft failure from rejection as the end-point.	88

		Page No.
Fig. 9.	HLA-DR antigen matching study: survival curves of grafts with 0 versus 1 and 2 antigen mismatches, showing first graft rejection episode as the end-point.	89
Fig. 10.	HLA-DR antigen matching study: survival curves of grafts with 0, 1, and 2 antigen mismatches, showing graft failure from rejection as the end-point.	90
Fig. 11.	HLA-DR antigen matching study: survival curves of grafts with 0, 1, and 2 antigen mismatches, showing first graft rejection episode as the end-point.	91
Fig. 12.	HLA-A,-B antigen matching study: survival curves showing graft failure from rejection as the end-point.	93
Fig. 13.	HLA-A,-B antigen matching study: survival curves showing first graft rejection episode as the end-point.	94
Fig. 14.	Animal model study: corneal vascularization induced by sutures.	102
Fig. 15.	Survival estimates for model: untreated and treated allografts.	105
Fig. 16.	Preoperative MLC results expressed as stimulation index in relationship to allograft survival in the three groups of rabbits.	116
Fig. 17.	Stratified survival estimates for unmodified corneal allografts in MLC study.	117

		Page No.
Fig. 18.	Stratified survival estimates for vascularized untreated corneal allografts.	118
Fig. 19.	Stratified survival estimates for vascularized corneal allografts treated with topical CSA.	119
Fig. 20.	UV-B Irradiation Study: Survival analysis of the three treatment groups.	130
Fig. 21.	Mechanisms of actions of cyclosporine.	140
Fig. 22.	Preliminary systemic CSA study. Case 1: Preoperative photographs.	152
Fig. 23.	Preliminary systemic CSA study. Case 1: postoperative photographs.	153
Fig. 24.	Preliminary systemic CSA study. Follow-up periods and dosage of CSA.	156
Fig. 25.	Systemic CSA study. Survival estimates for the three treatment groups.	170
Fig. 26.	Short versus long CSA: survival estimates for the 3 treatment groups.	181
Fig. 27.	Survival estimates for the 3 groups, end-point is a rejection episode.	191
Fig. 28.	Survival estimates for the 3 groups, end point is graft failure from any cause.	197
Fig. 29.	Final visual acuities in each group, shown as a percentage.	199
Fig. 30.	Mean number of lymphocytes in circulating blood following a single intravenous pulse of 500mg methylprednisolone.	213

		Page No.
Fig. 31.	Single pulse versus oral therapy: Survival graphs showing the risk of a subsequent rejection episode.	223
Fig. 32.	Single versus double pulse therapy: Survival graphs showing the risk of a subsequent rejection episode.	238
Fig. 33.	The number of lymphocytes in circulating blood following pulse therapy.	241

Index To Tables

		Page No.
Table I.	Tissue matching study: Clinical details.	77
Table II.	Numbers of tissue matched corneas.	79
Table III	Establishing an animal model: graft survival results.	103
Table IV.	Effect of treatments on graft survival compared to model.	104
Table V.	MLC study: graft survival results	115
Table VI.	UV-B irradiation study: graft survival results.	129
Table VII.	Systemic CSA study. Indications for keratoplasty.	162
Table VIII.	Systemic CSA study: patient data.	167
Table IX.	Systemic CSA study: graft outcome results.	168
Table X.	Comparison of treatment regimens.	169
Table XI.	Short versus long CSA: patient details.	175
Table XII.	Short versus long CSA: original corneal pathology.	177
Table XIII.	Short versus long CSA: grafts irreversibly lost from rejection.	180
Table XIV.	Short versus long CSA: number of grafts with rejection episodes.	190
Table XV.	Total number of rejection episodes.	193
Table XVI.	Number and outcome of rejection episodes while on CSA.	194
Table XVII.	Graft failure from other causes.	196

		Page No.
Table XVIII.	Final visual acuities.	198
Table XIX.	Preliminary pulse therapy study: clinical details I.	210
Table XX.	Preliminary pulse therapy study: clinical details II.	211
Table XXI.	Corneal thickness measurements.	212
Table XXII.	Oral versus pulse therapy: clinical details.	219
Table XXIII.	Graft outcome (total group).	220
Table XXIV.	Outcome In patients presenting early.	221
Table XXV.	Outcome in patients presenting late.	222
Table XXVI.	Central corneal thickness.	224
Table XXVII.	Double versus single pulse therapy: clinical details.	231
Table XXVIII.	Original diagnosis.	232
Table XXIX.	Graft outcome (total group).	233
Table XXX.	Graft outcome in early and late presenters.	235
Table XXXI.	Graft outcome in high and low risk corneas.	237
Table XXXII.	Circulating lymphocyte counts following pulse therapy.	240

Abbreviations Used In This Thesis.

i.m.	intramuscular
i.v.	intravenous
CSA	Cyclosporin A
D	Donor
R	Recipient
MLC	mixed lymphocyte culture
Dm	mitomycin-C treated donor cells
Rm	mitomycin-C treated recipient cells
SI	stimulation index
IFN	gamma interferon
IL-1	interleukin 1
IL-2	interleukin 2
CTL	cytotoxic T lymphocyte
DTH	delayed-type hypersensitivity
ACAID	anterior chamber-associated immune deviation

Papers Supporting This Thesis.

I. Papers Presented At Scientific Meetings.

II. Papers Published.

I. Papers Presented At Scientific Meetings.

1. Hill JC. **Corneal Graft Rejection.** Annual Ophthalmological Society of S.A., Bloemfontein, March 1985.
2. Maske R, Hill JC. **An New Animal Model for High Risk Corneal Graft Rejection.** Annual Ophthalmological Society of S.A., Bloemfontein, March 1985.
3. Hill JC. **The Use Of Cyclosporin in High Risk Corneal Grafting.** Annual Ophthalmological Society of S.A., Pretoria, March 1986.
4. Hill JC. **The Use Of Cyclosporin in High Risk Corneal grafting.** Ophthalmological Society of the United Kingdom, Annual Meeting. London, April 1986.
5. Hill JC. **Cyclosporin in Corneal Grafting.** International Meeting on Immunosuppression, Cape Town, May 1986.
6. Hill JC. **Techniques of Corneal Grafting.** Continuing Medical Education Meeting. Cape Town, Dec.1987.
7. Hill JC. **Diagnosis and Management of Corneal Graft Rejection.** Continuing Medical Education Meeting. Cape Town, Dec. 1987.
8. Hill JC. **High Risk Corneal Grafting.** Annual Ophthalmological Society of S.A. Sun City, March 1988.
9. Hill JC, Maske R, Watson P. **Pulse v. Oral Corticosteroid therapy in Corneal Graft Rejection.** American Academy of Ophthalmology. New Orleans, Nov. 1989.
10. Hill JC, Maske R, Watson P. **Pulse Therapy for Corneal Graft Rejection.** Annual Ophthalmological Society of S.A, Transkei 1990.

11. Hill JC. **Tissue matching for corneal grafts. Is it feasible in Southern Africa.** Annual Ophthalmological Society of S.A., Bloemfontein March, 1991.

12. Hill JC, Maske R, Watson P. **Pulse therapy in corneal graft rejection.** Annual Ophthalmological Society of S.A., Bloemfontein, March 1991.

13. Hill JC. **Short versus long term systemic cyclosporine therapy in high risk keratoplasty.** American Academy of Ophthalmology, Dallas, Nov. 1992.

14. Ivey A, Hill JC. **Double pulse therapy in corneal graft rejection.** Annual Ophthalmological Society of S.A., Cape Town Feb. 1993.

15. Hill JC. **Systemic cyclosporine in high risk keratoplasty: short versus long term therapy.** Annual Ophthalmological Society of S.A., Cape Town Feb. 1993.

16. Hill JC. **The use of systemic cyclosporine in high-risk keratoplasty.** 25th Annual Meeting of the Royal Australian College of Ophthalmologists. Hobart, Australia Nov. 1993.

17. Hill JC. **The use of pulse therapy in corneal graft rejection.** 25th Annual Meeting of the Royal Australian College of Ophthalmologists. Hobart, Australia Nov. 1993.

II. Papers Published.

1. Hill JC. **The use of systemic Cyclosporin in human corneal transplantation, a preliminary report.** Doc Ophthalmol 1986; 62: 337-44.
2. Hill JC. **Corneal graft rejection.** S Afr Arch Ophthalmol 1986; 12: 25-6.
3. Hill JC, Maske R. **An animal model for corneal graft rejection in high-risk keratoplasty.** Transplantation 1988; 46: 26-30.
4. Hill JC. **The use of Cyclosporin in high risk keratoplasty.** Am J Ophthalmol 1989; 107: 506-10.
5. Hill JC. **Systemic Cyclosporine in high risk keratoplasty.(Letter).** Am J Ophthalmol 1989; 108: 468-9.
6. Hill JC, Maske R, Watson PG. **The use of a single pulse of intravenous methylprednisolone in the treatment of corneal graft rejection.** Eye 1991; 5: 420-4.
7. Hill JC, Maske R, Watson PG. **Corticosteroids in corneal graft rejection: oral versus pulse therapy.** Ophthalmology 1991; 98: 329-33.
8. Sarvan J, Hill JC, Els WJ. **Preliminary report on the localization of Langerhans' cells in rabbit corneal epithelium.** Proc Electron Micr Soc S Afr 1991; 21: 239-40.
9. Hill JC. **The use of cyclosporine in high-risk keratoplasty: short versus long term therapy.** Ophthalmology 1994; 101: 128-33.

10. Maske R, Hill JC, Horak S. **Mixed lymphocyte culture responses in rabbits undergoing corneal grafting and topical cyclosporine treatment**. Cornea 1994; in print.

11. Hill JC. **The use of systemic cyclosporine in high-risk keratoplasty: long term results.** Submitted.

12. Hill JC, Ivey A. **Corticosteroids in corneal graft rejection: double versus single pulse therapy.** Cornea 1994; in print.

13. Hill JC, Sarvan J, Maske R, Els WJ. **UV-B irradiation decreases corneal Langerhans cells and improves graft survival in the rabbit.** Transplantation 1994; in print.

14. Hill JC, Isaacs S. **The benefit of ABO blood group and HLA antigen matching in penetrating keratoplasty.** Submitted.

Section One: Introduction.

1.1 An historical review of corneal grafting.

1.2 An outline of the clinical data used in this thesis.

1.1 An Historical Review Of Corneal Grafting

Descriptions of corneal disease and suggested treatments have been documented since the ancient civilisations of Egypt and Greece. However, in these cultures corneal surgery was limited to various types of superficial keratectomy. The idea of corneal grafting was suggested relatively recently[3,4]. Guillaume Pellier de Quengsy[5], a Frenchman, suggested in 1789 the complete replacement of the opaque cornea using a thin glass disc the size of the cornea and set in a silver rim. He suggested that it could be stitched in with cotton threads and designed instruments for the operation although he never performed the procedure himself. This idea was really the forerunner of the modern keratoprothesis. At the end of the 18th and beginning of the 19th century there was a stimulus for corneal grafting as a result of blindness from diseases such as smallpox, venereal disease, and trachoma brought back by soldiers in the Napoleonic wars. It is uncertain who first suggested the replacement of damaged corneas with living tissue, but the credit is usually attributed to Franz Reisinger[6] who also coined the term "keratoplasty". Reisinger confined his work to rabbits and chickens but most grafts failed either from opacification or sepsis. His work, however, did stimulate debate over the feasibility of keratoplasty, but many were extremely sceptical including one of Berlin's great surgeons of the time, Dieffenbach. He wrote in 1831[2] that Reisinger's idea of keratoplasty was "... certainly one of the most audacious fantasies, and it would be the highest reward of surgery if this operation succeeded".

In 1837 Dr. S. Bigger published a report[7] on his attempts at keratoplasty. While held prisoner in 1835 by Bedouins near Cairo he performed a homograft on his pet gazelle who was blinded by corneal scarring. He used tissue from another gazelle and the full thickness graft remained clear. He continued his work and his report rekindled interest in keratoplasty. One person inspired by this report was Richard Kissam[8] in New York. In 1838 he transplanted a pig cornea into a young man

blinded using just two sutures and no anaesthetic! This and other attempts failed because the grafts either became infected or opaque.

In the second half of the century the results of all types of surgery improved with the introduction of general anaesthesia, antiseptics and cocaine as a local anaesthetic. Henry Power in London published his experimental work in rabbits, dogs, cats and humans in 1872. He stressed the importance of aseptic techniques, fresh donor material, accurate placement, minimal surgical trauma and also the need for homograft material. In 1886 von Hippel[9] successfully transplanted a heteroplastic lamellar graft using a clockwork trephine. He used a full thickness rabbit cornea into a lamellar bed of a young girl and improved her vision to 6/60. This was the first successful corneal graft in a human. Recognizing the importance of the endothelium von Hippel concentrated his efforts on lamellar grafts. Edward Zirm[10] is credited with performing the first successful full thickness human graft in 1905. The donor was a boy who underwent an enucleation for an intraocular foreign body, the graft was performed on a patient who had bilateral vascularized opaque corneas following lime burns. Zirm used ether anaesthesia, trephines, preoperative miotics, iodoform antiseptics and overlay sutures.

The advantages of autografts were recognized in the early part of this century but widespread use of this technique was obviously limited. However, rotation autografts to move scars out of the visual axis were introduced by Maigitot and Morax in 1913. In 1935 Filatov[11] suggested using the membrane found inside the shell of a hard boiled egg to help fixate the graft, a technique that became widely accepted. He also rekindled interest in sutures to perform direct closure of the wound and showed that cadaver corneas were suitable for transplantation. Apart from egg membrane, various splint and straddling suture techniques were introduced to help fixate the graft; most were used with prolonged bed rest combined with the use of sand bags to keep the head immobile.

The prognosis for corneal grafts has improved considerably during this century, partially due to increased knowledge of corneal physiology and partially because of advances in microsurgical techniques and instrumentation. The recognition of corneal rejection was made by Maumenee[12] in 1951, and the description and understanding of the process was advanced by the work of Khodadoust and Silverstein. Castroviejo designed a number of fine instruments and recommended closure by direct sutures. The surgical microscope was popularized by Troutman resulting in a new generation of microinstruments being introduced; designed by Troutman and others such as Dermot Pierse who also developed the diamond knife. More sophisticated manufacturing techniques allowed finer sutures to be bonded onto finer and sharper needles. Improved collection and storage of donor corneas was improved by the setting up of eyebanks, the first being the New York Eye Bank founded by Townley Paton in 1959. Greater understanding of the corneal endothelium was aided by the development of the specular microscope by Maurice. The introduction of storage mediums helped preserve corneas for longer periods and prevent waste: they also allowed easier transportation. The first and still widely used medium was introduced by McCarey and Kaufman (MK solution) in 1974.

These factors together have resulted in primary graft failure becoming an uncommon cause of graft failure. The commonest cause of corneal graft failure is now graft rejection.

1.2 An Outline Of The Clinical Data Used In This Thesis.

The clinical data presented in this thesis was obtained from the clinical records of the 818 penetrating corneal grafts performed by myself during the period 1982 to 1992 inclusive. Data from patients who had lamellar corneal grafts or therapeutic patch grafts are not included in this thesis. Also excluded are details from patients who had autografts either from the contralateral eye or rotation grafts to move scarring out of the visual axis. I have included data from patients referred from other centres within South Africa or from neighbouring countries such as Malawi, Zimbabwe or Namibia. Usually these patients returned home soon after their operations and long term data is therefore not available. Attempts were made to obtain clinical information from the referring doctor or ophthalmologist whenever possible. When no such information was available the patient was excluded from the long term analysis. The clinical data was stored in a computer using a database program, the information was transferred and retrieved by myself.

The animal experiments were performed in the microsurgical laboratory of the Department of Surgery, University of Cape Town. The animals were procured from the Animal Unit, and permission to undertake the work was approved by the Animal Users and Ethics Committee of the University of Cape Town.

Section Two: Corneal Graft Rejection.

2.1 An overview of transplantation immunology.

2.2 Cornea transplantation immunology.

2.3 Corneal graft rejection.

2.4 High-risk keratoplasty.

2.5 Treatment of graft rejection.

2.1 An Overview Of Transplantation Immunology.

If tissue is transplanted from one identical twin to another, or within inbred strains of laboratory animals, the recipient recognizes the graft as "self" and an immunological attack is not mounted. Clinically, this situation is rare because recipient and donor usually do not share the exact tissue antigens. When the recipient becomes exposed to transplanted tissue, a series of immunological responses occur and the recipient becomes sensitized to the donor tissue[13]. The first response is that antibodies and immunologically active cells, specific for the graft, appear in the recipient even in the absence of a rejection response. This is known as the afferent arc of the immune cycle. When these immune reagents come into contact with the graft the efferent arc of the cycle is activated resulting in the process known as rejection.

The afferent arc.

The most important antigens to initiate rejection are the major histcompatibility antigens. In humans the major histocompatibility complex (MHC) consists of multiallelic genes located on the short arm of chromosome 6, this area codes for the human leukocyte antigens (HLA)[13]. These antigens are glycoproteins and are found on most cells, red blood cells are an important exception. The function of the MHC and HLA antigens is self recognition, in transplantation they lead to the graft being recognized as non-self. The HLA system is thought to be the most important factor influencing the "take" of transplanted tissue. Similar twins possess similar antigens and hence transplanted tissue is not recognized as non-self.

Ten loci have been found in the MHC and since the genes in this complex are multiallelic, the resultant gene products (i.e. HLA antigens) are variable. The HLA antigens in humans are encoded by genes in the A, B, C, and D-DR regions of the MHC. The HLA antigens are divided into two main classes[14]. Class I antigens were originally serologically defined and are known as SD antigens, and include the

HLA-A, HLA-B and HLA-C antigens. These antigens are polypeptides and have a heavy chain of molecular weight 44,000 daltons that possess a single asparagine-linked oligosaccharide unit, which carries the allotypic determinant[15]. These products are transmembrane glycoproteins associated with a smaller peptide, a nonpolymorphic beta$_2$-microglobulin and are expressed on the surfaces of virtually all nucleated cells[16], the concentration of antigen varies with cell types. Class I antigens elicit strong antibody responses and T-cell proliferation during rejection. Class II antigens have a more restricted cell distribution and were originally defined by mixed lymphocyte culture and are known as lymphocyte-defined (LD) antigens, these are called HLA-D antigens. Class II antigens consist of two polypeptide chains of molecular weight 28,000 and 34,000 daltons, the transmembrane alpha and beta chains, the latter chain contains the alloantigenic determinant[15]. Class II antigens exist on B lymphocytes, subsets of T lymphocytes, macrophages and Langerhans' cells. However, under certain conditions class II antigens can be expressed by other cells, including vascular endothelium and epithelial cells[17]. Closely associated with the HLA-D locus is the HLA-DR locus; other class II antigens are now recognized and are called DQ and DP, the clinical significance of these is not yet known. Class II antigens, like class I evoke strong antibody responses. Donor HLA antigens are responsible for recipient sensitization and act as targets for the immunological response. However, some grafts reject despite HLA compatibility and this is thought to be due to the presence of minor histocompatibility antigens. These are less potent than their major counterparts but nevertheless add to the overall antigenicity of the graft[18]. Most of the work on minor histocompatibility antigens has been done in animals, but they have been recognized in humans although their role is unknown as little clinical data is available[19-21].

The mechanism of recipient sensitization is complex and many aspects are still not fully understood. Pure fibroblasts only express class I antigens[22,23]: they do not stimulate proliferation in mixed lymphocyte cultures which is an in vitro correlate

of recipient sensitization[24]. Also skin grafts containing pure dermal fibroblasts are not rejected[25]. It therefore appears that donor cells possessing only class I antigens are not capable of sensitizing recipients. Antibody production appears to require two antigenic signals[26-28] and the unique group of cells that carry both class I and class II antigens are thought to be important as antigen-presenting cells[29-32]. Recent studies[19,33] indicate that recipient sensitization can be caused by grafts only when viable antigen-presenting cells bear class II antigens plus class I and/or minor histocompatibility antigens. These antigen-presenting cells come into contact with recipient lymphocyte subpopulations that are capable of generating an immune response, with the result that the recipient's serum contains antibodies directed against donor histocompatibility antigens[34], and cytotoxic T cells specific for donor cells[28]. If these antibodies and/or sensitized cells come into contact with the graft, then a series of effector responses result that in turn may lead to graft rejection. In the more natural situation of infections, class I antigens are required for the interaction of killer T lymphocytes (CD8+) with self cells that have foreign antigen (often viral) on their surface. Killer T cells react with self plus antigen ("dual recognition") to produce cell lysis; foreign class I antigens in a transplant can be treated in a similar way with killer T cells recognizing them as "self plus X" with consequent damage to the graft[35]. The natural function of class II antigens is to guide helper T cells (CD4+) in their interaction with antigen presenting cells, again there is "dual recognition" as both foreign antigen and host class II antigen (HLA-DR) are required to activate helper cells. In turn helper T cells release of lymphokines which promote the differentiation of killer T cells, which in turn destroy the graft[35].

Understanding the HLA system has led to an improvement in the success of renal transplantation. If a renal transplant is performed between siblings with identical HLA-A and B antigens, the result is a surgical success rate of 98%[36]. Using cadaver kidneys the results are less definite; European studies indicated a beneficial effect of

HLA-A and B matching[37] whereas studies in the United States showed little difference. The differences are probably the result of the heterogenicity of the population in the USA compared to Europe. When HLA-DR antigens were discovered a significant improvement in survival in cadaveric renal transplantation was found[38]. Now with improved methods of immunosuppression the role of tissue matching is again under debate, but it is generally agreed that matching should still be attempted when feasible[39]. Recent studies of the results of HLA-B and HLA-DR matching in renal transplantation indicate that graft survival at one year is 71% for those with four mismatched antigens compared to 88% for no mismatched antigens[40,41].

Apart from tissue matching, other methods of reducing donor immunogenicity have been found. An early finding was that donor passenger leucocytes played an important role in the recipient sensitization process[42]. The antigenicity of various endocrine grafts can be reduced by prolonged culture in oxygen or a shorter culture in hyperbaric oxygen. This in turn leads to better survival of the graft[43-47]. Culture appears to cause the disappearance of class II antigen-bearing interstitial dendritic cells and probably of the vascular endothelial cells which also express class II antigens[44,45,48,49]. Since the endocrine cells, which express class I antigens[50], survive it follows that the improved graft survival probably results from eradication of passenger leucocytes and other cells bearing class II antigens. Ultraviolet B radiation also has the ability to reduce the antigenicity of grafts, again by reducing the number of class II bearing cells[51]. These findings point to the theory that two antigenic signals are required from the donor to initiate recipient sensitization[27,28,52-54]. One signal is provided by class I antigens which are present on all nucleated cells. The second signal results from class II antigens. Under certain conditions class II antigens and other antigens, including minor histocompatibility antigens, can provide both antigenic signals[53,55].

Efferent arc.

The introduction of an antigenic substance sensitizes the recipient and initiates an immune response. This is a series of complex events resulting in the synthesis of specific antibodies and effector lymphocytes that react specifically with the immunizing antigen[13]. The immune system consists of lymphoid tissues (spleen, lymph nodes, and Peyer's patches), central lymphoid elements (thymus and bone marrow), and a recirculating pool of lymphocytes. The immune response is carried out by lymphocytes, monocyte/macrophage cells, and other inflammatory cells such as Mast cells, polymorphs, and eosinophils and by soluble mediators including products released by immune cells[13].

Lymphocytes originate from stem cells found in the bone marrow, they are antigen specific and only react with a single or closely related group of antigens. There are two broad groups of lymphocytes: T lymphocytes (T cells) differentiate within the thymus, whereas B lymphocytes (B cells) are associated with the bone marrow. Another group of lymphocytes are termed null cells.

T cells: these are responsible for cell-mediated immune responses. There are a number of T cell subsets, including cytotoxic cells, helper cells and suppressor cells. These cells do not produce antibodies but are able to regulate their production. The initial step in T cell activation is specific antigen binding which occurs at receptor molecules. Subpopulations of T cells interact with other subpopulations and with B cells. Helper T cells interact with B cells to aid antibody production, they also interact with cytotoxic T cells to generate more effective killer cells. The subsets can be recognized and studied by the use of monoclonal antibodies[32]. Human helper cells have the $T4^+$ marker, while the cytotoxic/suppressor T cells express both $T5^+$ and $T8^+$ antigens. The subsets are studied by the use of the mixed lymphocyte culture (MLC) and cell-mediated lympholysis. In the former, lymphocytes from the recipient (the responder) are

mixed with lymphocytes from the donor (the sensitizer) which have been prevented from proliferating by the prior addition of mitomycin C. When the histocompatibility antigens differ between the two individuals the responder cells proliferate, this is measured by the amount of tritiated thymidine that is incorporated into the activated cells. This is known as the one-way MLC[28,52,56-58]. In cell-mediated lympholysis a MLC reaction is set up which results in the production of responding cytotoxic T cells. These are assayed by their ability to lyse radioactive chromium-labelled target cells. If the target cells carry the same antigens as the sensitizing cells, then the former are lysed[28,57,59-61]. HLA-A, B and C antigens can serve as target antigens in cell mediated lympholysis[52,62].

B cells: these differentiate into plasma cells and produce antibodies. Like T cells they bind specific antigens on their surface. To become active they require the assistance of helper T cells[17]. The role of antibodies in graft rejection is unknown, but there is little evidence to implicate them in most types of rejection, except possibly in some tissues such as the kidney. Passive transfer of serum from an animal that has rejected a skin graft does not initiate or accelerate a rejection response in another animal with a similar graft. However, transfer of lymphocytes is effective in shortening graft survival[18].

Null cells: 5 to 10% of lymphocytes in the blood are null cells. In humans this subpopulation contains many cytotoxic cells including killer and natural killer cells. In the presence of antibody, under certain circumstances killer cells can destroy cells. Natural killer cells do not need the presence of antibodies and are directly cytotoxic without prior sensitization[63].

Monocytes and macrophages: like polymorphs these are phagocytic cells and are derived from stem cells in the bone marrow. Monocytes travel in the blood stream to tissues where they reside and are known as macrophages. They were originally thought to be scavenger cells but are now recognized as important immune system

cells. Macrophages are thought to have at least five distinct but related functions[64,65]; a) processing and presenting antigen, b) inducing T-cell proliferation, c) regulating the immune response, d) phagocytosis and killing of infectious agents, this ability is aided by plasma membrane receptors such as the Fc portion of IgG, e) monokine production. T cells in culture require contact with an antigen presenting macrophage before they proliferate or secrete mediators[66]. This interaction between T cell and macrophage is dependent on the two cells possessing the same class II antigens. The macrophage determines the portion of the antigen against which the T cell clone will react[67].

Cytokines: these are hormone-like chemicals produced by a wide variety of cells such as lymphocytes, macrophages, platelets, and fibroblasts. In the immune system monokines and lymphokines regulate the immunological and inflammatory host responses by acting as intercellular messengers that modulate cellular functions[68]. There appear to be both positive and negative feedback mechanisms involved in the regulatory process. Interleukin 1 (IL-1) is a monokine that augments T cell proliferation and promotes T helper cell functions and B-cell antibody production[69]. Interleukin 2 (IL-2) is produced by T cells in response to antigenic or mitogenic stimuli and stimulates the growth and proliferation of other T cells.

The exact mechanism of graft rejection remains uncertain and may vary with different tissues. It was originally thought that cytotoxic T cells played a vital role, but animal studies in which animals were depleted of certain lymphocyte subpopulations indicate that helper T cells are required but not necessarily cytotoxic T cells[70-73]. In some tissues at least antibody-dependent cell-mediated cytotoxic mechanisms are important. Fc-receptor positive non-T cells are able to lyse IgG-coated target cells. These effector cells are killer cells and have been found in rejecting grafts together with other lymphocytes[74]. Macrophages are also found in rejecting grafts but their effector role is uncertain, initially they may be responsible for recognition and antigen presentation and later may be important phagocytes[73,74].

Antibodies may play a role in some tissues especially vascularized tissues, a process requiring complement[75]. As would be expected in immunological reactions, the second contact of a recipient with an antigen is a more dramatic event with an accelerated response[18]. The effect of this second set rejection is seen in three or four days compared to the first set reaction that takes a minimum of 10 to 14 days.

2.2 Corneal Transplantation Immunology.

The relative success of early corneal grafts suggested that the cornea possessed immunological privilege compared to other tissues. Medawar[76], in 1948, proposed four reasons for this high success rate:

1. The amount of tissue transplanted is too small to evoke an immunological response.
2. The immune response may be too slow or "feeble" to have a clinical effect, or alternatively cell replacement may be able to keep up with destruction.
3. Corneal tissue is unable to initiate immune reactions, even if it succumbs to them.
4. Corneal tissue, being avascular, cannot succumb to immune reactions even if it initiates them.

Medawar[76] used heterotopic skin allografts to investigate these suggestions. He found that skin grafts were destroyed in the anterior chamber of the eye if they became vascularized. He concluded that explanation (4) was the most likely reason for the low rejection rates of corneal grafts. Billingham and Boswell[77] used similar techniques of heterotopic skin allografts but inserted them into intrastromal pockets within the cornea. They also found that grafts were only rejected if they became vascularized, and concluded that survival was improved because the efferent arc of the immune response was hindered by the lack of blood vessels. Other workers also recognized that the relative privilege was due to the avascularity of the cornea and to the lack of lymphatics in this tissue[12,78]. Studies have indicated that the lack of vessels interferes with both the recognition of non-self antigens and also their destruction by sensitized cells[79], conversely in vascularized corneas there is a greater chance that the host will recognize the foreign antigens and mount a successful immune attack. It is now widely accepted that corneal immunological privilege is dependant on the avascularity of the cornea[80]. The absence of blood

vessels and lymphatic channels prevents corneal alloantigens from reaching regional lymphoid tissues, and the graft although potentially immunogenic is sequestrated from the immunological system. This privileged position could thwart graft rejection by preventing the induction of an alloimmune response (afferent blockade) and by serving as a barrier to immunological effectors (efferent blockade). The afferent blockade theory is supported by the work of Maumenee[12] mentioned above, who reported that clear grafts in rabbits rejected following a subsequent skin graft, indicating that sensitization had not occurred and that the efferent pathways were not blocked. A similar conclusion was reached by Callanan and co-workers[81] who found that in orthotopic rat allografts, the appearance of donor specific cytotoxic T lymphocyte responses coincided with graft rejection. The role of efferent blockade is not clear, the work by Maumenee[12] suggests that it is not important in graft survival. The work by Billingham and Boswell[77] discussed above, suggests that the efferent blockade does play a role in immunological privilege, their work is supported by the findings of Khodadoust and Silverstein[79]. These workers found that a high percentage of corneal grafts survived despite the recipient animal having been sensitized by prior skin grafts. Clinically it has been shown that patients can be sensitized, as proved by the presence of circulating antibodies against donor antigens, without rejecting their grafts[34]. It would appear that the avascular corneal graft bed is responsible for the immunological privilege; it protects the graft by a partial, albeit significant, barrier for both the afferent and efferent arcs of the immune process[80].

Therefore the original suggestion that the cornea is not antigenic has been disproved with HLA antigens being demonstrated on all three layers of the cornea and each layer being vulnerable to immunological attack. However, there is significant variation in the antigenicity and immunogenicity of the corneal components[80]. The epithelium expresses large amounts of class I antigens[82], yet fails to induce detectable anti-class I cytotoxic T lymphocyte responses when grafted

heterotopically in the rat[83]. In contrast the endothelium expresses minimal amounts of class I antigens and, under normal circumstances, no class II antigens[82], yet this layer stimulates vigorous cytotoxic T lymphocyte responses when grafted heterotopically in the rat[83]. Another hypothesis to explain the privilege of corneal grafts held that donor tissues were rapidly replaced by recipient constituents before rejection could occur. This suggestion has been disproved by studies of sex-chromatin markers[84,85] and by studies using tritiated thymidine-labelled corneal grafts[86,87]. Clinical reports in which rejection has been reported up to three decades after transplantation also refute this hypothesis[13,88].

Other factors may also be operative in maintaining the immunological privilege of the cornea. The presence of allogenic antigens in the anterior chamber of the eye appears to elicit specific tolerance through the suppression of delayed-type hypersensitivity[89-93]. This response, anterior chamber associated immune deviation (ACAID), appears to be an active response rather than just immunologic ignorance of the antigens existence, and appears despite the concurrent stimulation of cytotoxic T lymphocytes and the production of circulating antibodies[94,95]. This process requires an intact spleen[89] and is thought to be mediated by direct exposure to the venous system via the aqueous outflow through the canal of Schlemm. Antigen processing by this route appears to bypass the lymphatic system where destructive responses are initiated. Associated with ACAID is an inability to reject challenged orthotopic skin allografts from the donors of the alloantigenic cells inoculated into the anterior chamber. Presumably antigens or cells could gain access to the anterior chamber from a corneal graft and so this mechanism could play a role in maintaining the immunological privilege of the cornea. However, the only process of ACAID that could conceivably affect the fate of orthotopic corneal allografts is the down-regulation of systemic delayed-type hypersensitivity (DTH). Because corneal graft rejection occurs in the absence of demonstrable systemic DTH, it is unlikely that suppression of this arm of the alloimmune response would

promote corneal graft survival. In fact the development of the cytotoxic T lymphocyte (CTL) response associated with ACAID may jeopardize graft survival[94]. However, it has been recently reported that orthotopic corneal allografts elicit a deviant systemic immune response in mice that correlates positively with long term graft acceptance[96].

The first report of corneal graft rejection was by Paufique et al[97] in 1948. They described late clouding of a graft for no apparent reason after an initial interval in which the graft was clear. They suggested that the cause was due to an allergy by the recipient to foreign protein in the graft and called the process "maladie du greffon" or graft sickness. Studies by Maumenee[12,98] demonstrated that donor corneal grafts could elicit an immunological reaction and that the process was accelerated by prior or simultaneous skin grafts. Khodadoust and Silverstein[99,100] reported that the epithelium, stroma and endothelium could reject individually or simultaneously. They also brought attention to the rejection lines that are pathognomonic of corneal rejection. The clinical features of corneal allograft rejection were described by Maumenee[101] in 1962. Subsequently Polack and Kanai[102] showed that sensitized lymphoid tissue was the cause of the rejection process that resulted in destruction of the graft endothelium and graft oedema.

A and B blood group antigens were found in minced preparations of the cornea by Nelken et al in 1956[103]. Later studies isolated these antigens to the corneal epithelium[104,105] and endothelium[105,106]. More recent work using the sensitive avidin-biotin-complex immunoperoxidase technique and monoclonal antibodies directed against ABO blood group antigens indicate that these antigens are expressed by the corneal epithelium but not by the endothelium or keratocytes[107]. It has also been found that the expression of the A antigen on corneal epithelial cells increases with age and, as with erythrocytes, it proceeds with maturation from H precursors[107]. ABH antigens are important histocompatibility antigens in vascular organ transplants but their role in the cornea is unknown. A recent report has shown

improved graft survival in high-risk cases receiving ABO compatible donor corneas[108].

The presence of HLA antigens are demonstrated using immunohistologic techniques: monoclonal antibodies directed against the monomorphic determinants of the antigens map out the location of these antigens in tissues. Class I antigens have been found in all layers of the cornea and these antigens are identical to those found on other tissue cells[109,110]. Within the epithelium staining increases towards the limbus[111]. In the stroma class I antigens are expressed by the keratocytes[107,111-113]. Originally it was believed that the endothelial cells did not express class I antigens in vivo[112,113], except in children less than two years of age[111], but recently they have been detected in all ages using a sensitive immunoperoxidase technique[107]. Class II antigens have been found on leucocytes and Langerhans' cells (LC) within the corneal epithelium[107,113-115]. LC are more abundant in the limbal area of the cornea and in the conjunctiva; following inflammation these cells can be found more centrally in the epithelium and also in the stroma[116]. Normally endothelial cells have not been found to express class II antigens[107,111-113], but they can be detected on cultured endothelial cells exposed to gamma interferon both in the rabbit[115] and in humans[117]. During graft rejection endothelial cells have been found to express class II antigens[118], whether this is the result of the associated inflammatory response, the release of lymphokines or gamma interferon, or to the rejection reaction itself is not known[119]. The clinical observation that rejection often follows an inflammatory disease, such as herpes simplex, can be explained by the increased expression of class II antigens that would accompany the infection. Likewise a mild rejection episode may become amplified by the resulting increase in class II expression. The relative contribution of class I and class II antigens to the immunogenicity of corneal allografts is not fully understood. Treseler and Sanfilippo[120] working with congenic rats have shown that corneal grafts mismatched for only class I antigens are capable of giving rise to cellular immune responses.

There was, however, a marked increase in the immunogenicity of class I MHC antigen-disparate grafts by the addition of a class II MHC antigen difference; this suggests that Langerhans cells make an important contribution to the immunogenicity of MHC-disparate corneal allografts[120]. Work by Katami et al[121] in the rat indicates that minor transplantation antigens may also play a role in corneal graft rejection.

LC were first described in the epidermis of the skin[122], but are also found in many mammalian epithelia including that of the conjunctiva and cornea[114,123,124]. LC are usually situated in the suprabasal or basal layers of the epithelium and are recognized by their dendritic appearance and by the presence of Birbeck granules in the cytoplasm, visible by electron microscopy[125]. Quantitative studies on LC are made by staining for ATPase[114,124,126-128] or other markers on the cell surface[114,124,129,130]. The presence of various receptors and antigenic sites on the surface of LC, revealed by immunocytochemical studies have helped to elucidate the role of these cells. LC possess Fc (IgG) and C3 receptors[131] and express the Class II major histocompatibility complex (MHC) antigens[132,133]. Radiation induced bone marrow chimeric experimentation has confirmed the mesodermal origin of these cells from bone marrow[134] and functional studies have classified LC as a tissue leucocyte with a similar role to that of macrophages, having the ability to phagocytose foreign material and to stimulate a mixed lymphocyte reaction in vitro[135]. The generally accepted view is that LC act as antigen presenting cells. They are probably the critical cells for the generation of skin-graft rejection[135] and it is likely that LC of the cornea play a similar immunological role. Apart from their role in processing and presenting foreign antigens to stimulate the host's immune system, these cells are themselves highly immunogenic and are potent stimulators of an immunological response when they are transplanted[136]. The number of leucocytes in the recipient graft bed also appears to be important. Williams et al[137], using monoclonal antibodies and immunohistochemical techniques, have shown that the

presence of large numbers of macrophages, dendritic cells, granulocytes, and to a lesser degree T cells in damaged recipient corneas before grafting, is associated with subsequent corneal graft failure. The presence of more than 50 leucocytes/mm^2 of central recipient cornea, at the time of surgery provided an excellent prognostic indicator for graft loss.

Recently there has been a renewed interest in the "passenger cell" theory first proposed by Snell over 30 years ago[138] He proposed that donor leucocytes present in transplanted tissues were an important source of tissue immunogenicity and that ridding tissues of these passenger cells would improve transplant survival. Callanan and co-workers[81] have found that in orthotopic rat allografts disparate for class I and class II MHC antigens, the presence of graft borne LC almost doubled the risk of rejection. Interestingly, a similar experiment in mice with grafts disparate only for class II MHC antigens, showed that donor-derived LC can render the graft immunogenic and elicit alloimmune cytotoxic T lymphocyte responses[129]. However, in the absence of constitutive expression of class I MHC antigens on the corneal components such grafts are antigenically invisible - allospecific cytotoxic T lymphocyte effectors have no target cells to attack and the graft survives. Depleting tissues of Ia positive cells by a variety of techniques has improved survival in a number of allograft models[139,140]. One method is the use of ultraviolet (UV) irradiation which has been documented to have an immunosuppressive effect[141-143]. The use of UV radiation to improve survival of transplanted tissue has been suggested[144,145] and shown to be successful in a number of animal corneal graft models[146-148], including our own animal model reported later in this thesis. In the cornea, like other transplanted tissues, rejection is thought to be triggered by antigens coded by the major histocompatibility complex, especially class II antigens. Under normal conditions the corneal epithelial, stromal and endothelial cells do not express class II antigens[111,112,149] but they are found in LC present within the corneal epithelium[150]. Although LC are more abundant in the corneal periphery,

scattered cells are found in the central part of the corneal and become passenger cells in corneal grafts. The beneficial effect of UV radiation on graft survival is most likely due to its effects on LC; possibly the antigen presenting ability of these cells is decreased[151-154] or it has been suggested that a tolerogenic signal is produced that activates donor-specific suppressor cells[155,156]. Because LC are found predominantly in the periphery of the cornea, with very few in the centre[114,124], another potential method of reducing antigenicity is to transplant small grafts. In a rat model, Katami has shown that small (2mm) grafts are not rejected, whereas standard sized grafts (3mm) are rejected acutely[157]. Furthermore, small grafts taken from the peripheral cornea and transplanted centrally in the recipient had a 40% rejection rate. These findings suggest that the incidence of graft rejection may be linked to the number of LC transplanted as passenger cells. Clinical studies on the effect of graft size on graft survival have been inconclusive, in most of the studies the grafts were within a narrow size range. Often the larger grafts were used in cases in which the prognosis is poor, such as infected eyes or perforated corneas. Also the studies that considered graft survival did not differentiate between failure from rejection or from other causes. Some studies reported no association between graft size and the incidence of rejection[158,159], some reported lower rejection rates with smaller grafts[160] or larger grafts[161], while others reported lower survival with larger grafts[162,163]. Likewise little work has been published on the use of eccentric grafts and the incidence of rejection, again the results are inconclusive because of the poor prognosis of the primary condition being treated in most cases[164,165].

Other methods have been suggested to reduce the antigenic load or reduce the antigenicity of transplanted corneas. The presence of class I and II antigens and LC in the epithelium has led to the suggestion that this layer should be removed at the time of surgery to reduce the incidence of rejection. Tuberville et al[159] reported that, in both retrospective and prospective studies, the incidence of rejection was significantly reduced when the epithelium was removed at surgery. Other studies

have shown no difference in the incidence of rejection[166,167], the incidence of irreversible rejection[167] or graft survival[167] between patients with or without removal of the epithelium; the results therefore remain inconclusive. Two studies have shown that there is a reduction in the number of class II antigen bearing LC in corneas preserved by organ culture at 37°C for one to three weeks[168,169], however no data exists to show that this leads to improved graft survival or a reduction in the incidence of rejection.

Surprisingly little is known about the precise process of corneal allograft rejection, but it likely that, as in other tissues, the effector response is predominantly cell mediated[170]. Passive transfer of sensitized lymphocytes into the anterior chamber of rabbits causes a picture analogous to endothelial rejection[170,171]. Histological studies of rejecting corneal grafts show accumulations of lymphocytes and monocytes[100,172-174]. Other evidence of the cellular mechanism is demonstrated by in vitro studies of leukocyte migration inhibition and macrophage migration inhibition factor activity[175,176]. However, as mentioned earlier the relative importance of the different subsets of lymphocytes in the rejection process is still uncertain[170]. It is generally agreed that solid organ transplants such as skin and corneas are rejected by cytotoxic T lymphocyte (CTL) or delayed-type hypersensitivity (DTH) mediated processes[177]. Because *central* corneal allografts fail to induce DTH yet undergo immune-mediated rejection at either orthotopic or heterotopic sites it is safe to assume that DTH is not important in central grafts. Moreover, in T cell-deficient mice the transfer of lymphoid cell suspensions depleted of CD4+ (i.e., T helper/DTH) but containing CD8+ (CTL/suppressor population) cells induced heterotopic corneal allograft rejection[178]. In corneal graft rejection T helper cells are stimulated and these in turn initiate the generation of cytotoxic T killer cells. In the case of LC deficient grafts, corneal alloantigens must be reprocessed by host antigen-presenting cells[80]. In contrast, grafts containing donor LC are at greater risk because the mobile alloantigenic LC of the donor, in addition to expressing large quantities of class II antigens, are capable of migrating to peripheral lymphoid tissues where they can

evoke an alloimmune response[80]. The importance of donor LC in initiating rejection in the clinical situation, in which small central grafts which are virtually devoid of LC are used, is not known. The role of the humoral immune system in corneal graft rejection is thought to be minimal. However, plasma cells are found histologically and lymphocytotoxic antibodies have been demonstrated in rejecting patients[34,109,179]. Possibly these antibodies are involved in antibody-dependent cell-mediated cytotoxic reactions.

2.3 Corneal Graft Rejection.

In the literature there is some confusion regarding the term corneal graft rejection. Some authors use this term for any rejection episode regardless of outcome, while others use the term to refer to graft failure from rejection. It is now becoming standard to use the term rejection to refer to any rejection episode regardless of outcome. When failure occurs then this is specified e.g. graft failure from rejection[180].

It is widely accepted that the diagnosis of graft failure from rejection should be made only in a graft that is technically successful and has remained clear for at least 10 days[181]. Prior to this time it is difficult to exclude primary graft failure, although rejection can occur before this time in a patient who has been previously sensitized[182]. Rejection can occur at any time after this ten day period, with cases being reported 20 years[13] and 31 years after surgery[88]. The symptoms of rejection can be minimal in the early stages, and include reduction in visual acuity, irritation, mild pain, photophobia, redness, and tearing. As the rejection reaction progresses the symptoms become worse and eventually severe loss of vision can occur. Patients who have had a graft must be warned of the symptoms and be encouraged to seek early medical treatment. In a recent American collaborative histocompatibility study in corneal grafting[108] it was suggested that the single most important factor in graft survival was early detection and treatment of rejection episodes. A rejection episode, especially those involving the endothelial layer, results in large numbers of endothelial cells being destroyed[183] and even if the rejection process is reversed sufficient cells may not survive to maintain graft clarity[184]. Rejection episodes should therefore be reversed as quickly as possible to preserve the maximal number of endothelial cells[185]. Patients should be particularly aware in the early postoperative period, at which time the eye is still irritable and the vision still poor. In grafts with a good prognosis the incidence of rejection has been reported to be between 2.3% and 35%[78,99,186] and this compares to high-risk grafts in which the

incidence is between 40 and 68%[12,99,179]. Most episodes of rejection occur within the first year[187-190] and especially within the first six months[34,88,191]. In a study of 37 high-risk keratoplasty patients reported later in this thesis, 27 grafts (62.2%) were lost from rejection at a mean time of 11.2 months, and a median time of 8 months, after operation. Of the 23 rejected grafts, eleven rejected during the first six months and sixteen during the first year.

The rejection episode can involve either a single layer or more than one layer of the cornea. Each layer exhibits a distinct clinical picture, during the rejection process an these resemble the experimental findings of Khodadoust and Silverstein[78,88,99,191,192]. Four types of corneal rejections have been described: epithelial rejection, subepithelial infiltrates, stromal, and endothelial rejection[88,192,193].

Epithelial rejection is characterized by a rejection line appearing as a raised ridge of epithelium that stains with fluorescein or rose bengal. This ridge is a zone of destruction of the donor epithelium, and is composed of donor epithelial cells and recipient inflammatory cells, mainly polymorphonuclear leucocytes. The line often starts at an area where blood vessels invade the donor-host interface; at the corresponding limbal area there is an infiltration of lymphocytes, plasma cells and polymorphonuclear leucocytes. If untreated the line advances across the graft in approximately six to ten days, but may take as long as a few weeks. Recipient epithelium grows in behind the ridge to heal the denuded Bowman's layer; in the early stages the cells are large with prominent microvilli but eventually they mature into normal epithelial cells. In a study by Alldredge and Krachmer[88], in which patients were followed for a minimum of a year, the incidence of epithelial rejection was 10% occurring at an average time of onset of three months (range 1 to 13 months). Arentsen[190] found an incidence of 14%. The incidence may, however, be much higher than these two studies indicate as the process is often asymptomatic. Of the 27 cases of epithelial rejection noted in these two studies, 20 occurred before

or with other types of rejection (subepithelial infiltrates and/or endothelial rejection).

Subepithelial infiltrates were first described as a form of corneal graft rejection by Krachmer and Alldredge[194] in 1978. They are seen as small white dots, 0.2 to 0.5 mm in diameter, located in a random fashion immediately below Bowman's layer in the donor cornea[180]. The infiltrates resemble the infiltrates seen in adenovirus epidemic keratoconjunctivitis except that they are restricted to the donor cornea and are not associated with conjunctivitis. There may be associated inflammation in the anterior chamber and other types of graft rejection may co-exist, especially epithelial rejection. The infiltrates disappear rapidly with topical corticosteroid treatment although residual scarring may remain. This type of rejection can occur from six weeks to 21 months following surgery, with a mean of ten months. The incidence is reported to be 15% but the reaction may be asymptomatic and undetected in many cases and they may occur more commonly in younger patients[194]. Arentsen[190] found an incidence of subepithelial infiltrates of only 2.4% in his study.

Stromal rejection was described in rabbits by Khodadoust and Silverstein[99] and has been described in humans[192]. It is difficult to diagnose as it usually appears in association with endothelial rejection. Clinically there is a sudden onset, peripheral, full-thickness haze associated with circumcorneal injection The haze has an appearance of an arc which progresses towards the centre[192]. Blood vessels invade the stroma and they spread to involve the whole cornea; there is no thickening of the graft unless the endothelium is involved. The cellular infiltrate at the limbus and around the vessels consists of lymphocytes, plasma cells and polymorphonuclear leucocytes. Within the donor stroma there is an intense, full-thickness infiltrate of polymorphonuclear leucocytes and lymphocytes[13]. Donor keratocytes are killed in the process. The reaction takes a few days to weeks to progress and on recovery

ghost vessels remain. Stromal rejection usually occurs within two years of keratoplasty[13].

Endothelial rejection is perhaps the most severe form of corneal rejection as this layer does not have regenerative capabilities and significant cell loss can lead to chronic graft oedema. Two forms of endothelial rejection are described. The first is an advancing rejection line, known as a Khodadoust line. This usually originates at a vascularized area of the cornea[99] or at the site of an anterior synechiae[195]. Over a period of a few days the line extends across the donor cornea, destroying endothelial cells, depositing keratic precipitates and causing graft oedema and folds in Descemet's membrane within the affected area. The rejection line and keratic precipitates are composed of lymphoid cells[99,196], and the process is accompanied by inflammatory signs in the anterior chamber. Symptoms of rejection include pain, redness and loss of vision. Arentsen[190] found that 45% of his patients with endothelial rejection had a Khodadoust line. In the other type of endothelial rejection, diffuse keratic precipitates are scattered across the donor endothelium although often they are not readily visible. The resultant diffuse damage to the endothelium in turn leads to diffuse graft oedema[88,99,191]. It is not known what factors are responsible for the two types of rejection. It has been suggested that the vascularization associated with the rejection line acts as a route for the afferent and efferent arcs of the rejection process. In the diffuse type it is suggested that the immunological route is via the anterior chamber. Thus differing routes of immunological contact may be responsible for the differences seen clinically[180]. The reported incidence of endothelial rejection varies, partially because the studies used different immunosuppressant regimens and also because the study groups contained different proportions of high-risk cases. Gibbs et al[158] reported an incidence of 44%, Offret et al of 30%[197] and Polack of 12%[198]. Alldredge and Krachmer[88] in their series reported an incidence of endothelial rejection of 21%, with an average onset at eight months and a range of two weeks to 29 months. The incidence of

rejection was higher in younger patients (less than 50 years), a finding similar to that found in the other types of rejection. A higher incidence of rejection was also found in vascularized corneas, this finding is discussed in the section on high-risk keratoplasty.

Often the clinical picture of rejection does not fall neatly into any of the above groups, and there is frequent overlap between the types of rejection. Furthermore, one type of rejection may precede another; epithelial rejection or subepithelial infiltrates may be followed by the more severe endothelial type of rejection[88]. Isolated keratic precipitates or fine stromal oedema should be regarded as signs of early rejection and treated accordingly unless another diagnosis is apparent[190].

It is often difficult to differentiate the graft oedema associated with rejection, from other causes of endothelial cell loss, especially in the absence of other signs of rejection such as inflammation or the typical Khodadoust line. This can be particularly important in herpes simplex keratitis, a common reason for keratoplasty. In disciform keratitis there is inflammation, with graft oedema and keratic precipitates. In herpetic uveitis endothelial dysfunction is common and leads to graft oedema. In these cases a careful search must be made for a rejection line and if there is any doubt as to the diagnosis then corticosteroids and antivirals must be given. Other conditions which may be confused with rejection include sterile or infective endophthalmitis, in this condition the inflammation is usually more severe and a hypopyon and vitreous infiltrate may be present, signs that are rare in rejection[181]. The line found in epithelial downgrowth may cause confusion; however, the accompanying signs of clumps of material in the anterior chamber, an iris membrane, and severe glaucoma should help in the diagnosis.

Because of the difficulties sometimes encountered in the diagnosis of rejection, Stulting et al[167] have suggested a terminology for rejection episodes. A ***definite*** rejection episode is defined as one producing corneal oedema and an endothelial rejection line, in a previously clear graft. A ***probable*** rejection episode is one with

corneal oedema and inflammation (stromal infiltrate, keratic precipitates, cells in the anterior chamber, or ciliary injection) without a rejection line in a previously clear graft. A ***possible*** rejection episode is one with diffuse oedema (often occurring gradually) with no signs of inflammation or other diagnosable cause. If a graft never became clear after operation it should be regarded as a primary failure.

2.4 High-risk Keratoplasty.

A number of factors are known to be associated with a higher incidence of graft failure. In this section I will concentrate predominantly on factors that are known to influence the incidence of graft failure from immunological rejection. Recipients that have one or more of these adverse factors are regarded as being at high-risk and special measures may be required in an attempt to reduce the high incidence of rejection. The exact definition of high-risk has not been universally agreed, but recently some consensus has been emerging.

Vascularization

A number of studies have documented the increased incidence of rejection and reduced graft survival in recipient corneas that are vascularized[160,162,163,187,199-201]. Moreover, the degree of vascularization also appears to influence the outcome[88,190,202]. In avascular corneas the incidence of failure from rejection is reported to be between 0 - 10% within the first year compared to heavily vascularized where immunological failure occurred in 25 - 50%[162,163,199,201]. In a series of 400 grafts, Khodadoust[191] classified the incidence of rejection according to the degree of vascularity. In avascular corneas the incidence was 3.5%, in mildly vascularized corneas 13.3%, in moderately vacularized 28% and in heavily vascularized corneas 65%. The overall incidence was 23%. Gibbs et al[158] also found a higher incidence in vascularized corneas and also noted that the reversal rate of the rejection episodes was lower in these patients.

The type of vessels may also be important, most investigators feel that superficial vessels are less important than deep stromal vessels[88,180]. Possibly these latter vessels are accompanied by lymphatic vessels which may provide a more direct route for the afferent and efferent arcs of the rejection cycle[203-205]. There may be other associated factors that occur in conjunction with vascularization: these include previous graft rejection, persistent epithelial defects, infections and chemical

injuries, all of which have a negative effect on graft survival. In the animal model for high-risk keratoplasty, presented later in this thesis, corneal vascularization is induced by corneal sutures. The presence of vessels leads to a high failure rate from rejection. However, our studies showed that vascularization was associated with an increase in the number of Langerhans cells (LC) and if these were reduced, e.g. by ultraviolet irradiation, the failure rate fell to that found in nonvascularized corneas. This indicates that the increased number of LC associated with vascularization were possibly a more potent cause of rejection than the vessels themselves.

Most of the studies on vascularization and rejection are concerned with the pre-operative condition of the recipient. Less data exists regarding the effect of postoperative vascularization on rejection. Thicker sutures and some materials such as braided silk are more prone to cause vascularization, compared to finer sutures and materials such as monofilament nylon[206]. Loose sutures are potent causes of vessel ingrowth and should be removed; this is easy with interrupted sutures but may be a problem with a continuous suture, especially if part of the wound appears unhealed. In vascularized corneas, interrupted sutures should be used to allow individual sutures to be removed if vessels begin to invade the graft[207]. It is important to bury the knots of sutures as this is less likely to induce new vessels, compared to sutures with exposed knots[180]. In corneal grafts for herpetic keratitis, the use of interrupted sutures has resulted in increased survival by reducing both rejection and recurrent viral infection[208]. Other factors can cause postoperative neovascularization, including the use of contact lenses[209-212], trauma, infections, and persistent epithelial defects associated with dry eyes or previous chemical burns.

It is difficult to quantitate the number of vessels in a cornea. In most pre-operative assessments only stromal vessels and only those that are likely to reach the recipient-donor interface, are considered. Usually the cornea is mentally divided into quadrants and the number involved estimated. However, some observer judgement is required as vessels lying close to each other should not be classified as

being in separate quadrants (figure 1, page 54). Recent studies such as the collaborative corneal transplantation study (CCTS) into the role of tissue matching in high-risk keratoplasty[108] and the collaborative topical cyclosporine study in high-risk keratoplasty have both used the criteria of two quadrants of corneal vascularization and/or previous graft failure from immunological rejection, to constitute high-risk in keratoplasty.

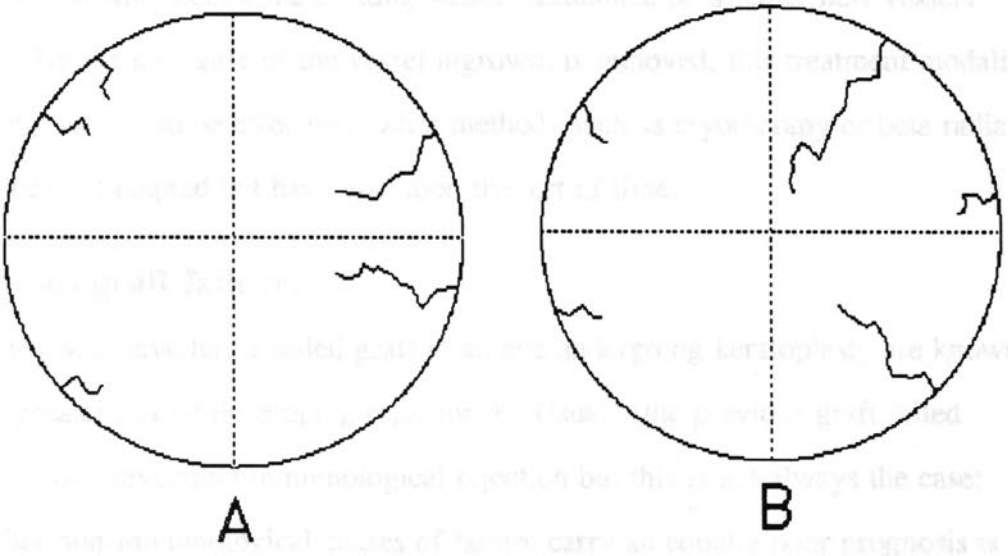


Figure 1: Assessment of corneal vascularization.

In the cornea on the left (A), the peripheral vessels are ignored, the two vessels reaching the proposed graft junction are close together and therefore the cornea would be classified as having one quadrant of vascularization. In B the vessels are separated and this cornea is regarded as having two quadrants of vascularization.

Apart from measures to alleviate the causes of neovascularization, little can be done to reduce the number of vessels. Argon laser ablation has been attempted both pre- and post-operatively but although the vessels are closed they invariably recur[213,214]. It is not known whether the existing vessels recanalize or whether new vessels occur. Unless the cause of the vessel ingrowth is removed, this treatment modality does not appear to be effective. Other methods such as cryotherapy or beta radiation have been attempted but have not stood the test of time.

Previous graft failure.

Patients who have had a failed graft in an eye undergoing keratoplasty are known to be at greater risk of developing rejection[180]. Usually the previous graft failed because of irreversible immunological rejection but this is not always the case; whether non-immunological causes of failure carry an equally poor prognosis is unknown. Few studies exist regarding graft survival in second grafts[160-163], in one study the survival rate in first grafts was 83.4% compared to 74.6% in second or subsequent grafts, the difference was statistically significant[162]. Whether the higher failure rate is a result of immunological sensitization or other factors is not clear. Certainly graft failure is often associated with vascularization or other factors such as anterior synechiae[195], which are risk factors in their own right. As mentioned above, the degree of vascularization strongly correlates with the survival of corneal grafts. The quasi importance of transplant number is due to the strong correlation between this factor and the degree of recipient cornea vascularization which increases with each successive transplant[215]. Likewise the apparent importance of indication for grafting is also a confounding effect of recipient vascularization, as there is a strong inverse correlation between corneal diseases with a good prognosis and the degree of vascularization[215]. Apart from these associated risk factors, it is possible that patients are sensitized to previously transplanted antigens; the resultant rejection would therefore be a second set reaction. However, the number of HLA antigens are large and the chances are small that an antigen is transplanted a second

time, unless the patient has had multiple grafts[216,217]. Sensitization from other sources of antigens, such as pregnancy or blood transfusions, may be important although obviously this would also affect first time grafts. However, Völker-Dieben et al[162,163] reported that neither of these potential methods of sensitization has an adverse effect on the incidence of rejection in grafts in normal or vascularised corneas. Khodadoust and Silverstein[79] have shown in rabbits, that even if an animal is systemically sensitized to a transplanted antigen, rejection does not invariably occur. This indicates that the efferent arc of the rejection cycle is unable to operate, emphasising the importance of avascularity of the cornea in maintaining immunological privilege. A previously rejected graft with vascularization has lost this immunological privilege and is therefore more likely to undergo a rejection episode. In clinical practice it would be expected that a keratoplasty patient with lymphocytotoxic antibodies, would benefit from not having a graft, with the corresponding antigens, transplanted. Therefore, with direct crossmatching of patient and donor to exclude the presence of these antibodies, a negative result should be beneficial, whereas a positive result should indicate a higher chance of subsequent rejection. However, the recently published Collaborative Corneal Transplantation Study (CCTS)[108], has shown no significant difference in the number of rejection episodes or graft survival, in high-risk keratoplasty patients with positive or negative crossmatches.

The evidence, therefore, indicates that the high failure rate in second and subsequent grafts may be related to the associated factors such as preoperative corneal vascularization rather than to immunological sensitization. However, previous graft rejection is still generally regarded as a risk factor in its own right and two recent studies, the CCTS in high-risk keratoplasty[108] and the collaborative topical cyclosporine study in high-risk keratoplasty have both used previous graft failure from immunological rejection as one criteria for defining a keratoplasty

patient as being at high-risk. In this thesis I have used similar criteria to these two studies to define high-risk keratoplasty, namely:

Deep stromal vascularization in two or more quadrants.

and/or

Previous graft failure from immunological rejection.

These criteria were used to maintain conformity and to allow comparison with other studies. However, these criteria were not regarded as severe enough when considering high-risk patients for systemic cyclosporine (CSA). In these patients high-risk was defined as:

Three or four quadrants of stromal vascularization.

and

>16 vessels reaching the proposed donor site.

This stricter definition is dependant on the degree of vascularization of the recipient cornea, and ignores previous grafts; although the majority of the patients given CSA had had previously rejected grafts in the affected eye.

Bilateral grafts

The question as to whether a graft in the second eye will lead to rejection in either the first or second graft has not been resolved. Donshik[186] reported that in patients that had grafts for keratoconus, patients with unilateral grafts had a 13% incidence of rejection. In patients that had bilateral grafts the incidence was 27%, but interestingly the incidence was not uniform. In the first graft, before the second graft was performed, there were no rejection episodes until the second graft and then the incidence was 16.7%. The incidence in the second graft was 38%; the statistical significance was not reported, but the second graft would appear to be at greater risk. Although some studies have reported a greater risk[218], most recent studies have reported no increased risk of rejection in either eye after the second

graft[88,187,190,219] and no decrease in graft survival[220]. There is, therefore, no strong evidence that the first graft can be adversely affected by a second graft in the fellow eye, and consequently a second graft should not be withheld for this reason.

Other risk factors in high-risk keratoplasty exist but are not well documented. Alldredge and Krachmer found that all types of rejection were more common in younger patients, defined as those under the age of 50 years[88]. This may be attributable to the more active immune system in younger patients or conversely the immune senescence found in the older population. Mention has been made before of the worse prognosis associated with large grafts or those that approach the limbus. It has been suggested that this is a result of the close proximity to the limbal vessels, but no data exists to support this suggestion[181]. Another suggestion is that the graft is being placed in that part of the recipient bed that contains the most Langerhan's cells[116,150]. Arentsen[190] reported a higher incidence of rejection is grafts with postoperative synechiae, and suggested that they should be broken down whenever possible because they lead to vessel ingrowth, and rejection lines can be seen to originate in the area of the synechiae.

2.5 Treatment Of Graft Rejection.

Corticosteroids, administered locally and systemically, remain the mainstay in the treatment of graft rejection. Although one standard regimen is not in universal use, most studies use similar dosage regimens of locally administered corticosteroids[13,88,180]. Some authors rely entirely on the use of local steroids whereas others use oral corticosteroids for severe or advanced rejection[88,184,190,221]. Epithelial, subepithelial, or stromal rejection is not usually associated with graft loss unless there is also endothelial involvement; although stromal scarring with some loss of vision can occur[88]. These forms of rejection do, however, indicate that recipient sensitization has occurred and may be harbingers of impending endothelial rejection. Therefore, all episodes of rejection should be treated vigorously as early as possible. In a questionnaire review of the members of the Castroviego Corneal Society performed recently[222], the treatment preferences of surgeons were presented. For subepithelial infiltrates and epithelial rejection 93% of surgeons gave topical steroids (dosage range was 0 to 24 drops per day), approximately 10% gave subconjunctival steroids and approximately 10% gave systemic steroids. For endothelial rejection 100% gave topical steroids (dosage range 4 to 24 drops per day), approximately 55% gave subconjunctival steroids and approximately 35% gave systemic steroids. It is apparent that the intensity of treatment given for rejection varies tremendously between surgeons. The following regimen has been suggested by Wilson and Kaufman[180], and is similar to regimens advocated by other studies[13,88,184]. For non-endothelial types of rejection topical 1% prednisolone acetate or 0.1% dexamethasone sodium phosphate drops are given six times daily for approximately a week and then tapered of over a period of 3 to 5 weeks. For endothelial rejection the drops are given hourly during the waking hours with dexamethasone sodium phosphate 0.05% or betamethasone 0.1% ointment at night. In addition a subconjunctival injection of 2 mg dexamethasone sodium phosphate is given. The drops are administered hourly for three days, then two hourly for several

days and then slowly tapered off over several weeks or months. The ointment is usually stopped after two or three weeks. If there is little response to treatment a repeat subconjunctival injection can be given after two or three days[184]. As an alternative to subconjunctival injections, it has been suggested that collagen shields in conjunction with corticosteroid drops be used, the shield acting as a reservoir for the drug[223]. In aphakic patients without steroid induced glaucoma it has been suggested that long term topical steroids be used on a daily basis for patients with vascularization or other risk factors[180]. In alkaline burn and other high-risk patients, higher maintenance doses of topical steroids may be required[184,224].

Although some authors use only topical corticosteroid drops[160,180,208], others treat the more severe rejection episodes involving the endothelium with systemic steroids[88,184,190]. In a recent study Boisjoly et al[160] reported that 17 of 23 grafts (73.9%) that developed a single episode of endothelial rejection failed when only topical corticosteroids were used. We have also found that the use of topical corticosteroids alone, in cases of definite and probable rejection, has a low success rate (39%) in reversing graft rejection (unpublished data). Because of the lower success rates in endothelial rejection many authors prefer to give systemic corticosteroids to these cases providing that there are no contraindications. The usual dose is 60-80 mg daily (depending on body weight) for three days, tapering off over a week or two. Although corticosteroids remain the mainstay in the treatment of graft rejection, these preparations are frequently ineffective especially in high-risk cases[225,226], and serious complications may result from their use. Later in this thesis the use of a single 500 mg intravenous pulse of methylprednisolone for the treatment of endothelial rejection is discussed. This form of corticosteroid administration appears to be at least as effective as oral administration and avoids the potential side-effects of prolonged oral medication.

In exceptional cases other immunosuppressant agents such as azathioprine (Imuran)[227-232] have been used to help prevent rejection and to treat rejection

episodes when they occur. But the results show little advantage over corticosteroids and their use requires great care because of potentially serious side-effects. Recently, interest has been shown in the use of cyclosporine (CSA), used either topically or systemically, to help prevent rejection. The role and actions of CSA are described in a later section of this thesis.

Regimens for reversing graft rejection have variable success rates, with values of between 50 - 91% being reported. This variation results from the studies reporting rejection reactions of different severity, using different routes of corticosteroid therapy depending on the severity of the immune reaction, the degree of corneal vascularization and other risk factors, and the delay in presentation and treatment[78,88,158,191,225,233-235]. Fine and Stein[225] found that in avascular corneas the reversal rate was 67%, but in vascularized corneas only 50% of rejection episodes were successfully reversed. In cases of previous graft rejection it is possible that the recipient has already been sensitized to a transplanted antigen, a subsequent rejection could then be "a second set reaction" which may become established quicker and be more difficult to reverse. However, this probably does not occur and the poor prognosis is more likely to be related to the vascularization associated with the previous rejection episode[181]. Furthermore, it has recently been shown that the presence of circulating antibodies (indicating prior sensitization) is not associated with a higher graft failure rate[108].

An extremely important aspect of rejection management is patient education and awareness of the advantages in treating rejection early. Patients should be informed about the symptoms of rejection and encouraged to report them early. Particular emphasis should be placed on the three "danger signals" of rejection: namely redness, decreased vision and pain[181]. These symptoms should be reviewed with the patient at each visit, especially in the early postoperative period and urgent consultation should be encouraged if these symptoms occur and persist for more than 24 hours. This sort of patient education is thought to decrease the proportion of

rejection episodes that result in graft failure[181]; in the recently published collaborative corneal transplantation study[108] good patient education was thought to be responsible for the lower rejection and failure rates found in this study compared to previously published results. In our studies using a pulse of corticosteroid for treating rejection, patients that presented early (within 8 days of onset of symptoms) did significantly better in terms of graft survival compared to patients who presented later; these results are presented later in this thesis.

Section Three: The Role Of Tissue Typing In Preventing Corneal Graft Rejection.

3.1 Introduction.

3.2 Results from other centres.

3.3 Clinical studies.

3.1 Introduction.

The absence of rejection in autografts and in grafts between identical twins suggests that the most effective method of preventing rejection would be to graft tissue with an identical, or as near identical as possible, complement of HLA and other transplantation antigens. The high success rate of unmatched grafts in corneal transplantation, especially in avascular corneas, led to the concept of the cornea having immunological privilege. We now know that this privilege is not absolute and that the original beliefs that the cornea was non-antigenic and the amount of tissue transplanted in keratoplasty was insufficient to evoke an immune response, are both untrue. The immunology of rejection has been discussed earlier in this thesis and mention was made that the cornea has both class I and class II antigens and is able to evoke an immunological response when transplanted.

Tissue typing is performed to determine which HLA antigens a patient genetically possesses. The procedure involves using the microlymphocytotoxicity complement-dependent test introduced by Terasaki and McClelland[236] in 1964. In this test lymphocytes are separated from the patients blood by density-gradient centifugation. T lymphocytes express class I antigens and B lymphocytes express both Class I and Class II antigens on their cell surfaces. To determine class I antigens the patients T lymphocytes are tested against a large panel of known antisera which define each of the HLA alleles. Likewise class II antigens are tested by using B lymphocytes against a panel of known antisera. After incubation, in the presence of complement, the number of cells that have been lysed are counted and a high number indicates that the corresponding antigen is present on the cell surface. Although tissue typing is now routinely done in many laboratories, the test is expensive and time consuming. Obtaining fresh lymphocytes from patients is relatively easy but from cadavers this may present a problem. Waiting for the tissue typing to be done, then selecting a patient, and arranging for surgery all involve delays; however, with the improved methods of corneal storage available today the delays should not be

detrimental to the graft. The best source of tissue typed corneas come from donors of kidneys and other organs and the tissue typing is known before the organs are harvested. To obtain these corneas cooperation with the transplantation team and in particular the transplant coordinating sister is essential.

Another test used clinically in transplantation is crossmatching. Sensitization of a patient to either class I or II antigens can result in circulating antibodies[34,76,237]. Sensitization can result not only from previous transplants, but also from blood transfusions and pregnancies. In renal transplantation the presence of these circulating antibodies can result in hyperacute rejection[238]. In this condition antigen-antibody complexes are deposited in the blood vessels of the donor kidney, leading to lack of circulation in the graft and usually graft death. In renal transplantation it is mandatory to detect these circulating antibodies and this is done by crossmatching. The technique is similar to that used for tissue typing, namely the microlymphocytotoxicity test. The patients serum is tested against a panel of typed lymphocytes to detect specific antibodies. If one only needs to know whether antibodies are present, then lymphocytotoxic antibody screening can be performed using the Amos modified method on a screening panel of cells from at least 50 donors[239], if the patients serum is consistently cytotoxic to at least 20% of cells in a panel, then antibodies are considered detectable. The patients serum is also stored frozen and tested against the lymphocytes of a potential donor to see if there has been prior sensitization, this test is called the direct crossmatch. The role of circulating antibodies in corneal grafting is not known and has been discussed previously; however, their presence may indicate prior sensitization and may be a marker for cellular sensitization[240,241]. Certainly hyperacute rejection cannot occur in corneal grafting as the donor tissue is avascular[116]. Stark et al[216,242] have reported good results using negative crossmatched grafts, but the recently published collaborative corneal transplantation study[108] has not confirmed these findings. This and other clinical studies are discussed in the following section.

3.2 Results From Other Centres.

There have been many studies published on the effect of HLA antigen matching on corneal graft rejection and graft survival. The conclusions reached by these studies are difficult to interpret as the studies are limited by one or more of the following factors: small numbers of patients, retrospective designs with poor degrees of HLA match, lack of appropriate controls, variable follow-up and inappropriate statistical methods[180]. Many of the studies include variable numbers of patients regarded as being at high-risk, so it is difficult to compare studies. Indeed some studies only include these high-risk patients, because smaller patient numbers are required to study the effects of HLA typing, as the rejection rate is higher and any statistical significance would be achieved earlier. Another reason is that tissue typing is often reserved for these high-risk patients in the hope that matching will improve the prognosis. The length of follow-up is an important factor; although rejection occurs more frequently in the early postoperative period the graft never stops being at risk of rejection. Therefore, in designing studies it is important, as Siegel[243] has pointed out, that either the follow-up periods are kept identical for the groups being compared or else actuarial analysis methods must be used[244-248]. Many of the studies discussed below have not used either of these statistical methods.

A few studies have found no benefit from HLA matching, both in respect of graft rejection and for graft survival[109,216,217,249]. Other studies, however, have reported some benefit of HLA matching and in some reports the incidence of graft rejection and graft failure seemed to correlate with the degree of HLA matching[161,162,188,189,199,201,234,242,250-257]. It can be seen that numerically more studies support the hypothesis that HLA matching offers some benefit in corneal grafting. However, recently the results of *The Collaborative Corneal Transplantation Studies* (CCTS) have become available[108]. This was a double-masked prospective study to investigate the effect of donor-recipient histocompatibility matching and crossmatching on the survival of corneal transplants in high-risk patients. The

results were analysed using survival probability estimates and the study concluded that HLA matching did not offer any statistical benefit in high-risk keratoplasty. Before this study is discussed in more depth, some of the earlier HLA matching studies will be reviewed. In many of these earlier studies, emphasis was placed on matching class I antigens, especially HLA-A and -B. Nowadays workers in other fields of transplantation initially try to match HLA-DR antigens[258,259], preferably with a HLA-B match as well. Although sometimes amounting to the same result, most workers now prefer to refer to the number of antigen mismatches rather than the number of matches.

A large series of tissue matched corneal grafts has been performed at East Grinstead, England; 450 cases were matched over a 14 year period[116,119,158]. The patients were graded as low (n=142), medium (n=156) or high risk (n=152) according to the degree of pre-operative vascularization and the presence of a previous rejected graft. The statistical method was to look at graft survival at one and two year follow-up periods. Both the low and medium-risk patients did well. In the low-risk group graft survival rates were 88%, 94%, 91% and 100% at one or two years for 0, 1, 2 and 3 antigen matches, respectively. In the medium-risk risk patients survival rates were 76%, 77%, 90% and 100% at one or two years for 0, 1, 2 and 3 antigen matches, respectively. If the patients were grouped for 0 and 1 matches versus 2 and 3 matches, a difference of 76% versus 92% graft survival existed at one and two years ($p = < 0.1$). The high-risk patients fared worse: at one year graft survival was 65%, 75%, 74% and 92% for 0, 1, 2 and 3 antigen matches, respectively. At two years graft survival dropped to 52%, 51%, 74% and 69% for 0, 1, 2 and 3 antigen matches, respectively. Comparing patients with 0 or 1 antigen matches to those with 2 or 3 antigen matches, the graft survival at one year was 72% versus 76%, and at 2 years 50% versus 76%: the difference is significant ($p = < 0.01$). More recently Boisjoly et al[189] have shown in a prospective study of 438 patients, that grafts with 0 or 1 mismatched HLA-A or -B antigens had

a lower rejection rate than if there were 2 mismatched antigens. The study considered corneal vascularization and graft size and found that the chances of rejection in the 2 antigen mismatched group were double. Two mismatches resulted in a 60% survival at 36 months compared to 81% in 0 or 1 mismatch ($p = < 0.002$). Völker-Dieben et al[162], in a prospective study of 497 patients, found highly significant improvement in graft survival in HLA-A and -B matched grafts in both normal corneas ($p=0.0008$) and vascularized corneas ($p=0.0001$). These studies all point to a significant benefit from matching class I antigens in keratoplasty.

The results of HLA-DR antigen matching are not clear. Völker-Dieben et al[162], in their study quoted above, retrospectively looked at HLA-DR matching and found no significant effect. Likewise Beekhuis et al[260] found significant benefit from HLA-A and B matching but no benefit from HLA-DR matching. Boisjoly et al[189] gave priority to HLA-DR matching over HLA-A and B matching; overall HLA-A, B and DR matching improved prognosis but no specific conclusions could be made regarding the benefit of HLA-DR matching alone. Mayer and Casey[119] tissue matched 20 high-risk patients, priority was given to HLA-DR matching. Five patients had both DR antigens matched and 15 had a single DR match. Two of the grafts with both HLA-DR antigens matched rejected, one failed from other causes, and two remained clear, the authors felt that overall HLA-DR matching conferred some benefit although the numbers are too small to make any meaningful conclusions. Other studies have supported[188,257,261] or refuted[262] the importance of HLA-DR matching in keratoplasty.

Humoral immunity is not believed to play an important role in corneal graft rejection, however lymphocytotoxic antibodies may be important. These can be detected preoperatively by crossmatching patients serum to donor lymphocytes. The degree of cytotoxicity of recipient serum is indicative of the presence of preformed circulating antibodies to donor antigens; a positive crossmatch would indicate previous recipient exposure and reaction to an antigen possessed by the donor.

Development of lymphocytotoxic antibodies has been demonstrated following corneal grafting[34] and other organ transplants[263-265], and it is rare to detect them without a prior history of sensitization[34]. Sensitization to HLA antigens, as evidenced by the presence of lymphocytotoxic antibodies, has a deleterious effect on renal transplants[264]. It is not known whether the same negative influence occurs in corneal grafting. A number of studies have suggested that a negative crossmatch improves graft survival[189,192,216,242]. Stark[192] found a lower incidence of rejection and failure in high-risk cases with negative crossmatch, compared to a similar group in which crossmatching was not done. Other studies in which HLA matching was undertaken in the presence of a negative crossmatch are difficult to interpret, some have suggested an improved prognosis[161,188] or no definite conclusion could be reached[266]. However, as all these studies used retrospective or historical controls and positive crossmatched controls were not included, no definite conclusions can be made.

Only a few studies exist on the role of ABO matching on corneal graft survival and these have produced mixed results. Nelken et al[267] in 1961 reported corneal clouding accompanied by high serum isoagglutination titres to donor ABO antigens. Mehri et al[268] and Mailäth[269] found a better graft survival when donor and recipient were ABO compatible and Allansmith[270] found a small, but not significant, decrease in failure rates with ABO compatible grafts. Völker-Dieben and co-workers[163,271] reported that O recipients who received an A donor cornea had a better graft survival after one year (89%) as compared to A recipients who received an A graft (74%), the difference was not significant; other combinations of mismatch showed no difference in graft survival. It has been suggested that non-O ABO blood groups, or genes linked to them, can exert a significant, adverse effect on graft survival especially in vascularized recipients in whom a positive crossmatch or HLA mismatches exist. This finding has also been reported in cardiac and renal transplants[215]. Foulks and co-workers[272] have reported that in high-risk patients

ABO incompatibility resulted in a greater chance of graft rejection, however, the group was too small to draw conclusions. Most other studies have shown that ABO incompatibility has little effect on graft prognosis[161,199,266,273].

The reports outlined above have attempted to evaluate the effect of HLA matching, crossmatching, and/or ABO compatibility on preventing rejection episodes and irreversible graft failure from rejection. The results have varied and interpretation has been difficult because of the use of historical controls, variable numbers of low and high-risk recipients, differences in follow-up times, unmasked postoperative assessment and treatment, variable and often low postoperative steroid usage, and different end-point criteria with some studies taking any rejection episode and others graft failure from rejection as the end-point. Therefore, despite considerable research into the efficacy of antigen matching and crossmatching, the role of these factors in the management of keratoplasty is still uncertain[274].

However, most studies seem to indicate that antigen matching and probably crossmatching, are beneficial. Conversely ABO compatibility does not seem to be an important factor. To initiate a program of tissue matching would be expensive and would result in extended waiting periods for patients needing surgery. To clarify the role of tissue matching in high-risk keratoplasty the Collaborative Corneal Transplantation Study (CCTS) was initiated in 1985 and commenced the following year. The object was to answer the following:

1. Does matching of HLA-A and -B antigens reduce the risk of immune reactions and irreversible rejection among high-risk patients?
2. Does matching of HLA-DR antigens reduce the risk of immune reactions and irreversible rejection among high-risk patients?
3. What are the joint effects of matching for HLA-A, -B and -DR antigens?
4. Is a negative donor-recipient crossmatch important?

5. Is ABO blood group compatibility important?

In addition, the role of a number of potential risk factors and the development of posttransplantation lymphocytotoxic antibodies were of secondary interest[274].

To answer these questions posed by the CCTS research group, two separate studies were initiated [274]:

A. The antigen matching study (AMS).

B. The crossmatch study (CS).

In both studies, patients at high-risk were recruited, these were defined as patients with corneas having two or more quadrants of stromal vascularization and/or a history of allograft rejection in the study eye.

The antigen matching study.

The AMS was designed to assess the effect of HLA-A, B and DR antigen matching on graft failure from rejection in high-risk patients who had no detectable lymphocytotoxic antibodies. It was a prospective, double-masked, nonrandomized clinical trial of patients assigned corneas with different degrees of HLA matching. A small proportion (initially 10%, later 20%) were randomly selected to be controls and were given the next available cornea, this would most likely be one of low match. The remaining patients were given a high antigen match which was either a 3 or 4 HLA-A or -B antigen match or a double HLA-DR match. If a patient was not allocated a cornea by 8 months they were allotted a medium antigen match of either 2 or more HLA-A or -B antigens. If a patient had not been allocated a cornea by 12 months the next available cornea was allocated. The total number of patients in this study was 419, of which 137 (33%) were in the high HLA-A and -B match group and 199 (47%) were in the high HLA-DR match group[108].

Outcome for HLA-A and -B matching. The incidence of graft failure, *from any cause*, was similar in the high and low HLA-A and -B matched groups; at three years the failure rates were 33% and 37% respectively. The rates of graft rejection

episodes were also similar with values at three years of 64% and 66% respectively. The rates of graft failure *from rejection* at three years were 21% and 26% respectively (p=0.34).

Outcome for HLA-DR matching. The incidence of graft failure, *from any cause*, was nearly identical in the high and low HLA-DR matched groups. The proportion of grafts having a rejection episode were also similar. The rates of graft failure *from rejection* at three years were 25% and 24% respectively (p=0.6).

Outcome for ABO compatibility. The ABO incompatible group had slightly more graft failures, *from any cause*, than the ABO compatible group; at three years the failure rates were 41% and 34% respectively (p=0.5). The rates of graft rejection episodes were similar with values at three years of 68% and 64% respectively. The rate of graft failure *from rejection* at three years was greater in the ABO incompatible group, with values of 30% and 22% respectively (p=0.004).

Crossmatch study.

In the crossmatch study (CS) patients were entered if, on initial entry into the CCTS, they were found to have lymphocytotoxic antibodies. They were then randomly assigned to having either a positive or negative crossmatch cornea. The positive and negative groups had a similar incidence of graft failure from any cause; at two years the failure rate was 36% and 45% respectively. The rates of graft rejection episodes were also similar with values at two years of 55% and 61% respectively. The rates of graft failure *from rejection* at two years appeared much higher in the negative group, the values being 16% and 38% respectively, but the difference was not statistically significant (p=0.33).

It can be seen that the results of the CCTS[108] differ from the consensus of results from previous studies, and in this respect are surprising. There was no evidence of a large beneficial effect of HLA-A, -B or -DR donor-recipient matching on the rate of

graft failure, rate of rejection episodes, or on the rate of graft failure from rejection. Similarly crossmatching did not offer any advantage, and in fact a positive crossmatch seemed to correlate with improved graft outcome. One explanation is that CCTS used a more intensive topical immunosuppressive regimen than previous studies and any beneficial effect of HLA matching on graft survival may have been abrogated in the CCTS by postoperative steroid therapy. Another suggestion is that the degree of histocompatibility matching/mismatching may not have been accurately assessed. In the CCTS the ethnic composition of donor and patient groups was broader than many other studies, differences at the molecular level that cannot be detected serologically can lead to graft failure[275]. Other antigens, such as the HLA-DQ and -DP antigens and minor histocompatibility antigens, may play an important role in corneal graft rejection. The lack of benefit of HLA-DR matching on graft survival can be explained by the minimal class II antigen expression by non-inflammed corneas (aided in the CCTS by the intense postoperative steroid regimen), compared to vascularized organs which demonstrate improved survival with HLA-DR matching.

The finding that a positive crossmatch did not adversely affect graft outcome was another surprise finding. Solid organ transplants induce cellular immunity and antibody formation, and these effectors predict the accelerated rejection of subsequent grafts. However, not all encounters with alloantigenic tissues evoke antibody formation with accelerated graft rejection. In humans there is evidence that pretransplant blood transfusions impairs subsequent kidney graft rejection and promotes acceptance. As discussed in an earlier section, the presence of allogenic antigens in the anterior chamber of the eye appears to elicit specific tolerance through the suppression of delayed-type hypersensitivity[89-93]. This response, anterior chamber associated immune deviation (ACAID), appears to be an active response rather than just immunologic ignorance of the antigens existence, and appears despite the concurrent stimulation of cytotoxic T lymphocytes and the production of

circulating antibodies[95]. This process requires an intact spleen[89] and is thought to be mediated by direct exposure to the venous system via the aqueous outflow through the canal of Schlemm. Antigen processing by this route appears to bypass the lymphatic system where destructive responses are initiated. Presumably antigens could gain access to the anterior chamber from a corneal graft and this mechanism could theoretically play a role in maintaining the immunological privilege of the cornea. Many of the patients in the CCTS had had previous exposure to antigens from previous grafts and possibly this type of tolerance could have been induced. However, the main effect of ACAID in producing tolerance is down-regulation of systemic DTH, and as this does not play a role in graft rejection ACAID is not thought to be important. However, it has been recently reported that orthotopic corneal allografts elicit a deviant systemic immune response in mice that correlates positively with long term graft acceptance[96]. Patients in the CCTS with a positive crossmatch, indicating prior sensitization, had fewer corneal graft failures from rejection; however, the differences were not statistically significant.

The beneficial effect of ABO matching on graft survival, resulting from a lower failure rate from rejection, was a surprise finding in the CCTS. Further statistical studies on the data in which other risk factors were controlled, indicate that the effect of ABO matching was not an artifact. ABO incompatibility is associated with higher transplant rejection rates in vascular organs such as the kidney, heart and liver. The mechanism by which ABO incompatibility increases the rate of rejection in the cornea is not clear. Although these blood group antigens have been found in the cornea, as discussed previously, recent reports show that they are only expressed by the epithelium. Being carbohydrates, it is unlikely that A and B group blood antigens themselves are the molecular targets of T lymphocytes as these cells have surface receptors that recognize peptides. However, the polymorphic cytoplasmic enzymes that create the A and B blood group carbohydrates may be the immunological targets. Because of the ubiquitous nature of the antigenic moieties of

the ABO system it has been shown that exposure to microorganisms can induce T cell *independent* antibody production. Therefore, incompatible A or B antigens do not require the participation of T cells to evoke an immunological response. Another possibility for the method of rejection, is that isohemaglutinins directed against ABH antigens may provoke an inflammatory response involving complement activation. This in turn would lead to macrophage migration and in turn to increased expression of alloantigens that would initiate T cell-mediated allograft rejection[108].

The results of the CCTS have a number of significant implications for corneal grafting. ABO matching is simple and cheap, and given that approximately 70% of donor recipient pairs match by chance, it is unlikely that matching would result in a significant delay for surgery for most patients. This can be compared to HLA antigen matching that is costly and involves considerable logistic problems and delay for patients awaiting a good match.

Another unexpected finding of the CCTS was the relative low incidence of graft failure, found to be 35% after three years. Previous studies have indicated a failure rate of up to 65%. The CCTS had estimated at the beginning of the study that there would be a failure rate from rejection of 45% at two years, whereas the rate was only 28% at two years. They attribute the relatively high success rate in the CCTS to the use of intensive topical steroid therapy postoperatively, close personal follow-up by the ophthalmologists and the study coordinators, excellent compliance and knowledge of the study medications by the patients, and the fact that patients were encouraged to report any unusual sign or symptom. Given the current state of knowledge of the mechanisms involved in corneal transplant failure, they argue that the most important conclusion to be drawn from the CCTS is that high-dose postoperative steroid therapy, good compliance, and close follow-up are the keys to successful corneal transplantation in the high-risk patient[108].

3.3 Clinical Studies.

Groote Schuur hospital serves a broad ethnic population of patients who often live in outlying areas and therefore find transport difficult. Because of these logistic problems and the fact that there is a diversity of HLA antigen genotypes and consequently a smaller chance of easily obtaining good matches, a regular tissue matching service for corneal transplantation has not been instituted. In the renal transplantation unit of this hospital an attempt is made to HLA match donors who are ABO compatible; preference is given to HLA-DR matching followed by HLA-B matching. However, because of the ethnic diversity of the population the average number of antigens matched is only 0.87 (Pontin, A: personal communication).

The corneas from multiple organ donors at this hospital, who have been tissue typed, are used when available for the patients first on our waiting list for corneal grafts. The corneas are therefore randomly assigned and as our patients have not been tissue typed, no matching is possible. Postoperatively the recipients are usually tissue-typed and the degree of matching is assessed, no crossmatching is undertaken. The results of the donor and recipient tissue typing is not recorded in the hospital notes, and therefore attending physicians are unaware of the degree of antigen matching. Since 1986, I have transplanted 104 tissue typed corneas, of these 13 were used for patients who also received systemic immunosuppression with cyclosporine and are therefore excluded from this analysis. The results of the remaining 91 grafts are presented.

Patients And Methods

The clinical details of the patients undergoing keratoplasty in this study are shown in Table I (page 77), it can be seen that they represent a broad cross-section of patients.

Table I

Tissue matching study: Clinical Details.

Mean age (range) years	40.7 (1 - 87)
Male / Female	42 / 49
Eyes with previous grafts	21
Vascularized corneas (2+ quadrants)	35
High-risk corneas	39
Low-risk corneas	52
Mean follow-up (range) months	42.5 (16 - 86)*

* this refers to the postoperative follow-up of clear graft only.

All the grafts were performed by myself, the keratoplasty technique was identical in all patients. All donor grafts were between 7.5 and 8.5 mm and were 0.25-0.5 mm larger than the recipient bed. Donor corneas were preserved as whole eyes, with the corneoscleral disc being removed just prior to grafting, or as discs preserved in culture medium (M.K. or KSol solution) at 4°C. A number of patients had simultaneous extracapsular cataract extraction, in most cases with placement of a posterior chamber intraocular lens. Sodium hyaluronate 1% (Healon) was used to protect donor endothelium and to dissect any iris adhesions. Sixteen buried interrupted 10/0 nylon sutures or a continuous 10/0 nylon suture were used to secure the graft. The anterior chamber was reformed with balanced salt solution at completion of surgery and sub-conjunctival injections of gentamicin 20 mg and

betamethasone (Celestone) 1.5 mg were given. Postoperatively all patients received topical dexamethasone 0.1% or prednisolone 1% drops four times daily initially, the frequency was reduced over the ensuing months and was stopped at 6 months unless persistent inflammation required extended therapy. Topical antibiotics and cycloplegics were given when necessary in the early postoperative period.

In the statistical analysis graft survival was compared by the Cox proportional hazard model[246] and log-rank analysis[247-8]. In the Cox proportional hazard model the following risk factors were considered:

ABO compatibility

ABC matching

DR matching

High or low risk status of graft

Outcome

Follow-up*

* This was defined as the period of follow-up of clear grafts or to the assigned end-point in the analysis,

Survival curves were calculated using an actuarial method.

Two studies were performed using two different end-points for outcome:

i) Graft failure study: the end-point was taken as graft failure from rejection.

ii) Rejection incidence study: the end-point was taken as the first (if any) rejection episode.

Results

Table II

Numbers of tissue matched corneas

Type of matching	Number	High Risk	Low Risk
ABO compatible	52	25	27
ABO incompatible	27	11	16
Data not available	12	2	10
HLA-DR 0 MM	11	4	7
HLA-DR 1-2 MM	80	34	46
HLA-A,-B 0-2 MM	24	11	13
HLA-A,-B 3-4 MM	67	27	40
HLA-DR + HLA-B *	35	16	19
HLA-DR + HLA-B >*	56	22	34

* HLA-DR 0 MM, or HLA-DR 1 MM and HLA-B 0-1 MM.

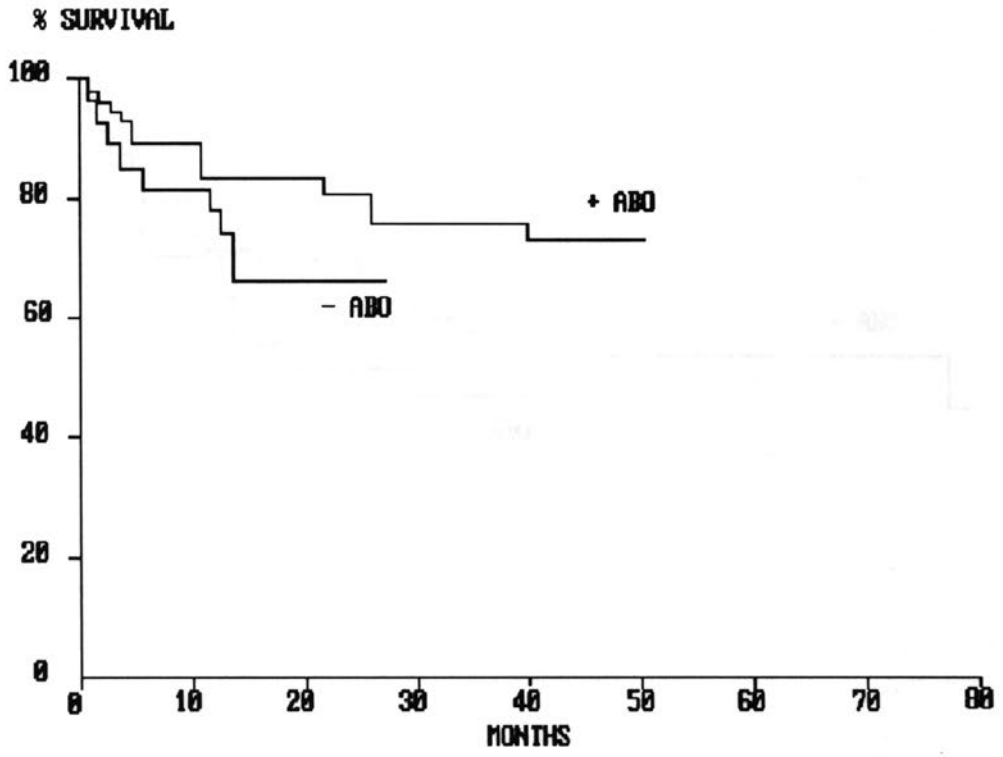
MM = antigen mismatch

ABO compatibility studies.

The ABO blood groupings of both donor and recipient were available for 79 grafts, 52 of these were ABO compatible and 27 incompatible. In the graft failure study ABO compatibility was not a significant factor in the Cox proportional hazard model. Graft survival curves are shown in figure 2 (page 81), the ABO compatible grafts appear to have improved survival compared to ABO incompatible grafts but logrank analysis indicates no significant differences in graft survival ($p=0.73$). If the grafts are divided into high and low risk groups then again ABO compatibility has no effect on failure rate ($p= 0.39$ and 0.25 respectively).

In the rejection incidence study, ABO compatibility was again not a significant risk factor in the Cox proportional hazard model. Survival curves are shown in figure 3 (page 82), the ABO compatible grafts have a lower incidence of rejection compared to ABO incompatible grafts but there was no significant difference on logrank analysis ($p=0.42$). When divided into high and low risk groups then again ABO compatibility showed no effect on rejection rate ($p= 0.25$ and 0.63 respectively).

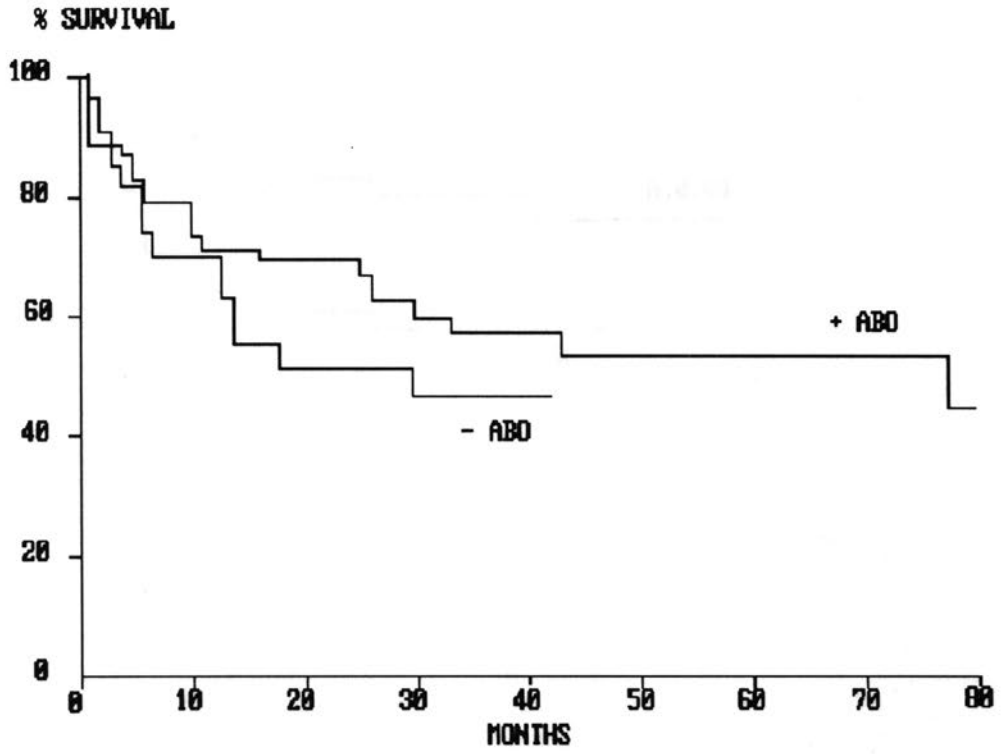
It has been reported that grafts with different blood groups have different survival rates. To investigate this the patients were divided into those with blood group O ($N=31$) and those with groups A, B, or AB ($N=43$). The failure-free survival curves are shown in figure 4 (page 83). It is seen that survival is worse in group O grafts and this difference is significant with logrank analysis ($p=0.05$). A similar result is found if rejection episodes are considered. The rejection-free survival curves are shown in figure 5 (page 84). The difference between the two groups does not reach statistical significance ($p=0.085$). The non-O group was divided into groups A ($N=24$), B ($N=15$) and AB ($N=4$). The failure-free survival curves are shown in figure 6 (page 85) and the rejection-free survival curves in figure 7 (page 86). In both it is seen that blood group A grafts appear to have the best prognosis but the differences between the non-O groups is not statistically significant.



+ ABO = ABO compatible graft.

- ABO = ABO incompatible graft.

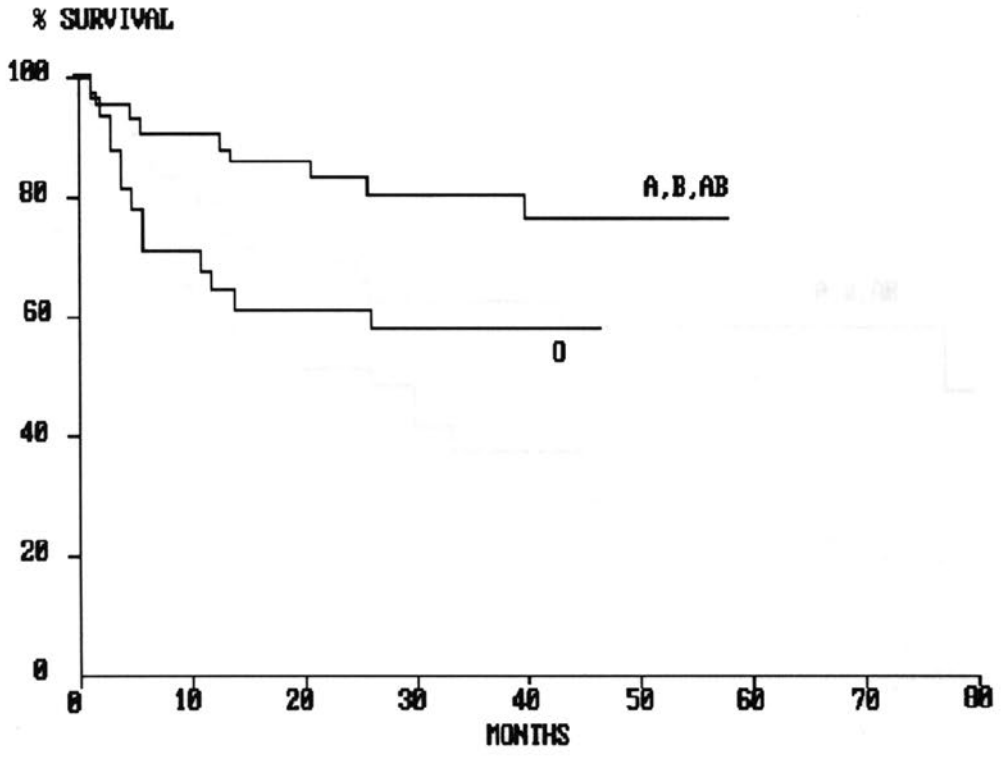
Fig. 2. ABO compatibility study: survival curves showing graft failure from rejection as the end-point.



+ ABO = ABO compatible graft.

- ABO = ABO incompatible graft.

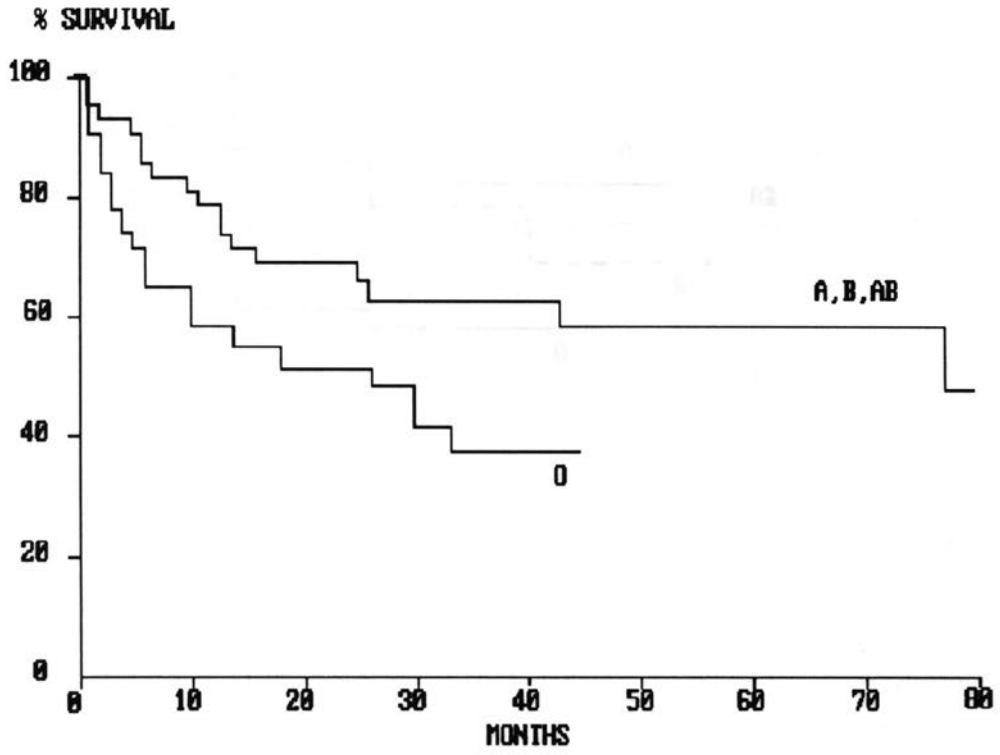
Fig. 3. ABO compatibility study: survival curve showing first graft rejection episode as the end-point.



O Grafts of recipients with blood group O.

A, B, AB Grafts of recipients with blood groups A, B or AB.

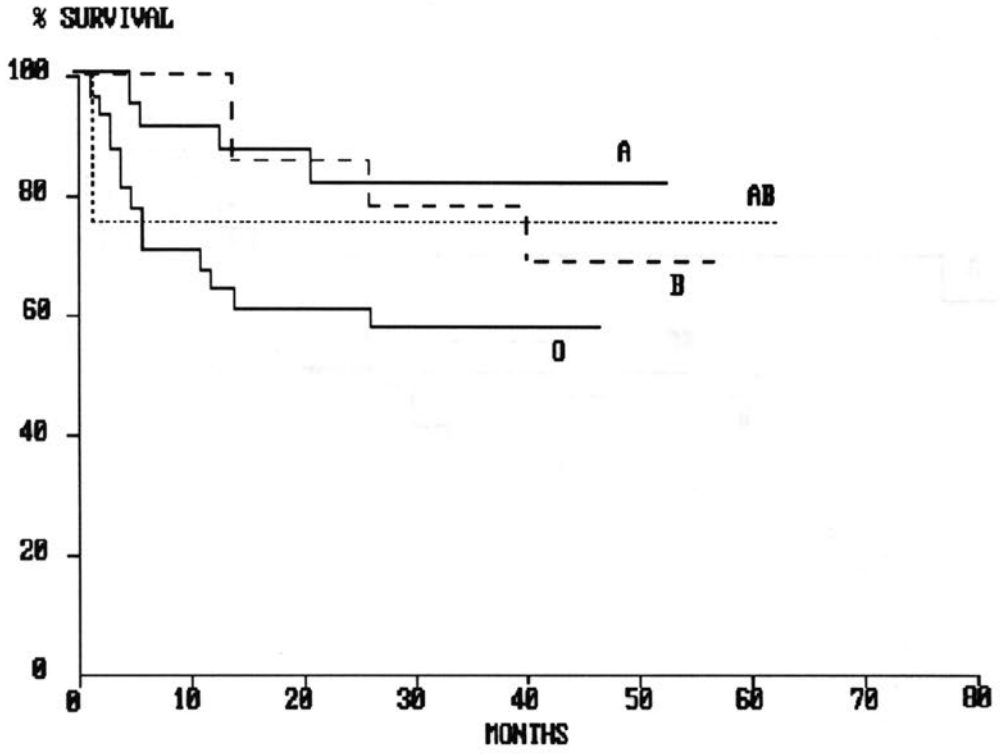
Fig. 4. ABO blood groups study: survival curves of group O versus groups A, B, and AB, showing graft failure from rejection as the end-point.



O Grafts of recipients with blood group O.

A, B, AB Grafts of recipients with blood groups A, B & AB.

Fig. 5. ABO blood groups study: survival curves of group O versus groups A, B, and AB, showing first graft rejection episode as the end-point.



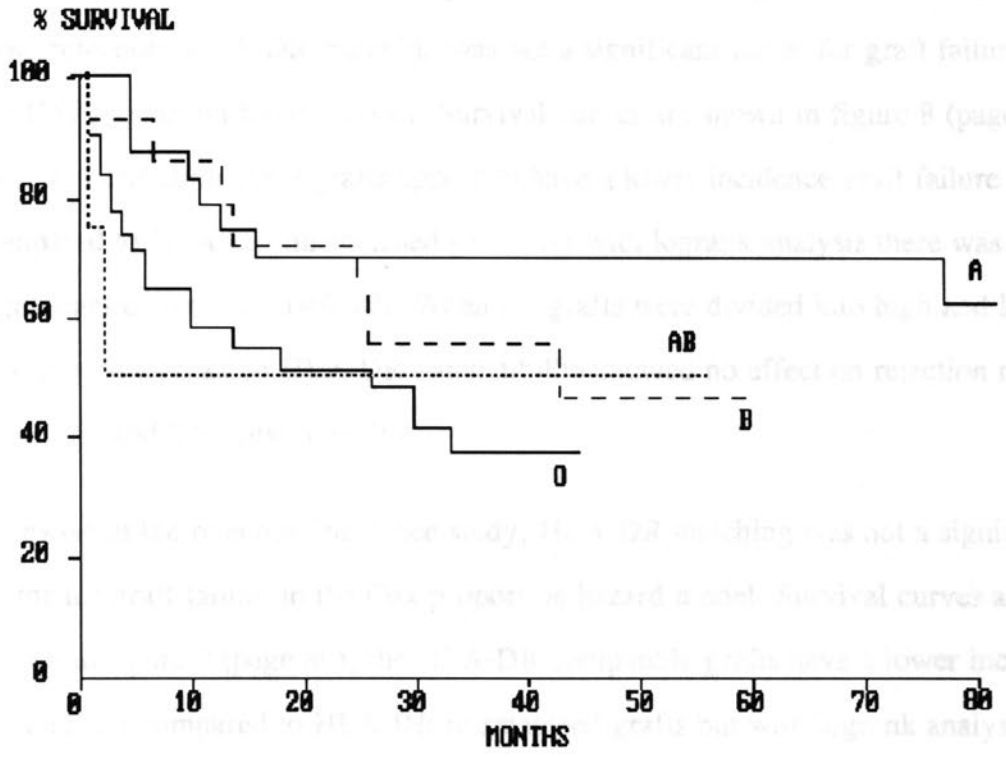
O Grafts of recipients with blood group O.

A Grafts of recipients with blood group A.

B Grafts of recipients with blood group B.

AB Grafts of recipients with blood group AB.

Fig. 6. ABO blood groups study: survival curves of groups O, A, B, and AB showing graft failure from rejection as the end-point.



O Grafts of recipients with blood group O.

A Grafts of recipients with blood group A.

B Grafts of recipients with blood group B.

AB Grafts of recipients with blood group AB.

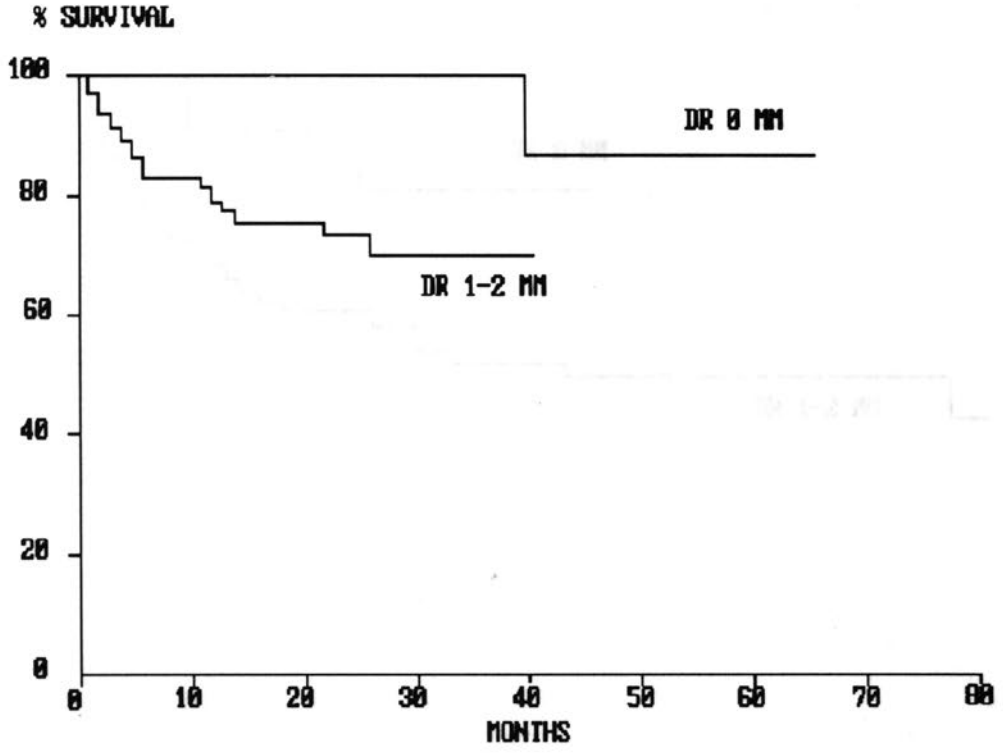
Fig. 7. ABO blood groups study: survival curves of groups O, A, B, and AB showing first graft rejection episode as the end-point.

HLA-DR compatibility studies.

Eleven grafts showed no HLA-DR mismatches at the DR loci, of these seven were low-risk grafts and none of these rejected and four were high-risk and one failed from rejection. HLA-DR matching was not a significant factor for graft failure in the Cox proportion hazard model. Survival curves are shown in figure 8 (page 88), the HLA-DR compatible grafts appear to have a lower incidence graft failure compared to HLA-DR mismatched grafts but with logrank analysis there was no significant difference ($p=0.17$). When the grafts were divided into high and low risk groups then again HLA-DR compatibility showed no effect on rejection rate ($p= 0.33$ and 0.32 respectively).

Likewise in the rejection incidence study, HLA-DR matching was not a significant factor for graft failure in the Cox proportion hazard model. Survival curves are shown in figure 9 (page 89), the HLA-DR compatible grafts have a lower incidence of rejection compared to HLA-DR mismatched grafts but with logrank analysis this difference did not reach statistical significance ($p=0.09$). When the grafts were divided into high and low risk groups then again HLA-DR compatibility showed no effect on rejection rate ($p= 0.33$ and 0.1 respectively).

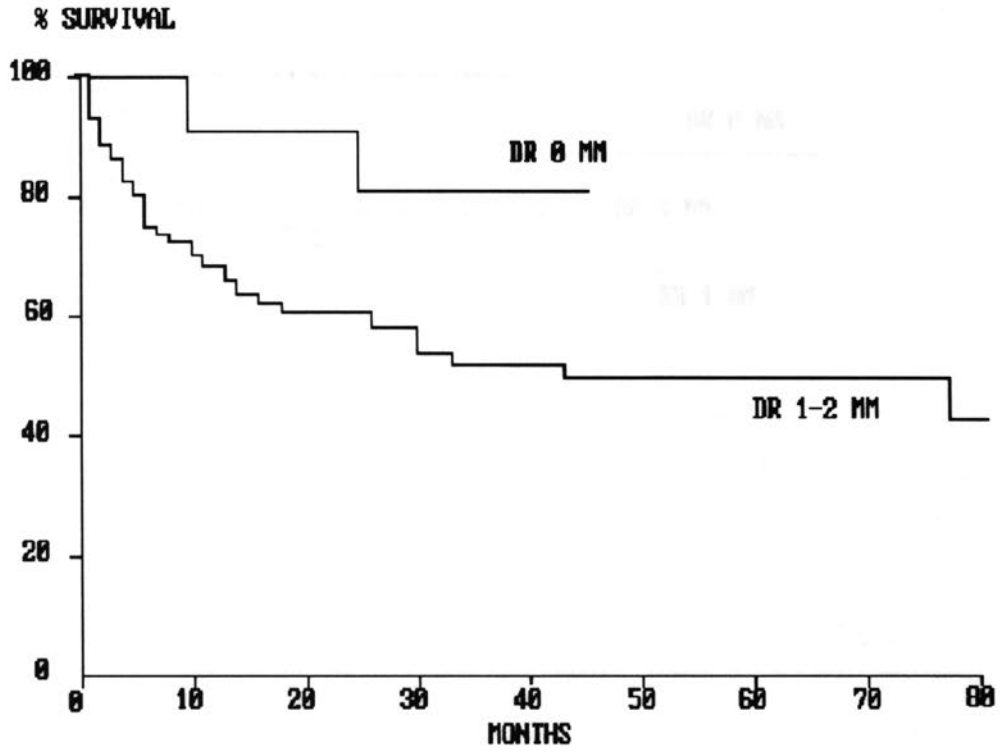
When the groups were divided into 0, 1 and 2 HLA-DR antigen mismatch groups no difference could be found between those grafts with one HLA-DR mismatch and those with two HLA-DR mismatches. Survival curves with graft failure as an end-point are shown in figure 10 (page 90), and with first rejection episode as the end-point in figure 11 (page 91).



DR 0 MM = HLA-DR 0 antigen mismatch grafts.

DR 1-2 MM = HLA-DR 1-2 antigen mismatches grafts.

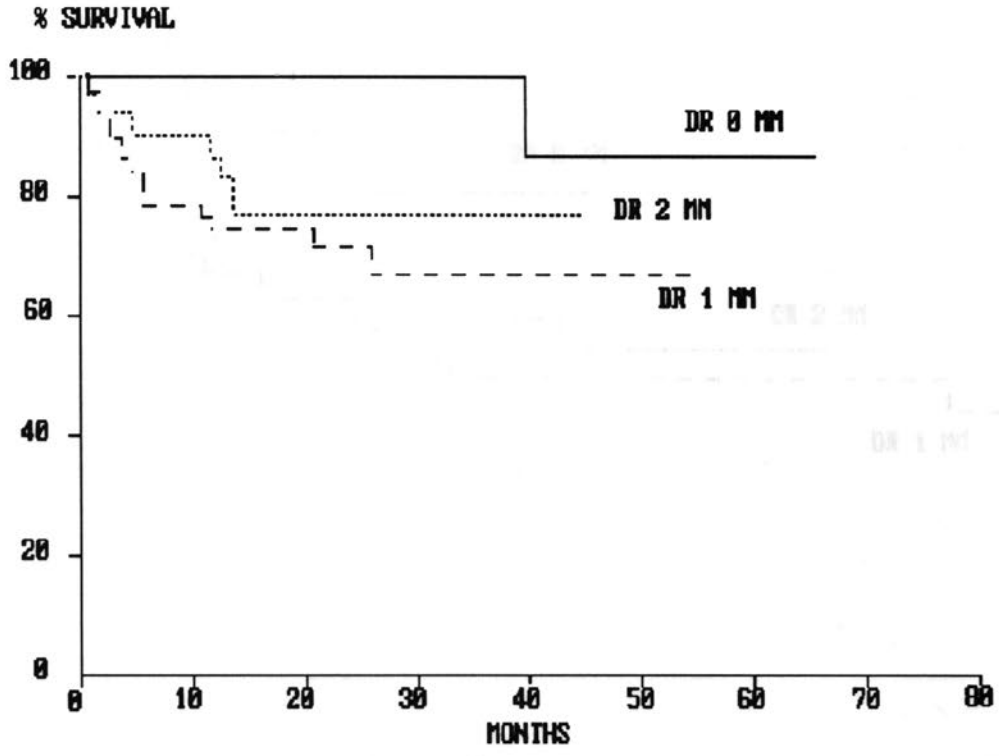
Fig. 8. HLA-DR antigen matching study: survival curves of grafts with 0 antigen mismatches versus 1 and 2 mismatches, showing graft failure from rejection as the end-point.



DR 0 MM = HLA-DR 0 antigen mismatch grafts.

DR 1-2 MM = HLA-DR 1-2 antigen mismatches grafts.

Fig. 9. HLA-DR antigen matching study: survival curve of grafts with 0 antigen mismatches versus 1 and 2 mismatches, showing first graft rejection episode as the end-point.

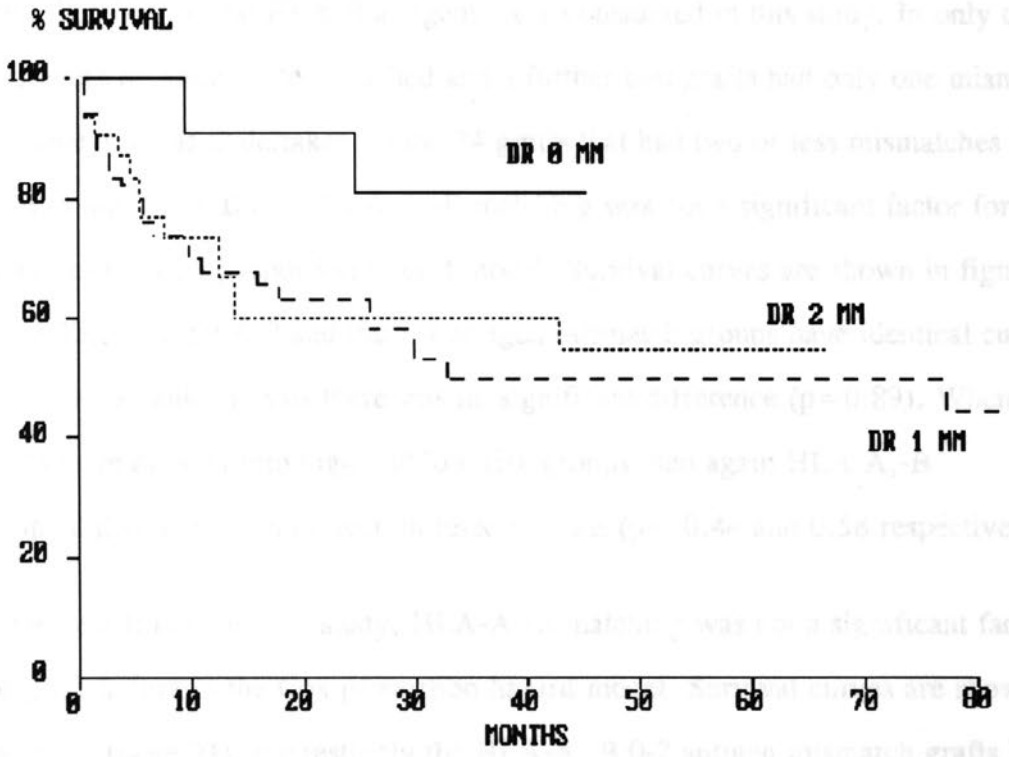


DR 0 MM = HLA-DR 0 antigen mismatch grafts.

DR 1 MM = HLA-DR 1 antigen mismatches grafts.

DR 2 MM = HLA-DR 2 antigen mismatches grafts.

Fig. 10. HLA-DR antigen matching study: survival curves of grafts with 0, 1, and 2 antigen mismatches, showing graft failure from rejection as the end-point.



DR 0 MM = HLA-DR 0 antigen mismatch grafts.

DR 1 MM = HLA-DR 1 antigen mismatches grafts.

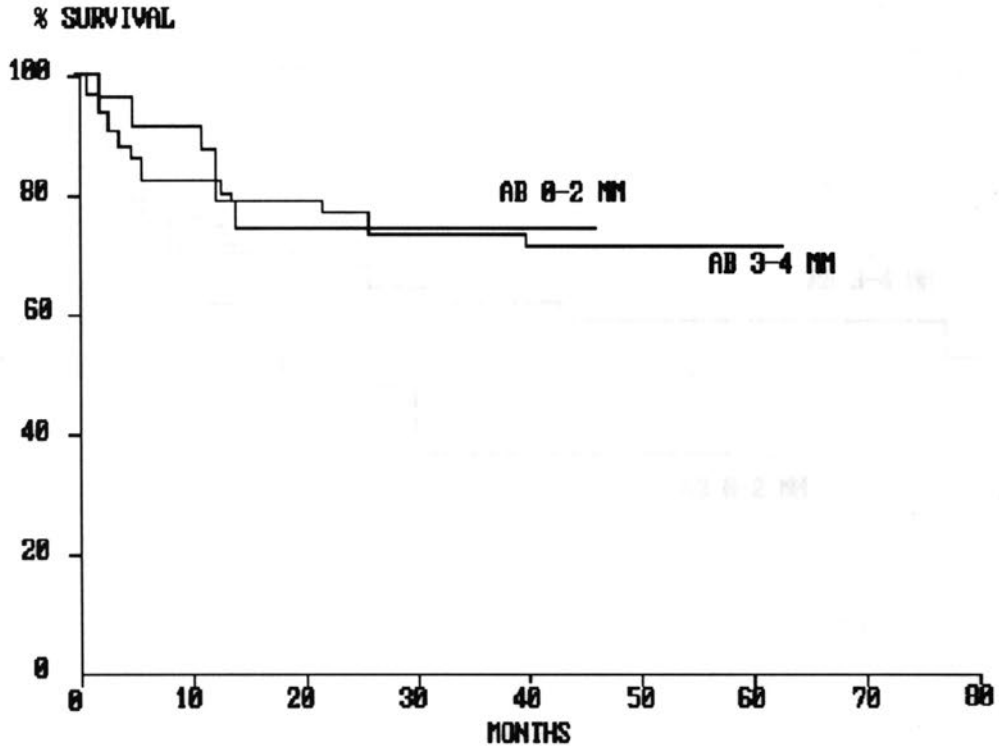
DR 2 MM = HLA-DR 2 antigen mismatches grafts.

Fig. 11. HLA-DR antigen matching study: survival curves of grafts with 0, 1, and 2 antigen mismatches, showing first graft rejection episode as the end-point.

HLA-A,-B,-C compatibility studies.

Only the HLA-A and HLA-B antigens were considered in this study. In only one graft were no antigens mismatched and a further two grafts had only one mismatch. The analysis was undertaken on the 24 grafts that had two or less mismatches at the HLA-A and HLA-B loci. HLA-A,-B matching was not a significant factor for graft failure in the Cox proportion hazard model. Survival curves are shown in figure 12 (page 93), both the 0-2 and the 3-4 antigen mismatch groups have identical curves and with logrank analysis there was no significant difference ($p=0.89$). When the grafts were divided into high and low risk groups then again HLA-A,-B compatibility showed no effect on rejection rate ($p= 0.44$ and 0.58 respectively).

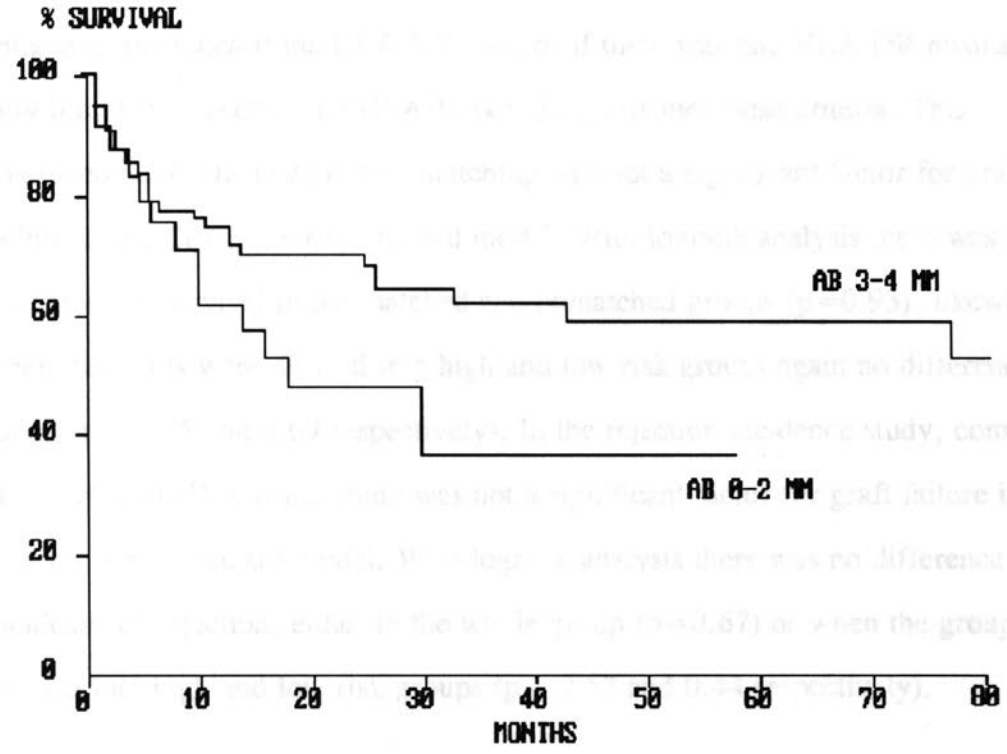
In the rejection incidence study, HLA-A,-B matching was not a significant factor for graft failure in the Cox proportion hazard model. Survival curves are shown in figure 13 (page 94), interestingly the HLA-A,-B 0-2 antigen mismatch grafts have a higher incidence of rejection compared to 3-4 antigen mismatch grafts but with logrank analysis this difference did not reach statistical significance ($p=0.073$). When the grafts were divided into high and low risk groups, there was no difference in the high-risk group between the 0-2 antigen and 3-4 antigen mismatch groups ($P=0.55$). In the low risk group the 0-2 antigen mismatch group fared worse than the 3-4 antigen mismatch but the difference failed to achieve statistical significance ($p=0.08$).



AB 0-2 MM = HLA-A,-B 0-2 antigen mismatch grafts.

AB 3-4 MM = HLA-A,-B 3-4 antigen mismatches grafts.

Fig. 12. HLA-A,-B antigen matching study: survival curves showing graft failure from rejection as the end-point.



AB 0-2 MM = HLA-A,-B 0-2 antigen mismatch grafts.

AB 3-4 MM = HLA-A,-B 3-4 antigen mismatches grafts.

Fig. 13. HLA-A,-B antigen matching study: survival curves showing first graft rejection episode as the end-point.

A further study was undertaken to investigate the combined effect of HLA-DR and HLA-B on graft survival and rejection. Grafts were selected if there were no antigen mismatches at the HLA-DR loci, or if there was one HLA-DR mismatch but only 0 or 1 mismatch at the HLA-B loci; 31 grafts met these criteria. This combined HLA-DR and HLA-B matching was not a significant factor for graft failure in the Cox proportion hazard model. With logrank analysis there was no difference in survival in the matched and unmatched groups ($p=0.93$), likewise when the grafts were divided into high and low risk groups again no difference was found ($p= 0.85$ and 0.09 respectively). In the rejection incidence study, combined HLA-DR and HLA-B matching was not a significant factor for graft failure in the Cox proportion hazard model. With logrank analysis there was no difference in the incidence of rejection, either in the whole group ($p=0.67$) or when the group was divided into high and low risk groups ($p= 0.53$ and 0.44 respectively).

Discussion

The results of this study tend to agree with the findings of the CCTS[108] in showing that in corneal grafting there is no significant improvement in either graft survival nor in the incidence of rejection episodes with antigen matching. However, unlike the CCTS we were not able to show any benefit with ABO blood group compatibility. Our study therefore agrees with previous studies that have shown no effect of ABO compatibility.

In the HLA-DR matching study the survival curves show a reduced failure rate from rejection and a lower incidence of rejection in the grafts with no HLA-DR antigen mismatches compared to one or two HLA-DR antigen mismatches. However, possibly because of the small number of HLA-DR compatible grafts ($N=11$), statistical significance was not found in either the Cox proportional hazard model nor in the logrank analysis. There was no difference in survival or the incidence of rejection if the graft was mismatched for either one or two HLA-DR antigens. In

the cornea class II antigens are usually only found on Langerhans cells within the epithelium, these cells are normally present predominantly in the periphery and therefore are not present in any great numbers in the transplanted donor cornea. In the presence of inflammation class II antigens can be expressed in all layers of the cornea including the endothelium. However, after keratoplasty local inflammation is usually suppressed by topical corticosteroids and the expression of class II antigens should be minimal and therefore a less important factor in initiating rejection. In renal transplantation greater importance is attached to matching HLA-DR antigens which are given priority followed by HLA-B antigen matching. In this study no beneficial effect of combined HLA-DR and HLA-B antigen matching was found. Possibly a greater degree of matching may confer benefit, but given the lack of benefit found with no HLA-DR antigen mismatch and with 0-2 HLA-A,-B antigen mismatch it seems unlikely that better matching would be of significant benefit. Also in our broad ethnic population it is likely that waiting for a high level of matching would significantly delay surgery.

In the HLA-A and -B matching study no difference in the incidence of graft failure from rejection could be found in either the high matched group (0-2 antigen mismatch) or the low matched group (3-4 antigen mismatch). This finding applied not only to the total group, but was also found when the grafts were divided into high and low risk corneas. In the rejection incidence study the high matched group fared worse having a higher incidence of graft rejection episodes compared to the low matched group. The difference did not reach statistical significance in the whole group ($p=0.073$). When the grafts were divided into high and low risk corneas, the former showed no difference in the incidence in rejection episodes ($p=0.55$) but in the low-risk group there was a higher incidence of rejection in the high matched group, although again the difference failed to reach statistical significance ($p=0.08$). No explanation can be given as to why in the low-risk corneas there was a higher incidence of rejection in the high matched group.

This study has failed to show any benefit of tissue matching, on either graft survival or on the incidence of rejection episodes, in corneal grafting. Given the cost of tissue typing, the logistic problems of distributing donor corneas and getting patients from outlying areas, and the inevitable delay that would result in surgery for many patients, it does not seem a feasible proposition to start a tissue matching service at present at our institution.

Our study adds to the diversity of results that have been presented and which differ from centre to centre, and from country to country. The advantage of antigen matching has been questioned by some renal transplantation units in the United States, and the advantage in corneal grafting must be more dubious. The impression is that the criteria that we are using for matching are inadequate or that hitherto undetected antigen systems, such as the minor histocompatibility antigens, play a more important role in the cornea. Our finding that corneas of non-O blood groups, especially group A, fare better, whereas in Holland the reverse is true is surprising. It has been shown that the ABO blood groups, or genes linked to them, appear to exert a significant effect on the survival of corneal grafts and similar findings have been reported for cardiac and renal transplants[215]. It is possible that in our ethnic population these adverse factors are more closely linked to blood group O whereas in Holland they are more closely linked to the non-O blood groups. The CCTS may have dampened the enthusiasm for tissue matching but hopefully research into its role in corneal transplantation will continue.

Section Four: An Animal Model For High-risk Keratoplasty.

4.1 Establishing the model and investigations into the effects of treatment regimens on graft survival.

4.2 Investigations into the effects of tissue mismatch on graft survival using mixed lymphocyte cultures.

4.3 The effect of preoperative ultraviolet B irradiation of donor corneas on graft survival.

4.1 Establishing The Model And Investigations Into The Effects Of Treatment Regimens On Graft Survival.

Corneal graft rejection remains a clinical problem and is the leading cause of late graft failure[12,161,191]. Especially at risk are corneas that are heavily vascularized[158,191] or in which previous rejection has occurred[12,276]. In these cases immunological privilege is lost, the corneal new vessels providing the route for the afferent and efferent arcs of the rejection process. The incidence of corneal graft rejection is substantially increased in these patients, exceeding 50% within the first year after transplantation[12,158,190,191,198,199,234,276]. To evaluate different treatment regimens and to increase our understanding of the rejection process in high-risk corneal transplantation, it is necessary to study an animal model that closely resembles the condition found in humans.

The success rate of corneal grafts in animal models has been reported to be 75-100%[198,277-279]. Because it is difficult to reproduce corneal graft rejection in animals, many models rely on methods of immunological sensitization that are additional to the transplanted corneal antigens to produce consistently high rejection rates. These include delayed skin transfer[12,277,280-282] or transfer of previously sensitized lymphocytes[171]. Other workers have used local measures that aim to promote vascularization, such as eccentrically placed grafts, irritating sutures[283] or delayed suture removal in central penetrating grafts. However, many of these grafts fail to reject[79,284,285]. Hunter et al[286] reported a model that relied on such local measures and the anterior chamber was left flat to encourage the formation of anterior synechiae and deep corneal neovascularization. All their control allografts rejected but there was a high incidence of surgical complications.

The models described, all differ from the human situation in one or more important aspects. Sensitization with skin grafts results in transplantation of Ia antigen[287], the precise role of which is unknown in corneal transplant rejection. The local measures

that cause post-transplant vascularization and rejection by deliberate "poor" surgical techniques, are not analogous to human surgery, when these complications are avoided whenever possible. We have found that corneas in which neovascularization has been induced consistently reject allografts; similar findings have been reported by other workers[288]. This research describes an animal model for high-risk corneal transplantation using pre-vascularized corneas and employing surgical techniques similar to those used in human keratoplasty. In this section the use of the model to investigate the effect of topical and systemic CSA therapy on graft outcome is described. In later sections of this thesis, I will present the results of experiments in which the model was used to study the role of tissue histocompatibility on graft rejection as assessed by the mixed lymphocyte culture (MLC) reaction, and to study the effect of ultraviolet B irradiation on graft survival.

Materials and methods

Outbred strains of New Zealand white rabbits, Swiss hares, and California white rabbits weighing 2-3 kg were used in this study. Preoperatively 10 mg/kg chlorpromazine (Largactil) was given, and anaesthesia was induced by inhalation halothane and intramuscular Ketamine 10 mg/kg. When keratoplasty was to be performed, heparin 2500 IU was administered intravenously after induction of anaesthesia to prevent aqueous clotting. Topical heparin 1000 IU/ml was used during the operation.

Corneal vascularization was induced by placing six 6/0 silk sutures into the upper 180° of the cornea to a depth of one-half to two-thirds the thickness of the cornea. After two weeks the upper cornea was well vascularized (Fig. 14, page 102), the sutures were removed and penetrating keratoplasty was performed. With allografts, corneal buttons were either exchanged between two rabbits or obtained from a donor rabbit eye. Whenever possible a donor graft from a different type of rabbit

was used to minimize the chance of a good histocompatible match. In control autografts the corneal button was removed, rotated 180°, and replaced.

The corneal grafts were placed centrally using a technique similar to human keratoplasty. After removing a 6 mm corneal button, sodium hyaluronate 1% (Healon) was placed on the lens and iris. An identically sized donor button was secured by four interrupted 8/0 virgin silk sutures that were removed at the end of the operation. A continuous 10/0 nylon suture was inserted with a single knot buried in the wound. Postoperatively the anterior chamber was reformed with balanced salt solution, and any anterior synechiae were broken down with Healon. On completion of the operation, 20 mg of gentamicin was given subconjunctivally and tobramycin ointment applied.

Postoperatively the animals were assessed regularly by slit-lamp examination. No routine postoperative medications were given. After two weeks the nylon suture was removed under anaesthesia. The time from operation to the day of rejection was noted. This was defined as the first day a rejection line appeared, or when oedema occurred in a previously clear thin graft. Grafts that were clear at 100 days post-transplant were regarded as long-term survivors. One group of allografts received topical CSA 2% drops in castor oil four times daily and a control group received unmedicated topical castor oil four times daily. Another group of allografted rabbits received intramuscular CSA daily. The dose of CSA given to the first four rabbits was 40 mg daily i.m. This dose (15-20 mg/kg/day) failed to achieve therapeutic blood values. All subsequent rabbits were given 75 mg CSA daily (25-30 mg/kg/day). This higher dose achieved whole-blood trough values that were consistently within the therapeutic range of 250-450 ng/ml. Blood was taken just prior to administration of the drug to obtain trough blood levels; estimations were performed by radioimmune assay[289]. In all these groups treatment was commenced at operation and was continued for 100 days unless rejection occurred. The medication was then stopped and the animal observed for a further 100 days without

medication. Animals were sacrificed and end-point histological examinations were performed when a graft was clinically rejected or when the observation period was completed.

Results

The survival of the corneal grafts in the various groups of rabbits is recorded in Table III (page 103). The differences in survival, comparing the treatment groups B, D, E, and G to the control group C are shown in Table IV (page 104). Survival estimates for the model group and the three therapeutic groups are shown in Fig. 15 (page 105). Data were analyzed by survival analysis methods using generalized Wilcoxon[290] and Kruskal-Wallis[291] tests. With the small sample sizes of the groups the significant findings are reported but no comment can be made about non significance.

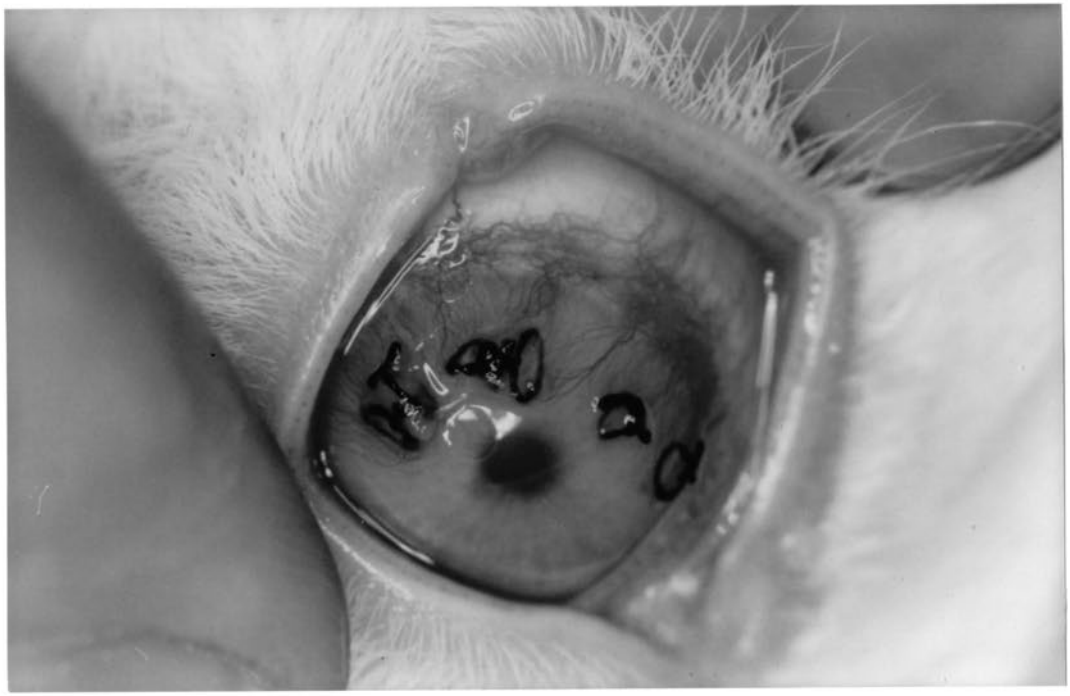


Fig. 14 Corneal vascularization induced by 6/0 silk sutures.

Table III

Establishing An Animal Model: Grafts Survival Results

Group	Type Of Graft	No.	Days To Rejection
A	Autograft	13	No grafts rejected.
B	Allograft (unmodified)	8	14, 22, 34, >100, >100 >100, >100, >100.
C	Allograft (vascularized)	11	12, 15, 16, 16, 17, 17, 17, 21, 25, 28, 30.
D	Allograft (topical CSA)	12	18, 19, 19, 20, 21, 43, 52, 86*, >100, >100, >100, >100.
E	Allograft (topical castor oil)	8	17, 18, 18, 18, 19, 20, 34, 40.
F	Allograft (low systemic CSA)	4	16, 41, >100, >100.
G	Allograft (high systemic CSA)	11	30, 31*, 38*, >100, >100, >100, >100, >100, >100, >100, >100.

* Rabbit died with clear graft.

TABLE IV

Effect Of Treatments On Survival Compared To Model

Group B	Unmodified allografts	P = 0.0091
Group D	Topical CSA	P = 0.0025
Group E	Topical castor oil	P = 0.1295
Group G	High systemic CSA	P = 0.0001

P values refer to statistical difference in survival compared to model (group C).

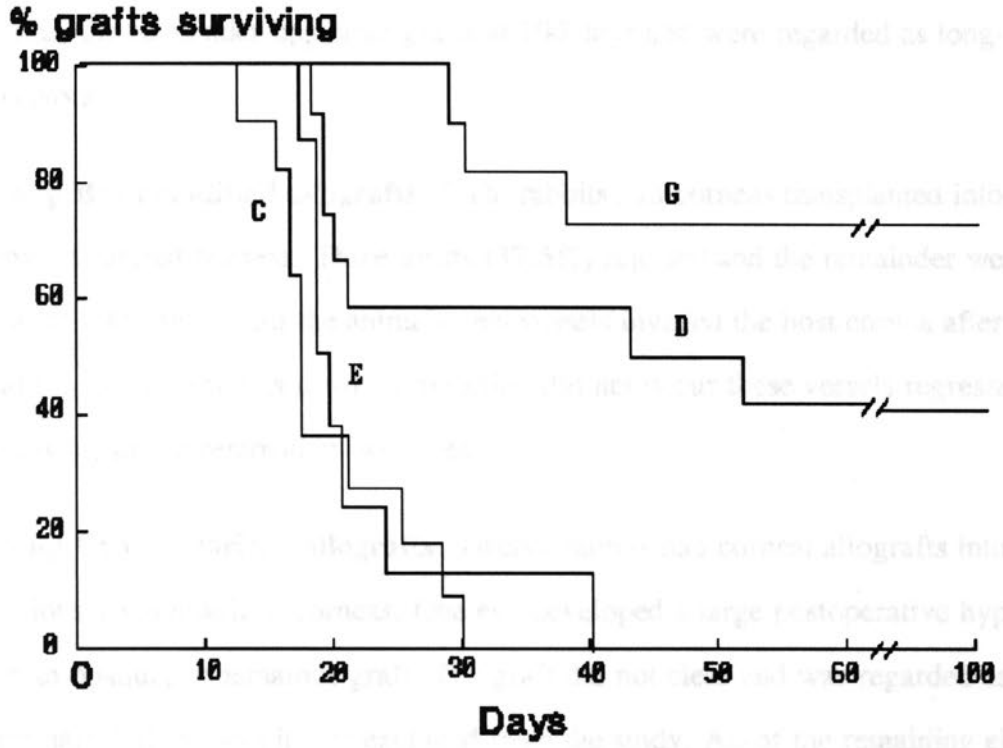


Fig. 15. Survival estimates for untreated (group C) and treated (groups D, E, and G) allografts.

Group A: autografts. Thirteen rabbits had corneal vascularization induced and then underwent an autograft. One rabbit died at 46 days of unknown causes with a clear cornea, the remainder had clear grafts at 100 days and were regarded as long-term survivors.

Group B: unmodified allografts. Eight rabbits had corneas transplanted into clear nonvascularized corneas. Three grafts (37.5%) rejected and the remainder were clear at 100 days. In all the animals new vessels invaded the host cornea after grafting. In the animals in which rejection did not occur these vessels regressed following suture removal at two weeks.

Group C: vascularized allografts. Twelve rabbits had corneal allografts into previously vascularized corneas. One eye developed a large postoperative hyphaema and an opaque, oedematous graft. The graft did not clear and was regarded as being a primary failure, and it was excluded from the study. All of the remaining eleven allografts subsequently rejected, with a mean survival time of 17 days. There was a significant difference between survival of grafts in groups B and C ($P=0.0091$).

Group D: allografts treated with topical cyclosporine. Twelve rabbits were treated with topical CSA drops after receiving an allograft into a previously vascularized cornea. A further rabbit assigned to this group was excluded at day 2 because of primary graft failure following a large postoperative hyphaema. Rejection occurred in seven of the twelve corneas (58.3%), and median graft survival time was 47.5 days. One rabbit with a clear graft died on day 86 of unknown causes. In the statistical analysis this graft survival time of 86 days was censored as it was thought that death was unrelated to therapy. In the remaining animals treatment was stopped on day 100 and the corneas observed for a further 100 days; all these grafts remained clear.

No CSA was detected in the blood of any rabbit receiving topical CSA. There was a significant difference (P=0.0025) between the survival of grafts in this group compared with the untreated animals (group C).

Group E: allografts treated with topical castor oil. Eight rabbits were treated with topical castor oil after receiving an allograft into a vascularized cornea. All the grafts rejected, and the median survival was 18.5 days. Graft survivals in this group and in the untreated rabbits (group C, mean survival 17 days) were similar (P=0.1295). There was a significant difference in graft survival (P=0.0097) between grafts treated with topical CSA in the castor oil (Group D) and those treated with the vehicle alone. Apart from some matting of the eyelid hair, no inflammation, baldness, or other problems were encountered with the eyes that received castor oil with or without cyclosporine.

Group F: allografts treated with subtherapeutic doses of systemic cyclosporine. The first four rabbits given i.m. CSA received a single dose of 40 mg daily, equivalent to 15-20 mg/kg/day. Repeated trough whole-blood CSA levels were consistently less than 62 ng/ml (therapeutic range 250-450 ng/ml). Two grafts (50%) rejected (on days 16 and 41); the other two were clear at day 100, and remained clear for a further 100 days after treatment was stopped. It was thought that the effect of systemic CSA on graft survival could only be assessed if therapeutic blood values were achieved. Subsequent animals were therefore given a higher dose of CSA. The number of rabbits in this group is too small to perform meaningful statistical analysis.

Group G: allografts treated with therapeutic doses of systemic cyclosporine. Eleven rabbits had corneal allografts into vascularized beds and received 75 mg CSA i.m. once daily. This represented a dose of 25-30 mg/kg/day. Trough whole-blood CSA levels were maintained within the 250-450 ng/ml therapeutic range. One graft rejected on day 38. Two rabbits died with clear grafts on day 30 and day 31,

respectively. Both these grafts were considered as failures in the statistical analysis, because death could have been related to treatment, although the cause of death in these two animals was not found. The other eight grafts remained clear until day 100, and then for a further 100 days after cessation of treatment. There was a highly significant difference (P=0.0001) between graft survival in this group and that of the untreated controls (group C).

Although the numbers in each group are small, an overall comparison between graft survival of the control grafts (group C) and the treated grafts (groups D and G) shows a highly significant difference (P=0.0001) using the Kruskal-Wallis test. End-point histology was unremarkable. Clear grafts showed healthy donor discs with minimal cellular infiltration. Rejected grafts had a typical histological picture with an oedematous stroma, mononuclear cell infiltration, keratoprecipitates, and loss of endothelial cells.

Discussion

The efficacy of the surgical technique is demonstrated by the grafts in group A. These were autografts into vascularized beds and all remained clear. One problem encountered was operative bleeding, encouraged by the intravenous heparin given to prevent aqueous clotting. Both cases of primary graft failure encountered in these experiments were associated with large operative hyphemas. Of the eight allografts placed centrally into clear, nonvascularized corneas, three rejected - giving a graft survival rate of 62.5%. This is a little lower than other series[198,277-279], but our animals received no postoperative medication and the likelihood of a good histocompatible match was small. The low incidence of rejection makes this a poor model for corneal graft rejection.

In contrast, when allografts were placed into vascularized beds (group C) all eleven were rejected, with a median survival time of 17 days (range 12-30 days). There is a significant difference ($P=0.0091$) between graft survival in this group and allografts placed into clear corneas. This indicates that corneal vascularization plays a significant role in graft rejection. The short interval from operation to rejection, and the reliability of its occurrence, make this model suitable for testing treatment regimens and for studying the rejection process. This animal model is analogous to the vascularized cornea encountered in human high-risk keratoplasty. Furthermore there is the advantage that no additional methods of sensitization are used to induce graft rejection. The surgery closely follows the techniques of human keratoplasty, in which good wound alignment, reformation of the anterior chamber, burying of knots, and the use of inert suture materials are all vitally important.

The model was used to test two treatment regimens involving topical and systemic CSA. The grafts that were treated with topical CSA in castor oil showed improved graft survival compared with the model ($P=0.0025$). Of the 12 topically treated grafts, 5 (42%) remained clear. All the grafts that received castor oil rejected, with

a median graft survival similar to the model group (group C). Other workers using topical CSA have reported similar survival data[286,292-294]. Problems have been encountered using CSA in an arachis oil vehicle. Excoriation, loss of hair, and conjunctival irritation occurred[286,288]; in some cases the animals had to be killed because of the severity of the reaction[288]. Williams et al[288]. did not find CSA in arachis oil to be beneficial, but when using an olive oil vehicle graft survival was improved. It is possible that the irritation from the arachis oil counteracts the beneficial effects of the CSA. Using castor oil as a vehicle, we experienced none of the above side effects and no animal had to be withdrawn from the study. The four rabbits that were clear on day 100 remained clear for a further 100 days after cessation of treatment. This finding is in contradiction to the results of Foets et al[295]. who reported no rejection episodes while topical CSA was being administered, but all the grafts rejected within four weeks when therapy was stopped at six months. Studies in which topical CSA was given for 28 days only have reported limited success with this drug[281,288]. Our study shows that a modest prolongation of graft survival occurs with some grafts having long-term survival. Blood CSA levels were subtherapeutic in all the rabbits receiving topical CSA. This points to a local immunosuppressive effect being in operation. Whether the afferent or the efferent limbs of the immune response was suppressed is not known and requires further investigation.

The absorption, excretion and metabolism of CSA varies among species[296,297]. In rabbits, most workers[280-282] have used a dose of 25 mg/kg/day, given parentally by a single daily injection, which is substantially higher that the dose for humans. We had hoped to use a smaller dose to prevent high peak blood levels and to reduce toxicity. CSA given in a dose of 15-20 mg/kg/day failed to maintain therapeutic blood levels and we were obliged to use a dose of 25-30 mg/Kg/day to achieve therapeutic levels. Using this dose there was a highly significant improvement in graft survival compared to the untreated model. Only one graft rejected, but the two

rabbits that died were regarded as failures despite their grafts being clear at the time of death. It is likely that the cause of death was related to CSA toxicity[298]. All eight grafts that were clear at day 100 remained clear for a further 100 days after cessation of therapy. It is not known whether the grafts regained a degree of immunological privilege or whether tolerance was induced. Which of these mechanisms is responsible for long-term graft survival, and the duration of CSA therapy required, are important questions in deciding the role of CSA in human high-risk corneal transplantation. The mechanism of action of CSA is dose dependant and the higher doses necessary to achieve trough therapeutic blood levels in the rabbit may have also induced higher peaks, thereby altering the mechanism of action. Therefore, the results in different species may differ making extrapolation to the human clinical situation difficult. In the rabbit a graft survival of 200 days is regarded as long term survival, because of the short life span. In the human, a proportional long term survival will not necessarily occur.

In conclusion, this is a reliable animal model for corneal graft rejection in which rejection is initiated solely by the transplanted corneal tissue. Using this model two therapeutic regimens have been evaluated. Because of the similarities to human high risk keratoplasty, this model is suitable for evaluating other treatment regimens and for the investigation of the corneal allograft rejection process.

4.2 Investigations Into The Effects Of Tissue Mismatch On Graft Survival Using Mixed Lymphocyte Cultures.

This study is an extension of the work that has been done using our animal model. Preoperative mixed lymphocyte culture (MLC) responses were performed using lymphocytes obtained from rabbits undergoing allogeneic corneal transplantation. The MLC reaction provides a measure of the degree of tissue matching between donor and recipient animals at the RHODE locus which is an integral part of the major histocompatibility complex in rabbits[299]. The MLC results, indicating the degree of tissue matching, have been correlated with the final outcome of these grafts.

Materials and methods

Operative techniques

The surgical techniques are identical to those described in the previous section. Outbred strains of New Zealand white rabbits weighing between 2 and 3kg were used in this study. Preoperatively 10mg/kg chlorpromazine (Largactil) was given intramuscularly, and anaesthesia was induced by inhalation halothane and intramuscular ketamine (10mg/kg). When keratoplasty was performed, heparin 2500 IU was administered intravenously after induction of anaesthesia to prevent aqueous clotting. Topical heparin 1000 IU/ml was used during the operation.

The corneal grafts were placed centrally using a technique similar to that for human keratoplasty. Corneal buttons (6mm) were exchanged between animal pairs that were MLC tested preoperatively. The graft was secured by a continuous 10/0 nylon suture with a single knot buried in the wound. Postoperatively the anterior chamber was reformed with balanced salt solution and any anterior synechiae were broken down with a viscoelastic material. Subconjunctival gentamicin 20mg and topical

tobramycin ointment were given on completion of the operation. No routine postoperative medications were administered.

Postoperatively the animals were assessed regularly by slit lamp examination. After two weeks the nylon suture was removed under anaesthesia. The time from operation to the day of rejection was noted. This was defined as the first day a rejection line appeared, or when oedema occurred in a previously clear thin graft. Grafts that were clear at 100 days post-transplant were regarded as long-term survivors. Animals were sacrificed and end-point histological examinations were performed when a graft was clinically rejected or when the observation period was completed.

Animal groups

Rabbits were divided into three groups as follows:

Group A - Unmodified allografts: These animals received corneas transplanted into clear non-vascularised corneas. No postoperative treatment was given.

Group B - Vascularized allografts: Corneal vascularization was induced by placing six 6/0 silk sutures into the upper 180° of the cornea to a depth of one-half to two-thirds the thickness of the cornea. After two weeks the upper cornea was well vascularized, the sutures were removed and central penetrating keratoplasty was performed partially into the vascularized area. No postoperative treatment was given.

Group C - Vascularized allografts treated with topical CSA: Corneas were vascularized and grafted as described for Group B. Immediately postoperatively, two drops of 2% CSA in castor oil were instilled into the conjunctival sacs of the grafted eyes in this group. Thereafter one drop of 2% CSA in castor oil was applied to the grafts four times daily for 100 days unless rejection occurred. The medication was then stopped and the animal observed for a further 100 days without

medication. During the treatment period, whole blood CSA levels were estimated by radioimmune assay[289].

Mixed lymphocyte culture

These cultures were performed by Sue Horak using facilities in the Department of Haematology. Blood was obtained preoperatively from the peripheral ear arteries of donor (**D**) and recipient (**R**) pairs and was processed for one-way MLC tests as follows[299]. After defibrination on glass beads, the blood was sedimented through 3% gelatin at 37°C. The leukocyte rich supernatant was pelleted, re-suspended and 5ml aliquots were layered over 4ml Percoll (approximate density 1.086). After centrifugation at 2000rpm for 30 minutes, the interface cell layer was removed and washed. Responder cells from the recipient (R) were cultured at a concentration of 2 x 10^6 cells/ml. Stimulator cells from the donor (D) and recipient (R) were treated with 25ug/ml mitomycin C (Dm and Rm respectively), washed and re-suspended at a concentration of 4 x 10^6 cells/ml.

Cultures were carried out in Linbro round bottom microtitre wells using 0,1ml of responder and stimulator cells in RPMI containing 5% pooled rabbit serum. Responder cells were also stimulated by phytohemagglutin (PHA) to verify their ability to respond and to stimulate in MLC. Cultures were incubated for 72 hours at 37°C in 5% CO_2 and humidified air and then 1uCi of ^{3}H-thymidine was added. After further culture for 18 hours, the cells were harvested on a Titertek cell harvester unit and the dried filters were counted. The results are presented as the mean +/- SD and MLC responses were expressed as Stimulation Index (SI) calculated as follows:

$$\textbf{Stimulation Index (SI)} = \frac{\textbf{RDm}}{\textbf{RRm}}$$

Data were analysed by survival analysis methods of Kaplan and Meier[244] and the

Wilcoxon[290] and Log rank tests[247-8]. Stratified analysis was performed with SI stratified into two groups:

SI ≤ 20 (low responders) and

SI > 20 (high responders).

This figure of 20 was the overall mean SI for the three groups of animals.

Results

The survival of the corneal grafts in the three groups of rabbits is recorded in Table V below and the preoperative MLC responses are shown in figure 16 (page 116). The stratified survival estimates are depicted in figures 17, 18, and 19 (pages 117-119).

Table V

MLC Graft Survival Results

Group	Type Of Graft	N.	%Rejected	Days To Rejection
A	unmodified allograft	13	38.5%	31,34,42,46, 54, > 100, > 100, > 100, > 100, > 100, > 100, > 100.
B	vascularised allograft	11	100%	12,13,14,15,15, 17,21,22,23,26, 31.
C	vascularised allograft & topical CSA	12	58,3%	18,19,19,20,21, 43,52,86*, > 100, > 100, > 100, > 100,

* rabbit died with clear graft.

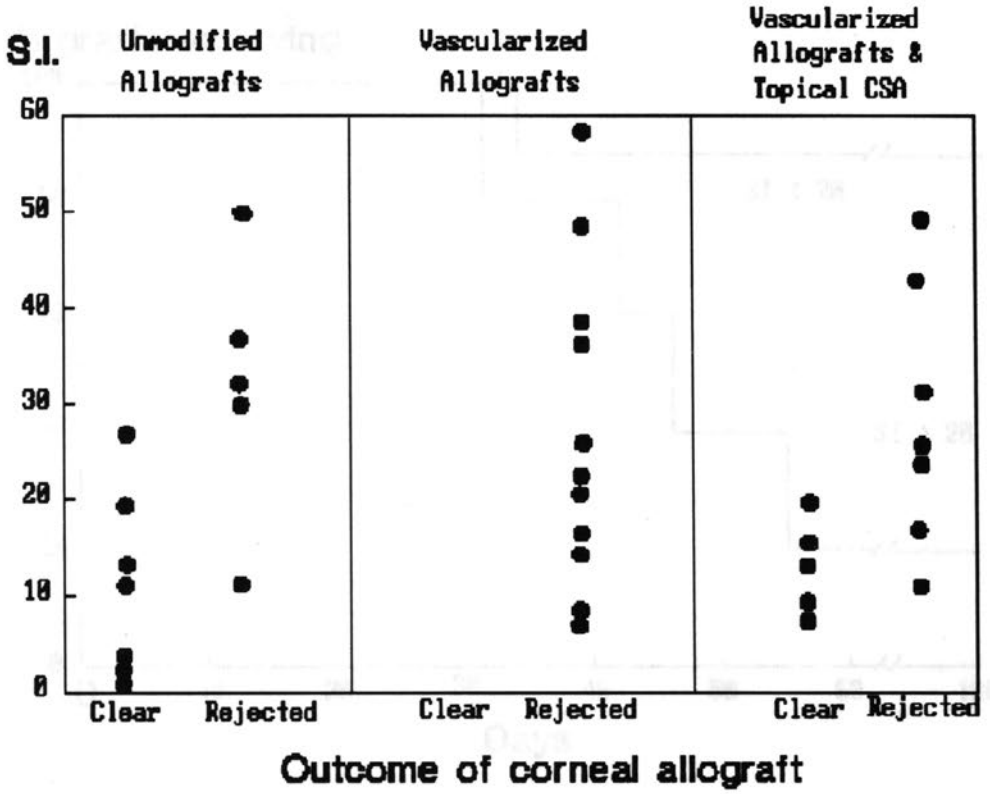


Fig. 16. Preoperative MLC results expressed as stimulation index in relationship to corneal allograft survival in the three groups of rabbits.
Y axis denotes Stimulation Index (**S.I.**).

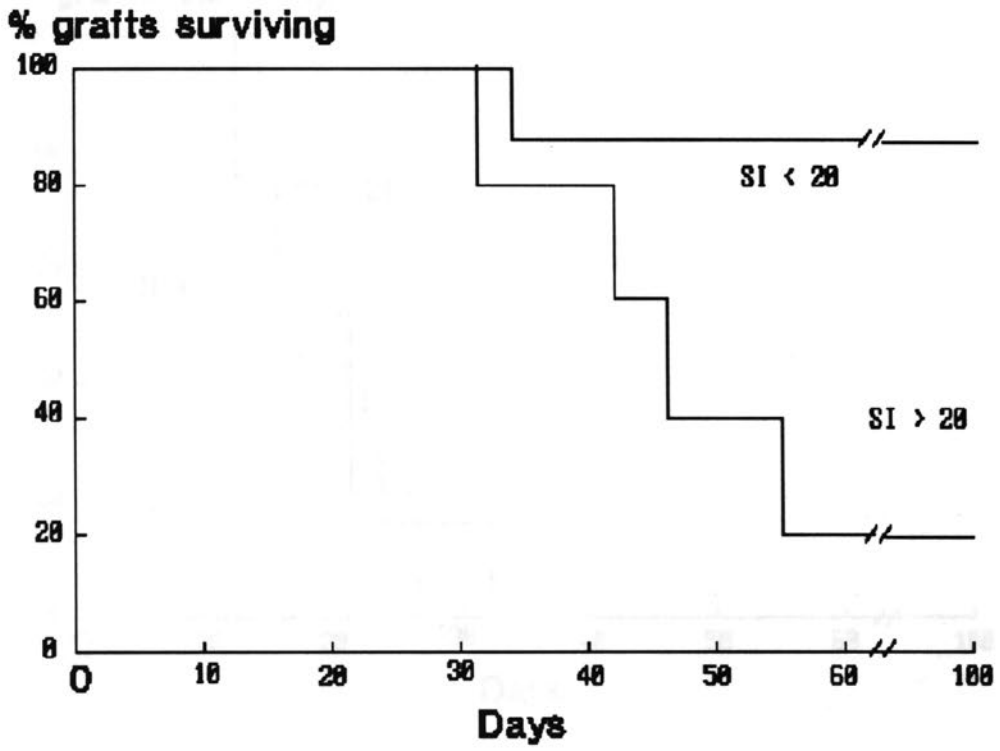


Fig. 17. Stratified survival estimates for unmodified corneal allografts (group A).

Stimulation index (SI) was stratified into 2 groups:

(i) greater than 20

(ii) equal or less than 20

(See text for details).

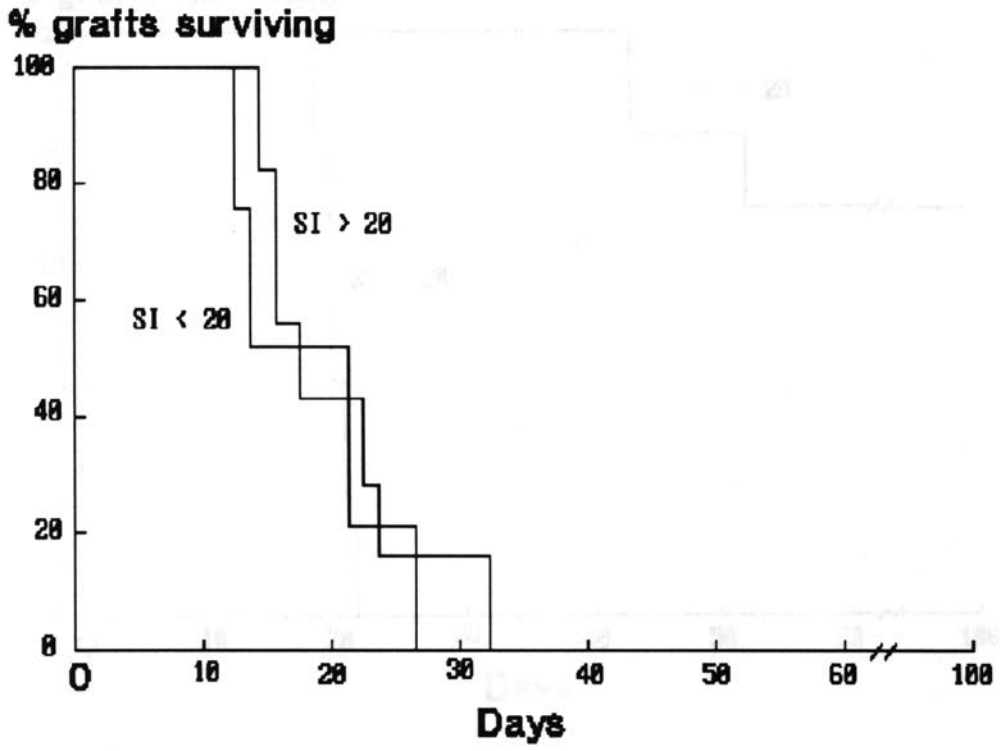


Fig. 18. Stratified survival estimates for vascularized untreated corneal allografts (group B).

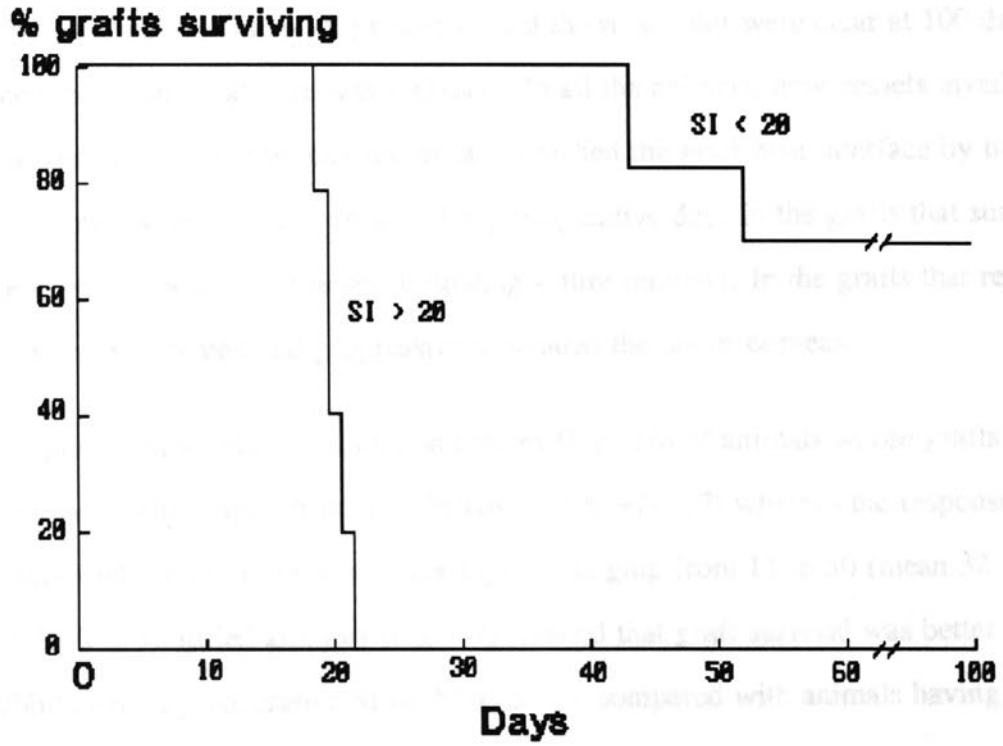


Fig. 19. Stratified survival estimates for vascularized corneal allografts treated with topical CSA (Group C).

Group A - Unmodified allografts

Thirteen control rabbits had corneas transplanted into clear non-vascularized corneas. Five grafts (38,5%) rejected, and the remainder were clear at 100 days. The median survival time was 100 days. In all the animals, new vessels invaded the host cornea from the limbus and usually reached the graft/host interface by the time the sutures were removed on the 14th postoperative day. In the grafts that survived, the vessels gradually regressed following suture removal. In the grafts that rejected, the vessels persisted and progressively invaded the donor corneas.

The preoperative MLC stimulation indices (Fig. 16) of animals whose grafts remained clear ranged from 1 to 26 (mean 9.6 +/- 9.7) whereas the responses of animals whose grafts rejected were higher, ranging from 11 to 50 (mean 32 +/- 13.8). The stratified analysis (Fig. 17) showed that graft survival was better in rabbits with a preoperative SI of 20 or below compared with animals having a preoperative SI greater than 20 ($p = 0.02$).

Group B - Vascularized allografts

Eleven rabbits had corneas transplanted into previously vascularized corneas. All 11 grafts rejected. The median survival time was 17 days. There was a significant difference between survival of grafts in groups A and B ($p = 0.0001$). The preoperative SI of these animals (Fig 16) covered the full range of responses from 5 to 59 (mean 26.6 +/- 16.5). The stratified analysis (Fig. 18) showed no significant difference between animals with a SI above or below 20.

Group C - Vascularized allografts treated with topical CSA.

Twelve rabbits were treated with topical CSA drops after receiving a corneal transplant into a previously vascularized cornea. Seven grafts (58.3%) rejected; five of these rejected within 21 days after grafting, but in the other two animals rejection was delayed to 43 and 52 days after grafting. One rabbit with a clear graft died of

unknown causes on day 86. In the statistical analysis, this graft survival time was censored as it was thought that death was unrelated to therapy. In the remaining four animals, the drops were stopped on day 100 and the corneas were observed for a further 100 days. All four grafts remained clear. The overall median graft survival time for this group was 47.5 days; this was significantly longer than for the untreated Group B animals ($p = 0.003$). No CSA was detected in the blood of any of these animals.

The preoperative SI (Fig. 16) for animals whose grafts survived ranged from 5 to 19 (mean 12 +/- 5) whereas the SI of animals whose grafts rejected were higher ranging from 10 to 48 (mean 28.1 +/- 13.8). The stratified analysis (Fig. 19) showed a significant difference in graft survival between animals with a preoperative SI of 20 and below and those with a SI above 20 ($p = 0.0003$).

Discussion

This rabbit model is analogous to the vascularized cornea encountered in human high risk keratoplasty and the surgery closely follows the techniques for human keratoplasty. No additional methods of sensitization, such as skin transplantation, are required to induce graft rejection as in other models[12,281,282,300]. When control allografts were performed into unmodified avascular corneas, five of the 13 grafts rejected giving a graft survival rate of 61,5%. In contrast, when allografts were placed into vascularized corneas, all 11 were rejected. There was a significant difference between graft survival in this latter group compared with the unmodified allografts ($p=0.0001$). These figures are identical to the findings in our original studies using this animal model. The findings confirm that corneal vascularization is a critical factor influencing graft rejection in this model.

The MLC reaction serves as a measure of the degree of genetic disparity between prospective donor/recipient combinations, although its predictive value in assessing the chances of graft survival in both humans and animals has been variable[301-304]. In

the rabbit, the MLC response is governed by the RHODE locus which is an integral part of the major histocompatibility complex[299]. Mismatching at the RHODE locus produces high and distinct MLC responses. Our results showed that all grafts into vascularized corneas rejected regardless of the preoperative MLC responses. However, in the control group of animals receiving grafts into avascular (unmodified) corneas, the mean preoperative SI was higher in the animals that rejected compared with the grafts that remained clear ($p<0.01$). This suggests that the degree of MLC mismatch was an important factor determining the graft outcome in the unmodified allograft group. During the early postoperative period in these animals, the graft sutures attracted blood vessels. High preoperative MLC reactions (high responders) indicating significant degrees of mismatch were associated with persistence of these vessels after suture removal with subsequent rejection. Lower preoperative MLC responses (low responders) indicating lesser degrees of mismatch were usually, but not invariably, associated with survival and recession of the vessels.

In the group of animals treated with topical CSA in castor oil, the survival was significantly better compared with the untreated vascularized allografts ($p=0.003$). Of the 12 grafts in this group, 5 (42%) remained clear. The mean preoperative SI was lower in the animals that remained clear compared with the grafts that rejected ($p<0.01$). As with the unmodified allograft group, this finding suggests that the degree of MLC mismatch was an important factor determining the graft outcome in the vascularized allograft group treated with topical CSA. Graft survival improved if there were mild degrees of mismatch (low responders) whereas all grafts rejected if there was marked degrees of mismatch (high responders). Although no CSA was detected in the blood of any animal treated with topical CSA, it is possible that values below the lower limit detectable by our assay (48 ng/ml) were present. It has been shown in vitro that the 50% inhibitory concentration of CSA is in the order of 10-20 ng/ml[305], and therefore theoretically a systemic effect could have been in

operation. However, the effect would probably have been operative in all the animals and should not have biased the results. In clinical practice higher concentrations of CSA are required to obtain benefits. In our laboratory the blood therapeutic range is 250-450 ng/ml and levels less than 48 ng/ml are likely to have only minimal effects. Furthermore, a study by Foets et al[295] indicated that topical CSA improved graft survival in rabbits if given to the grafted eye, but did not improve survival if applied to the fellow un-operated eye, indicating that a topical and not a systemic mechanism was in operation.

The results were further analysed by stratified survival analysis methods with SI stratified into two groups: $SI \leq 20$ and $SI > 20$. This figure was the approximate overall mean for the three groups of animals. Since all vascularized allografts rejected (group B), there was no difference in the stratified survival analysis of these animals (Fig.18) and the survival estimates were therefore identical in the two groups. However, in the unmodified control group (group A), the stratified analysis showed improved graft survival if the SI was 20 or below compared with the group of animals with SI above 20 ($p=0.02$). Stratified analysis of the vascularized allograft group treated with topical CSA (group C) showed a markedly significant improvement in survival in the $SI \leq 20$ group compared with the $SI > 20$ group ($p=0.0003$).

Our findings indicate that both corneal vascularization and the degree of MLC mismatch between donor and recipient play an important role in determining the final outcome of corneal transplantation. In the unmodified avascular corneas the greater the degree of mismatch, the greater were the chances of rejection. In this group, the high responders with marked degrees of MLC mismatch had virtually guaranteed rejection; on the other hand, however, the milder degrees of MLC mismatch in low responders did not ensure graft survival.

In the vascularized group treated with topical CSA, once again the greater the degree of mismatch, the greater were the chances of rejection. If there were marked degrees of MLC mismatch (high responders), then topical CSA did not prevent rejection. On the other hand, if there were only mild degrees of MLC mismatch (low responders), then topical CSA had a significant ($p=0.0003$) chance of preventing graft rejection.

Our findings suggest that CSA may have a regional effect in preventing rejection in high risk vascularized corneas since no CSA was detected in the blood of any animals[306]. This effect occurs providing the MLC mismatch is not too great. If there are marked degrees of MLC mismatch then topical CSA is ineffective, and in these cases a greater degree of immunosuppression may be required to prevent rejection and systemic rather than topical CSA may be necessary. The mechanism of action by which topical CSA exerts its effect is not known. Recent reports[307,308] have highlighted the role that accessory cells in both the donor button and graft bed play in promoting corneal graft rejection. These cells are present in increased numbers in high risk vascularized corneas. The accessory cells may function as antigen-presenting cells by taking up, processing and presenting foreign antigens to specific T lymphocytes. CSA has been shown to inhibit the capacity of accessory cells to process and present antigens to T lymphocytes[309,310] and topical CSA may inhibit antigen processing and presentation by local accessory cells. CSA also inhibits lymphokine production[311] e.g. interleukin-2 and gamma interferon, possibly reducing the expression of class II antigens by corneal cells and preventing the proliferation of immune cells and amplification of the immune response. Topical CSA produces high concentrations of the drug in the cornea, with mean peak values of 926 ng/ml being recorded three hours after a single application[312]. The actions of CSA are dose sensitive, but at these high concentrations in the cornea it is likely that a number of CSA actions are in operation.

In conclusion, these studies show that both corneal vascularization and the degree of donor-recipient matching determine the final outcome in corneal grafting and the prevention of rejection with topical CSA. This supports the findings that close matching in high-risk human keratoplasty is beneficial for graft survival[158,161,234]. A recent collaborative study on histocompatibility matching in high risk corneal transplantation did not show any significant improvement in survival with either tissue matching or crossmatching[108]. These findings are surprising as logically a good match should confer some benefit on graft survival. The authors of the study suggest that the minor histocompatibility antigens may play a role in corneal graft rejection, these were not matched for and very little clinical data exists concerning them. Mismatch of these antigens would have contributed to a high stimulation index in our study. All patients in the collaborative study received high doses of topical steroids and were followed up closely with good patient compliance. These factors may have "overcome" the disadvantages of mismatch in the control groups and indeed the overall failure rate was lower than that reported in other studies of high-risk keratoplasty; emphasising the importance of early diagnosis and treatment. In our model, although the animals were watched carefully, no treatment was given and the effect of a poor match (high responder) resulted in graft rejection.

4.3 The Effect Of Preoperative Ultraviolet B Irradiation Of Donor Corneas On Graft Survival.

The immunosuppressive effect of ultraviolet B (UV-B) irradiation has been well documented and it appears that the functional activity of immunocompetant cells can be modified without causing cell death[141-143]. The use of UV radiation to improve survival of transplanted tissue has been suggested[144,145] and shown to be successful in islet transplants in rats[51,313] and in a number of animal corneal graft models[146-148]. It is not fully understood how UV radiation exerts its immunosuppressive effects. In the cornea, like other transplanted tissues, rejection is thought to be triggered by antigens coded by the major histocompatibility complex, especially class II antigens. Under normal conditions the corneal epithelial, stromal and endothelial cells do not express class II antigens[111,112,149] but they are found in Langerhans cells (LC) present within the corneal epithelium[150]. Although LC are more abundant in the corneal periphery, scattered cells are found in the central part of the corneal and become passenger cells in corneal grafts. Studies have classified LC as a tissue leucocyte with a similar role to that of macrophages, having the ability to phagocytose foreign material and to stimulate a mixed lymphocyte reaction in vitro[135]. The generally accepted view is that LC act as antigen presenting cells. They are probably the critical cells for the generation of skin graft rejection[135] and it is likely that the LC in the cornea play a similar immunological role.

Recently there has been a renewed interest in the "passenger cell" theory first proposed by Snell over 30 years ago[138]. He proposed that donor leucocytes present in transplanted tissues were an important source of tissue immunogenicity and that ridding tissues of these passenger cells would improve transplant survival. Depleting tissues of Ia positive cells by a variety of techniques has improved survival in a number of allograft models[139,140]. The beneficial effect of UV radiation on graft survival is most likely due to its effects on LC; possibly the antigen presenting

ability of these cells is decreased[151-154] or it has been suggested that a tolerogenic signal is produced that activates donor-specific suppressor cells[155,156].
In this paper the effect of pre-operative UV-B irradiation on graft survival in our high-risk animal corneal graft model is investigated.

Materials and methods

The surgical techniques were identical to those described previously[314]. The following three groups of animals were compared:

Group A: UV-B irradiated donor discs into vascularized corneas (N = 13).

Group B: untreated donor discs into normal corneas (N = 8).

Group C: untreated donor discs into vascularized corneas (N = 11).

The last two groups (group B and group C) are the same animal groups that were presented in the earlier studies using this model. In group A a standard dose of UV-B radiation (75 mJ/cm^2) was given to donor corneas. Donor rabbits were killed by an overdose of intravenous ketamine and enucleations were performed. The eyes were then placed in holders so that the epithelium of the cornea faced the centre of the UV-B lamp at a distance of 10 cms. The output of the light source had been measured at an identical spot immediately prior to irradiating the corneas, and the dose of UV-B calculated from the formula[146]:

$$\textbf{mJ/cm}^2 = \textbf{meter reading in } \mu\textbf{W/cm}^2 \textbf{ x t (secs) x } 10^{-3}.$$

The time in seconds was calculated to give a dose of 75 mJ/cm^2 to the surface of the cornea, the usual time to achieve this was between 140 and 160 seconds. This dose has been shown previously to be effective in preventing rejection without causing significant cell damage[146]. The donor corneas were left in front of the lamp for the appropriate length of time to receive a dosage of 75 mJ/cm^2 of UV-B irradiation. The light source used in these experiments was a Westinghouse FS-20

fluorescent tube which emits radiation in the ultraviolet spectrum with a peak of 310 nm. The UV-B radiation was measured with a UVX digital radiometer equipped with a sensor reading at a peak of 310 nm (UV Products, San Gabriel, CA).

Postoperatively the animals were assessed regularly by slit lamp examination. After two weeks the nylon suture was removed under general anaesthesia. The time from operation to the day of rejection was noted. This was defined as the first day a rejection line appeared, or when oedema occurred in a previously clear thin graft. Grafts that were clear at 100 days post-transplant were regarded as long-term survivors. Animals were sacrificed and end-point histological examinations were performed when a graft was clinically rejected or when the observation period was completed. Statistical analysis was performed using the logrank test[247-8] and Kaplan Meier[244] survival analysis.

Results

Graft survival results are shown in Table VI (page 129). As noted in the previous experiments, all eleven of the untreated grafts placed into vascularized corneas (group C) rejected, in a median time of 17 days. Only four (30.8%) of the thirteen UV-B irradiated grafts placed into vascularized corneas (group A) rejected, with a median survival time of >100 days. There is a significantly better graft survival in this group compared to the untreated group ($p= 0.000014$). For comparison the results of untreated corneas into normal corneas (group B) are shown, three (38%) grafts rejected, median survival time was >100 days. A Kaplan Meier survival graph is shown in figure 20 (page 130).

Table VI

UV-B Irradiation Study: Graft Survival Results

Group	Type Of Graft	N.	Days To Rejection
A	Allograft vascularized + UV-B	13	21, 30, 42, 49, >100, >100, >100, >100, >100, >100, >100, >100, >100.
B	Allograft unmodified	8	14, 22, 34, >100, >100, >100, >100, >100.
C	Allograft vascularized	11	12, 15, 16, 16, 17, 17, 17, 21, 25, 28, 30.

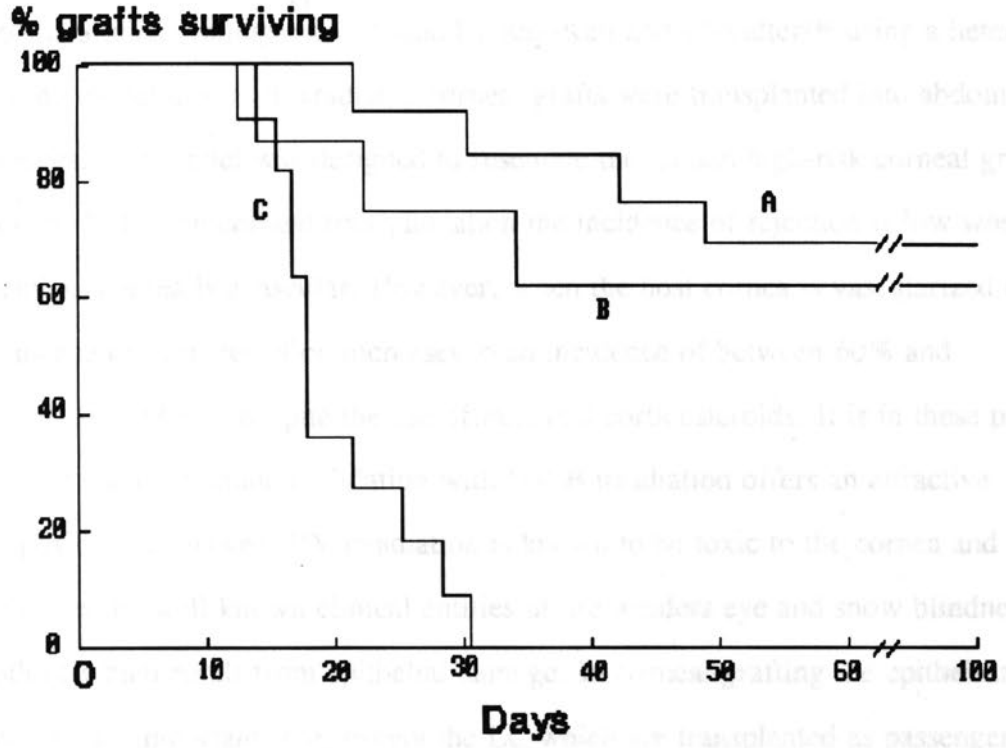


Fig. 20. UV-B Irradiation Study: Survival analysis of the three treatment groups.

Discussion

Preoperative irradiation of the donor corneas with 75 mJ/cm^2 UV-B produced a significant improvement in graft survival ($p = 0.000014$) in our high-risk animal model. Similar findings were found by Ray-Keil and Chandler[146] using a heterotopic murine model in which irradiated corneal grafts were transplanted into abdominal pouches. Our model was designed to resemble the human high-risk corneal graft patient. In human corneal transplantation the incidence of rejection is low when the recipient cornea is avascular. However, when the host cornea is vascularized the incidence of graft rejection increases to an incidence of between 60% and 90%[191,199,225,234,315] despite the use of maximal corticosteroids. It is in these patients that the use of immunomodulation with UV-B irradiation offers an attractive proposition. However, UV irradiation is known to be toxic to the cornea and produces the well known clinical entities of arc welders eye and snow blindness both of which result from epithelial damage. In corneal grafting the epithelium does not play an important role, except the LC which are transplanted as passenger cells. The endothelial cells are of paramount importance in human corneal grafting as they are unable to multiply and are of crucial for maintaining graft clarity. Our studies show (unpublished data) that giving a therapeutic UV-B dose to the epithelium (75 mJ/cm^2) results in only a small dose of UV-B being transmitted through the cornea to the endothelium: the mean reading on the endothelial side was 15.7 mJ/cm^2. All grafts in this series receiving UV-B were initially successful and no cases of primary graft failure occurred. Dana et al[148] found that when irradiating the endothelium directly with low-dose UV-B (110 mJ/cm^2) scanning electron microscopy revealed only minimal differences between treated and untreated endothelial cells, indicating that the doses received in our experiments were safe. Only one dose (75 mJ/cm^2) of UV-B radiation was used in this study, this dosage was based on the findings of Ray-Keil and Chandler[146]. In their heterotopic animal model, UV-B doses of 75 mJ/cm^2 and above significantly reduced rejection but doses of 300-600 mJ/cm^2

produced significant damage to corneal cells. Although a higher dose of UV-B may further improve graft survival, given the highly significant effect of this dose and its apparent safety it seems unnecessary to increase it. In human corneal grafting other forms of immunosuppression, such as corticosteroids, are used making the necessity of higher, potentially damaging, doses of UV-B unnecessary.

We have confirmed previous reports which indicate that UV-B irradiation profoundly reduces the number of ATPase positive LC in corneal epithelium (unpublished data), and has a beneficial effect on corneal graft survival. The exact mechanism is not known but most workers link these two findings and suggest that the immunological function of the LC is altered. One theory is that passenger leucocytes or LC in the donor tissue are responsible for initial allogeneic recognition and initiation of the rejection process. However, the central cornea which is transplanted has a small population of passenger cells and any increase in LC in the postoperative period are likely to be of recipient origin. In the clinical situation, removing the donor epithelium should have a similar beneficial effect. However, as discussed earlier the results have been inconclusive. Possibly all of the LC are not removed or the trauma induced by removal counteracts the beneficial effect. Another possibility is that the LC are only altered by the UVB and may induce tolerance, this is purely speculative and no evidence exists. Despite the uncertainty of action, the use of UV-B irradiation of donor corneas in human corneal grafting remains an attractive proposition especially in high-risk cases where additional forms of immunosuppression are required.

Section Five: The Role Of Cyclosporine In Preventing Corneal Graft Rejection.

5.1 Introduction.

5.2 The use of topical cyclosporine in high-risk keratoplasty.

5.3 Preliminary report on the use of systemic cyclosporine.

5.4 The use of systemic cyclosporine in high-risk keratoplasty: a prospective trial.

5.5 Systemic cyclosporine in high-risk keratoplasty: short versus long term therapy.

5.6 Systemic cyclosporine in high-risk keratoplasty: long term results.

5.7 Conclusion.

5.1 Introduction.

Many of the problems previously encountered in corneal grafting have been overcome by improved surgical techniques, greater understanding of the role of the corneal endothelium and the use of viscoelastic substances. Rejection now remains the leading cause of graft failure[119]. Patients at greatest risk are those in whom the corneas are heavily vascularized[119,191] or where previous graft rejection has occurred[316]. In these cases the immunological privilege of the cornea is lost and the incidence of graft rejection is between 60% and 90%[191,199,225,234,315].

Corticosteroids, administered locally and systemically, remain the mainstay in the treatment of graft rejection. However, these preparations are frequently ineffective especially in high-risk cases[225,226], and serious complications may result from their use. In exceptional cases other immunosuppressant agents such as azathioprine (Imuran) have been used[227-229] but the results show little advantage over corticosteroids and their use requires great care because of potentially serious side effects. Tissue matching has been shown to be of value in corneal transplantation[199,234], but logistic problems limit the widespread use of this method at present, especially in high-risk cases where a very close match may be necessary to ensure a reasonable chance of success. A recent multicentre trial has reported that neither tissue matching nor antibody screening improves graft survival[108]. Apart from the use of high dose steroids and tissue matching, very few other treatments have been available to patients with high-risk corneas who require a corneal graft to restore vision. In other fields of transplantation major advances have been made in methods of immunosuppression to prevent rejection. Because of the dangers of side-effects similar regimens and treatments have not been offered to high-risk keratoplasty patients who are blind from their disease, although patients with blinding uveitis are now frequently treated with immunosuppressant drugs if steroids are inadequate[317,318]. In patients who are blind from corneal disease and who constitute high-risk keratoplasty cases, we now offer treatment with systemic

immunosuppression providing they are free from systemic disease and are available for careful follow-up and monitoring.

Cyclosporin (CSA) is a potent immunosuppressant agent with a high degree of specificity for T lymphocytes: it is now widely used in other fields of transplantation. It was discovered in 1969 by workers at the microbiology unit of the pharmaceutical firm Sandoz, in Basel, Switzerland[319]. Two new strains of fungi imperfecti were isolated from soil samples taken from Wisconsin (USA) and Hardanger Vidda (Norway)[320]. One was *Cylindrocarpon lucidum* Booth, but because the organism only grows in surface culture it was later abandoned. The other strain was originally classified as *Trichoderma polysporum* (Link ex Pers) Rifai but its correct taxonomic name is *Tolypocladium inflatum* Gams. Both fungii synthesize cyclosporins, but since only the latter strain grows in submerged culture, it is the one now used for large scale production of CSA by fermentation. However, the cyclosporins are not released in the culture media but have to be extracted from the mycelia. The extract from these fungii showed a narrow spectrum of antifungal activity with low toxicity and was called compound 24-556. The reason the extract was put forward for pharmacological screening was its unusually low toxicity. The story became exciting in 1972 when Borel et al discovered the marked immunosuppressant effects of the compound[321].

CSA is a neutral, hydrophobic, cyclic, endecapeptide metabolite of the fungus *Tolypocladium inflatum Gans*; it contains eleven amino acids and has a molecular weight of 1202[322,323]. It is insoluble in water but can be dissolved in ethanol or, at a temperature of 60°C, in various oils such as olive or castor oils. These characteristics make administration of the drug difficult. The commercial oral preparation of CSA contains 100mg/ml of a mixture of 30 parts peglicol-5-oleate, an emulsifier, 60 parts olive oil, and 10 parts ethanol, the manufacturers suggest that it is ingested with milk or citrus juices. The absorption is variable with about 30% (range 4-60%) of the oral dose being absorbed, with peak blood levels reached

after 2-4 hours[324,325]. In addition it seems that absorption may increase with time in individual patients[326]. This variable absorption amongst patients makes it essential that blood levels are monitored and the dose adjusted to keep the blood level within the therapeutic range. This avoids the dangerously high levels in patients who absorb the drug easily. A radioimmune assay[289] is available to measure the blood levels of CSA (in nannograms per millilitre), the drug is largely bound to erythrocytes[327]. Because the binding is temperature dependant[328] the concentration is usually measured in whole blood although it can be measured in the serum if the temperature is held constant (at 25°C) for 2 hours. Because of its solubility in fat, CSA selectively concentrates in the fatty tissues such as the fat deposits, liver, kidney and lymphoid tissue[329,330]. The concentration in the fatty tissues is ten times that of the plasma and thus a large reservoir of the drug exists in patients. CSA is oxidised in the liver by the cytochrome P-450 microsomal enzyme system[324,331]; the majority of its metabolites are excreted in the bile. Only about 0.1% is excreted unchanged[332,333] and only 6% is excreted by the kidneys[332]. Because of the incidence of side-effects, notably nephrotoxicity, the recommended plasma concentration has been reduced from 100-300 ng/ml to 50-100 ng/ml or blood trough levels of 80-250 ng/ml[334]. The current method recommended is the specific whole blood method and the therapeutic range of our laboratory is a trough level of 130-250 μg/L. Drugs such as phenobarbitone or phenyltoin lower the plasma concentration because of their action of stimulating the activity of the hepatic microsomal enzymes[335,336]. Conversely ketoconazole inhibits these enzymes and tends to raise the plasma concentration of CSA[337]. In patients with endogenous uveitis, combined CSA and ketoconazole treatment allowed the dosage of CSA to be reduced by 70% and patients on the combined therapy had a better outcome with fewer relapses[338]. The renal toxicity effect is cumulative to the effects of other drugs that cause renal damage and thus gentamicin, amphotericin B, co-trimoxazole and non-steroidal anti-inflammatory agents should be avoided[339,340].

The drug selectively inactivates T cell dependent immunity[341] while retaining the integrity of other immunodefensive mechanisms fundamental to the protection of the host[342]. CSA is predominantly effective during the early stages of immune activation; in vitro studies indicate that there is a short time period for CSA to effectively inhibit T lymphocyte proliferation. Delay of only an hour or two in adding CSA to cultures results in significant reduction in the ability to suppress lymphoproliferation[305]. CSA inhibits monocyte/macrophage function by inhibiting antigen presentation, the mechanism is still unknown. CSA can alter interleukin 1 (IL-1) production but this is dose and stimulus dependent and is not thought to be important[343-345], but it may downregulate IL-1 receptors on T helper cells[305,346].

CSA appears to inhibit selectively the activation of cytotoxic T lymphocytes (OKT4)[347,358]. Of great interest is the effect of CSA on suppressor cells (OKT8), some workers found that these cells were spared[359] or relatively non affected[347], whereas others have found that these cells were induced by CSA[360]. Activation and amplification of the suppressor T cells results in the induction of specific immunological unresponsiveness to the stimulating alloantigen in vitro[305], and clinically this may lead to a degree of tolerance. CSA also inhibits the activation of helper/inducer T cells (OKT4)[347]. CSA may also bind to a cytoplasmic receptor inhibiting the influx of calcium and the associated activation of calcium-dependent protein kinases[305,361,362]. The exact mechanism of action of CSA at cellular level is not known. The activation of non-committed, resting T cells requires both specific antigen and nonspecific molecular signals[348]. Activation leads to the expression of various receptors on the T cell membrane. Of great importance is the lymphokine interleukin 2 (IL-2) which is thought to be responsible for the pathway by which T cells continue to recruit and activate new T cells. The production of IL-2 and other lymphokines by the T helper/inducer cell are prevented[346]. The expression of IL-2 receptors is critical for the T cells capacity to respond to this lymphokine. Larsson[349] suggested that CSA blocks the formation of IL-2 receptors but has no

effect on the proliferation of activated T cells which already possess IL-2 receptors. However, Bunjes et al[346] concluded from their work that CSA inhibits release of IL-2 whereas receptor formation was not affected. Other workers have suggested that CSA prevents the binding of an antigen to its appropriate receptor[350] or that its effect is mediated by preventing uptake of IL-1 by T cells[351]. The action of CSA is complex and probably depends on the nature of the T cell stimulus and the differentiation state of the cell itself[352,353]. Whatever the mechanism, it is clear that CSA mediates its action at a specific portion of the immune system in particular the production and action of IL-2 and probably also the lymphokine, gamma interferon (IFN)[353] at the level of messenger RNA transcription[354].

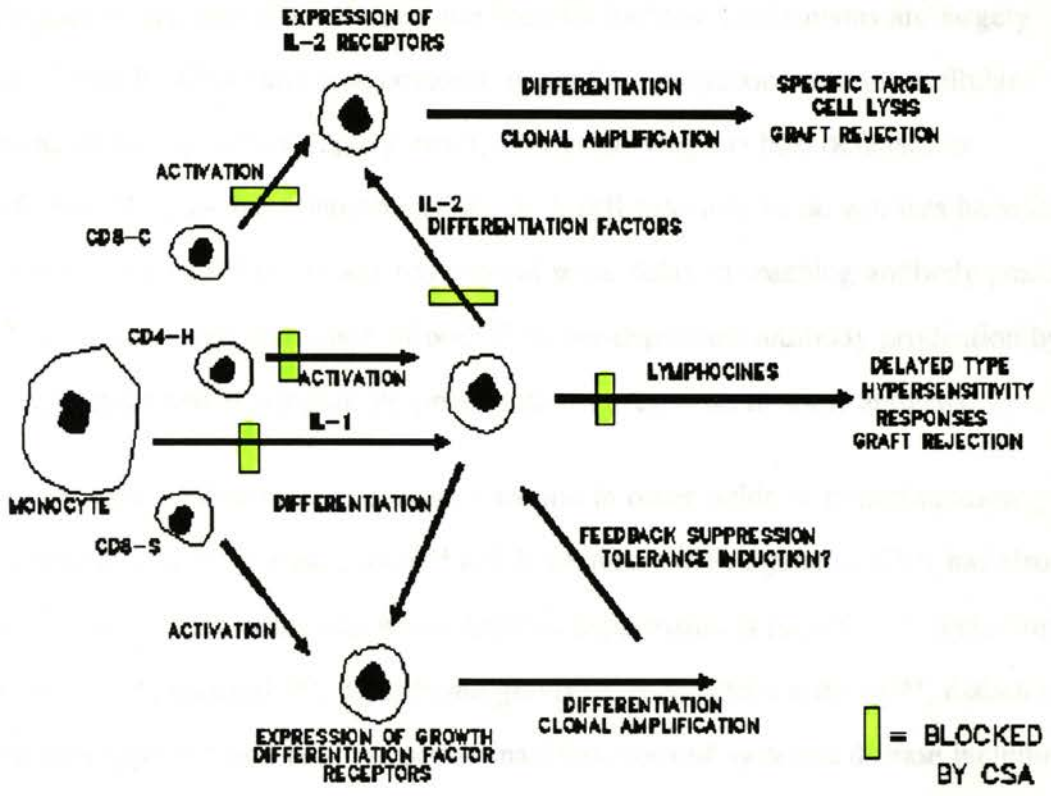
CSA does not inhibit the clonal expansion of activated cells expressing the IL-2 receptor in response to exogenous IL-2, but is thought to act on precursor cytotoxic T cells by inhibiting them from acquiring responsiveness to IL-2, possibly by inhibiting IL-2 receptor formation. Therefore, although clonal expansion of cytotoxic T lymphocytes is inhibited, once generated mature cytotoxic T cells are resistant to CSA even at high concentrations[347], indicating that CSA is not lymphotoxic[305] and should be given early to prophylactically prevent graft rejection. The effects of CSA are summarized in figure 21 (page 140).

The effects of CSA outlined above are extremely dose dependent[305]. IL-2 and other lymphokine production is the most sensitive of the lymphocyte functions (10-20 ng/mL), IL-2 receptor formation is less sensitive (100-200 ng/mL) and monocyte/macrophage function is least sensitive. Suppressor T activation (>1000 ng/mL) and IL-2 dependent proliferation (>1000 ng/mL) are relatively resistant. The figures given in brackets represent the 50% inhibitory concentration of CSA in vitro[305].

An important feature of CSA is that it spares the activation and amplification of suppressor regulatory T lymphocytes. These regulatory cells have been implicated

in the induction and maintenance of allograft tolerance[413]. Therefore, the imbalance between suppressor T cell activation and T helper and cytotoxic T cells induced by CSA should lead to tolerance induction or facilitated graft prolongation[305]. However, tolerance has not demonstrated, but in human renal transplant patients there does seem to be a correlation between the presence of donor-specific suppressor T cells and a low incidence of rejection[414].

Work from a number of laboratories suggests that CSA does inhibit antigen presentation by accessory cells, although the dose of CSA required is higher than that required to inhibit lymphokine production[305,415-417]. The mechanism of this inhibition is uncertain, one suggestion is that the expression of class II antigens is reduced by CSA but this has not been confirmed. Another suggestion is that CSA causes release of prostaglandin E_2, this is a powerful mediator and its functions include many of the suppressor functions that CSA is known to affect[418].



CD8-C = CD8 cytotoxic T cell subset.

CD4-H = CD4 T helper cell subset.

CD8-S = CD8 suppressor T cell subset.

Fig. 21: Mechanisms of actions of cyclosporine.

Effect of CSA on the immune response to alloantigens in the mixed lymphocyte response. Schematic representation of an allograft response and the primary effect of CSA.

Adapted from: Hess AD, Esa AH, Colombani PM.

Mechanisms of action of cyclosporine: effect on cells of the immune system and on subcellular events in T cell activation. Transplant Proc 1988; 20 (2 suppl.2): 29-40.

Despite its potent effect on T cell dependent immune responses, the integrity of the phagocytic cell and other antigen non-specific immune mechanisms are largely unaffected by CSA, and phagocytosis, oxyradical production and intracellular bacterial killing remain largely intact, thus preserving the host defenses to infection[355]. CSA has a variable effect on B cell function, some workers have found minimal changes[356] but others have found some delay in reaching antibody peaks[357]. There is, however, inhibition of both T helper-dependent antibody production by B lymphocytes and B lymphocyte proliferation in response to some antigens.

CSA is now used widely to prevent rejection in other fields of transplantation including renal[363], cardiac, liver[364] and bone marrow[365]. Systemic CSA has also been used in other situations where immunosuppression is required[318], including uveitis[317,366], psoriasis[367], myasthenia gravis[368], rheumatoid arthritis[369], diabetes mellitus type 1[370] and in some ocular manifestations of systemic disease including Grave's ophthalmopathy[371,372], Betçhets disease[373], peripheral corneal melting disease[374], Sjögren's syndrome and also in Moorens ulcer[375].

In laboratory studies CSA administered either locally or systemically has been shown to be of benefit in preventing corneal graft rejection[280-283,286]. Our studies using a rabbit model for high-risk keratoplasty demonstrated an improvement in graft survival using topical CSA but the results using systemic CSA were significantly better[314]. In this animal study CSA was administered for 100 days, treatment was then stopped and the animals were observed for a further 100 days. Eight of eleven grafts, in rabbits receiving systemic CSA, were clear at the end of the treatment period and these all remained clear during the observation period; indicating that CSA may confer long term benefit.

The use of systemic CSA is associated with a number of side-effects most notably renal damage[376,377]. The mechanism of nephrotoxicity is not fully understood, CSA appears to decrease renal blood flow, decrease glomerular filtration rate and

increase renal vascular resistance. CSA nephrotoxicity is now classified as functional, without significant morphological changes, or morphologic forms with tubular and/or vascular-interstitial changes[376]. Careful monitoring of the serum creatinine level is necessary to detect early renal damage which may be reversible by lowering the dosage. However, it has been shown that patients receiving high doses of systemic CSA for uveitis may develop biopsy proved irreversible histologic nephrotoxic changes despite normal creatinine levels[377]. Other side-effects of CSA are hypertension[378], hepatotoxicity[379], hirsuitism in females, skin rashes, propensity to infections and gum hypertrophy.

5.2 The Use Of Topical Cyclosporine In High-risk Keratoplasty.

Because of the potential side-effects of systemic CSA and also the cost of treatment, much interest has centred around the use of topical CSA to help prevent corneal graft rejection. Shepherd et al[281] in 1980 used CSA both intramuscularly and topically in a rabbit corneal graft model. The animals receiving systemic CSA showed a marked prolongation in graft survival compared to controls. There was no improvement in graft survival in the animals given topical CSA, however some workers using an analogous model did find some benefit[380]. The preparation used was a 1% solution of CSA in arachis oil, given five times daily for 28 days. Graft rejection in this model was induced by skin grafts 14 days after the corneal graft was performed. The authors felt that failure of topical CSA to improve graft survival was related to the model, CSA being "more effective if given with the antigen". In the light of present knowledge concerning the mode of action of CSA this statement is probably at least partially correct. CSA prevents recognition of foreign antigens and if given topically may work on the afferent arc of the immune cycle. Skin grafting produces a "second set" allograft reaction and would bypass this afferent block and initiate the efferent arc against which CSA would be less effective. In our rabbit model[314] rejection was induced by local factors, chiefly vascularization, and the rejection process was initiated by corneal antigens as a "single set" rejection. We found that topical CSA (2% CSA in castor oil given four times daily) did improve graft survival but not so effectively as systemic CSA. The presumption being that topical CSA prevented the initiation of the afferent arc. This suggestion had been proposed earlier by Hunter et al[286,293,381] who also used topical CSA 1% in arachis oil five times daily for 28 days. None of their rabbits rejected their grafts while on topical CSA although all eventually did rejected by the day 64; overall there was a significant improvement in graft survival. In their model rejection was encouraged by the peripheral placement of grafts to encourage

adhesions of iris to the cornea. Although this does not simulate the human situation, as our model does for high-risk keratoplasty, it does allow the corneal rejection process to be initiated by local causes and topical CSA would be able to suppress the afferent immune arc. The beneficial effect of topical 1% CSA on graft survival has been demonstrated by a number of other workers in rabbits using an arachis oil[288] or olive oil vehicle[288,295,382]. Williams et al[288] compared topical CSA to topical 1% prednisolone acetate and found that both prolonged graft survival, but that prednisolone was significantly more effective. They also found that anterior segment inflammation and graft vascularization were considerably less marked in animals treated with steroid. Like previous workers[281,286] they encountered problems using CSA in an arachis oil vehicle. Excoriation, loss of hair, and conjunctival irritation occurred and in some cases the animals had to be killed because of the severity of the reaction. Similar problems were encountered using an olive oil vehicle although graft survival was slightly improved. It is possible that the irritation from the arachis oil counteracts the beneficial effects of the CSA. Using castor oil as a vehicle, we experienced none of the above side effects and no animal had to be withdrawn from our animal study for these reasons.

It is not known exactly how topical CSA exerts its beneficial effects on graft survival, one uncertainty being the amount of CSA that is absorbed by the cornea and other ocular structures. Foets et al[295] found high plasma levels of CSA in their animals treated topically, mean values were 141 μg/l which is approaching therapeutic levels recommended in man. They did not consider that CSA was exerting a systemic effect because topical CSA applied to the contralateral normal eye did not prevent graft rejection in the operated eye. Similar CSA levels were found in the aqueous, when topical CSA was applied to the operated eye; mean CSA levels of 144 μg/l were found in the aqueous compared to 131 μg/l in the fellow untreated eye. CSA levels were measured by radio-immunoassay using the kit manufactured by Sandoz[289]. Similar plasma levels following topical CSA

treatment in rabbits were found by Bell and Hunnisett[383], but in this study aqueous levels were considerably lower. Conflicting results were found by Williams et al[288] who found undetectable levels of CSA in both the plasma and the aqueous of topical CSA treated rabbits, using the same immuno-assay technique. In our animal study[314] CSA was not detectable in the blood of rabbits treated with topical CSA. In a rabbit study in which a single application of 10% CSA ointment in a lanolin petrolatum vehicle was used, Mosteller et al[312] found peak corneal levels at 3 hours (926 ng/ml) but substantial levels were still present at 24 hours (672 ng/ml). Relatively low concentrations were measured in the aqueous humor and serum, peak levels measured were 100 ng/ml and <100 ng/ml respectively. This suggested that CSA tends to pool in the corneal stroma. Assuming total absorption of the drug delivered, the dose given to the rabbits was calculated to be 3 mg/Kg of body weight. A similar amount of topically applied 10% CSA ointment would provide only a 0.1 mg/Kg dose for a 70 Kg human, well below therapeutic systemic doses. In a study using human volunteers about to undergo cataract surgery, Diaz-Llopis and Menezo[384] found subtherapeutic aqueous CSA levels after topical application of 2% CSA in olive oil and after systemic administration. These results would indicate that CSA pools in the cornea and probably exerts an immunological effect in this organ, but that aqueous levels are lower than those necessary to be therapeutic. To overcome the problems associated with the irritating effects of the vehicles used, Mahlberg et al[385] used CSA-collagen shields. The shields contained 4mg of CSA and were changed every second day. These workers used the animal model developed by us: all their control rabbits rejected whereas all CSA treated grafts remained clear. The authors postulated that higher corneal and aqueous levels were obtained but no CSA levels were performed. A recent report showed that a significant improvement in graft survival was obtained in a rat model, when CSA was encapsulated in liposomes compared to solution in olive oil; this is another potentially useful vehicle for CSA administration.

Conflicting reports exist regarding the long term benefits of topical CSA. All studies that did not utilize skin grafting to initiate rejection showed improvement in graft survival compared to controls. Hunter et al[381] gave topical CSA for four weeks, no graft rejected while on treatment but all grafts subsequently underwent rejection by the 64th postoperative day. Likewise Foets et al[295,382] found that no animal rejected while on the four week course of topical treatment. Williams et al[288] reported that some grafts did reject while on treatment and similar findings were made in our study[314], in both studies topical CSA was continued for 100 days. After four weeks of topical CSA treatment given five times daily the grafts were treated with a less frequent dosage in the study by Foets et al[295]. One graft began to reject on a twice daily regimen but this was reversed by increasing the frequency to four times daily. These authors conclude that a three or four times daily regimen is optimum to prevent rejection. When treatment was stopped altogether at six months, all the grafts rejected within four weeks, implying that no long term benefit or tolerance was imparted. This is contrary to the findings of Hunter et al[286] who reported that the 44% of the grafts treated for three months remained clear. Two of the seven rabbits in this group rejected soon after the dose had been reduced from twice to once daily applications and the other three rejected soon after treatment was stopped altogether. This would also support the finding that topical CSA needs to be given at least twice daily. In our study[314] all grafts that were clear on treatment at 100 days, remained clear on no treatment for a further 100 days. In order to test whether the absence of rejection was due to the development of true immunological tolerance, Hunter et al[292] revascularized the grafted corneas of five long term survivors who had been treated with topical CSA. Four of these grafts rejected within 30 days. This would suggest that the prolonged graft survival, after treatment was stopped, is due to the return of a degree of immunological privilege of the cornea as the vessels regress, rather than the presence of specific immune tolerance.

No commercial topical CSA preparation is currently available, and in several of the ophthalmic studies published drops of the commercially available oral formulation were instilled into the eye. In other studies the active drug was dissolved in a variety of substances including mineral, corn and castor oils. Hoffmann and Wiederholt[386] first reported on the use of topical CSA in humans in 1985. They initially used it in four patients with glaucoma undergoing keratoplasty. The preparation used was 2% CSA in miglyol (derived from castor oil), this was administered initially eight times daily, tailing off to twice daily at six months, no concomitant corticosteroids were used. One graft was lost from rejection and all experienced severe burning and conjunctival irritation from the preparation. In the second part of their trial 2% CSA in castor oil was used in conjunction with topical corticosteroids. This preparation of CSA was better tolerated although all the eyes were bloodshot and exhibited punctate keratopathy. No mention is made in the report as to the status of the recipient corneas preoperatively; but overall, of the 25 grafts treated, four rejected and a further two underwent a reversible rejection episode. During the following year, 1986, the use of topical 2% CSA was reported in normal healthy adults[387] and in two patients with immune corneoscleral disease[388]. Sandoz, the manufacturers of CSA, undertook a multicentre trial in 1985 using a 2% CSA solution in castor oil in high-risk keratoplasty. The drops were used four times daily in addition to a normal regimen of topical corticosteroids. I participated in this trial but unfortunately the castor oil proved to be too irritating and this appeared to negate any possible benefit derived from the CSA. Three of the first four patients in the trial rejected their grafts and the medication was not well tolerated. I subsequently withdrew from the trial and soon after the trial was stopped because similar findings were reported by other participating centres. Goichot-Bonnat et al in 1987[389] reported on a randomized study of 54 eyes comparing topical 2% CSA in castor oil and dexamethasone 1%, to topical dexamethasone 1% alone. At follow-up of one year 88% of the grafts receiving topical CSA and dexamethasone were clear compared to 35% in the group treated

by dexamethasone alone. In 1989 Belin et al[390] reported on the use of topical 2% CSA in olive oil combined with topical steroid in eleven cases of high-risk keratoplasty. Both drops were given four times daily for three months, three times daily for three months and then twice daily until a year. High-risk was defined as a history of previous immunologic graft failure, greater than one quadrant of deep stromal vascularization or an opacity due to a severe alkali burn. Ten of the eleven remained clear, with an average follow-up of 16 months. These workers were able to detect blood levels of CSA but these were all below therapeutic values. In both these studies[389,390] a transient epithelial keratitis developed, but this seemed to spontaneously resolve after six to twelve months.

The success obtained by the above studies encouraged Sandoz, the manufacturers of CSA, to initiate other trials of topical CSA. One study (K 301) was commenced in the United States in early 1990 with the aim of recruiting 300 patients. The other study (OL K-204) commenced later in the same year and was conducted in Europe and South Africa; three hundred patients were to be entered; with each participating centre, of which we were one, recruiting 20 patients. Both studies were double-masked clinical trials to compare the safety and efficacy of CSA 2% ophthalmic ointment versus placebo in the prevention of graft rejection in high-risk corneal transplantation patients. The ointment was a new formulation of CSA in an ointment base with chlorobutanol as a preservative. The preparation was well tolerated in both humans and animals and resulted in significant concentrations of CSA in the anterior and posterior ocular tissues at 0.3 hours[391]. The greatest concentration was found in the cornea which appeared to act as a reservoir for the other ocular tissues. Minimal absorption into the systemic circulation was found with peak blood levels of <2 ng Eq/g being reported (equivalent intravenous dose gives typical levels of 100 ng Eq/g)[391]. The entry criteria and follow-up were strictly controlled and will not be dealt with at great length at this time. All patients admitted into the trial were high-risk keratoplasty patients defined as meeting one or both of the following

criteria;

1. two, three, or four quadrants of corneal stromal vascularization. Vessels were to be greater than 2mm in length and strict instructions as to how quadrants were to be counted were given.

2. a documented history of previous allograft rejection in the eye being considered for corneal transplantation.

The study medication was applied twice daily for the first year and thereafter once daily for a further six months. Concomitant topical corticosteroids were given with a tapering dose over a similar period. Strict criteria were given for diagnosing graft rejection which was further divided into mild or severe, according to the signs and symptoms of the episode. Treatment regimens that were to be followed in the event of either type of rejection episode were strictly laid down.

The USA study finished earlier than our study and unfortunately the results showed that there was no significant improvement in graft survival when topical CSA was used compared to placebo (personal communication, Sandoz Pharmaceutical). In view of these findings Sandoz considered it unethical to continue the trial and our study was terminated early. The treatment code in our study has not been broken yet nor have the results been analysed, but it is unlikely that our results will differ from the American findings. Why previous reports showed a beneficial effect of topical CSA whereas the double blind trial did not, has not been adequately explained. It is too early to know whether these studies will be regarded as definitive or whether further studies will be undertaken. It is extremely disappointing that this safe and potentially useful treatment has been shown, at least temporarily, to be valueless.

5.3 Preliminary Report On The Use Of Systemic Cyclosporine.

Introduction.

Although CSA was widely used in 1985 for other forms of transplantation, no published reports existed regarding its use in corneal grafting and no details were available from the manufacturers. As a preliminary study it was decided that the drug would be given to a small number of patients who were blind from severe corneal disease and who were otherwise systemically healthy. The CSA regimen used in the renal transplant unit at Groote Schuur Hospital was adopted, this included the concomitant use of systemic corticosteroids.

Patients and methods.

Four patients (six eyes) with severe corneal disease underwent corneal grafting with the concomitant use of systemic CSA. All were fit healthy males. Preoperative assessment revealed no systemic disease and serum urea and electrolytes, creatinine, liver function tests and blood counts were within normal limits. Urinalysis showed no abnormalities. All the patients, except case 4, were blind from severe corneal disease, and all eyes treated represented poor prognostic cases with respect to graft outcome. Every eye was heavily vascularized superficially and deeply in all four quadrants and four eyes had had rejection of previous corneal grafts. Informed consent for surgery and the use of CSA was obtained from all patients.

Case 1.

A forty year old male sustained alkali burns to both eyes. Uncontrolled glaucoma required bilateral filtering procedures on two occasions. Corneal grafts with extracapsular lens extractions had been performed but both grafts subsequently rejected at two and five months, despite high doses of systemic corticosteroids.

Vision was perception of light only, the corneas were opaque and highly vascularized (figure 22, page 152). Bilateral corneal grafts were performed (figure 23, page 153).

Case 2.

A 57 year old man with a blind right eye from a perforated Mooren's ulcer, developed a similar ulcer involving 270° of the limbus of his left eye. The central cornea was opaque and vascularized; the vision was hand movements. Peritomy and a patch graft failed to halt the disease. CSA was administered and further progression stopped and the ulcer slowly resolved. Trough CSA blood levels were measured[289] and maintained at approximately 200 ng/ml. Six weeks later a left central corneal graft was performed. CSA was increased to keep the whole blood CSA levels at between 200-400 ng/ml, this dose was continued for three months and then tailed off with treatment stopping a month later.

Case 3.

A 34 year old male sustained bilateral alkali burns, causing opaque corneas with neovascularization in all quadrants. Bilateral drainage procedures were required to control glaucoma. Vision was hand movements in both eyes. Bilateral corneal grafts with extracapsular lens extractions were performed.

Case 4.

A 21 year old male with bilateral keratoconus had a successful corneal graft to his left eye. Three corneal grafts to the right eye had rejected despite high doses of systemic corticosteroids. The cornea was now opaque and heavily vascularized with a vision of perception of light only. The patient requested a further corneal graft to obtain the binocularity necessary for his employment.



Fig. 22(a). Case 1: preoperative photographs of the right eye showing vascularization in 4 quadrants.

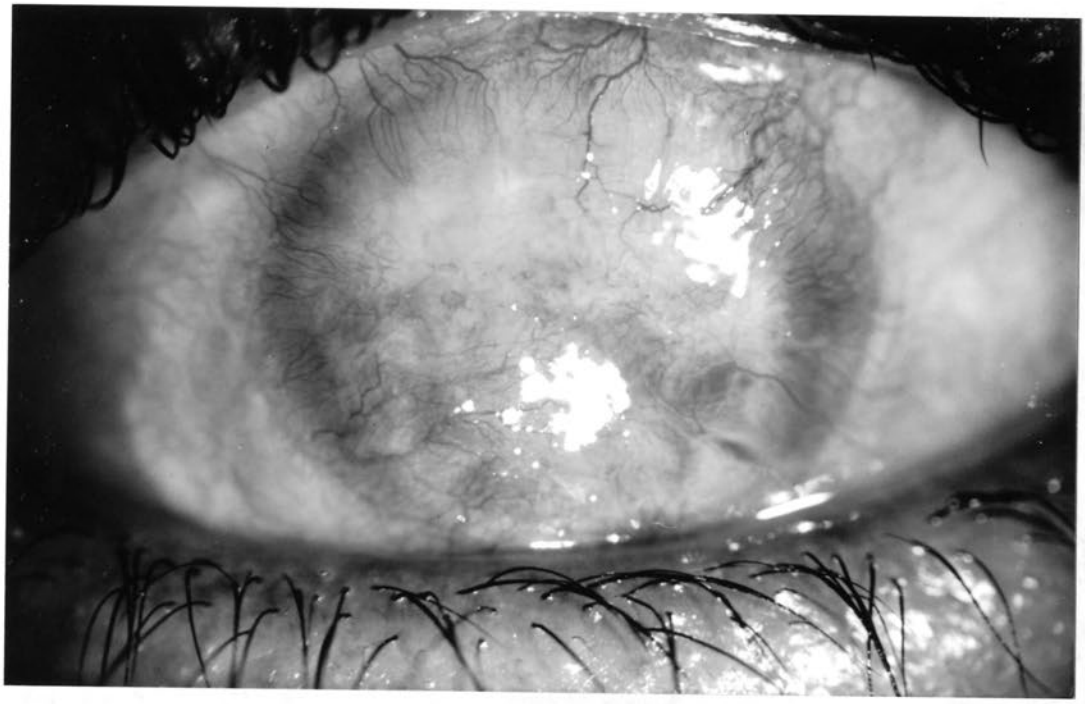


Fig. 22(b). Case 1: preoperative photograph of the left eye showing vascularization of 4 quadrants.

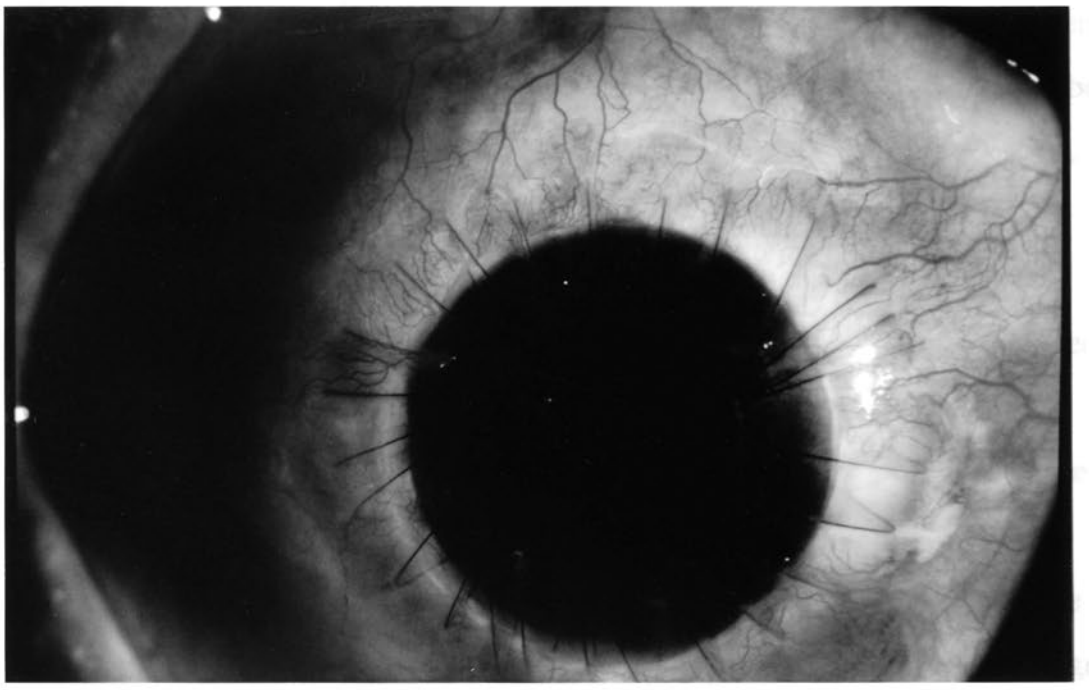


Fig.23(a). Case 1: postoperative photograph of right eye.

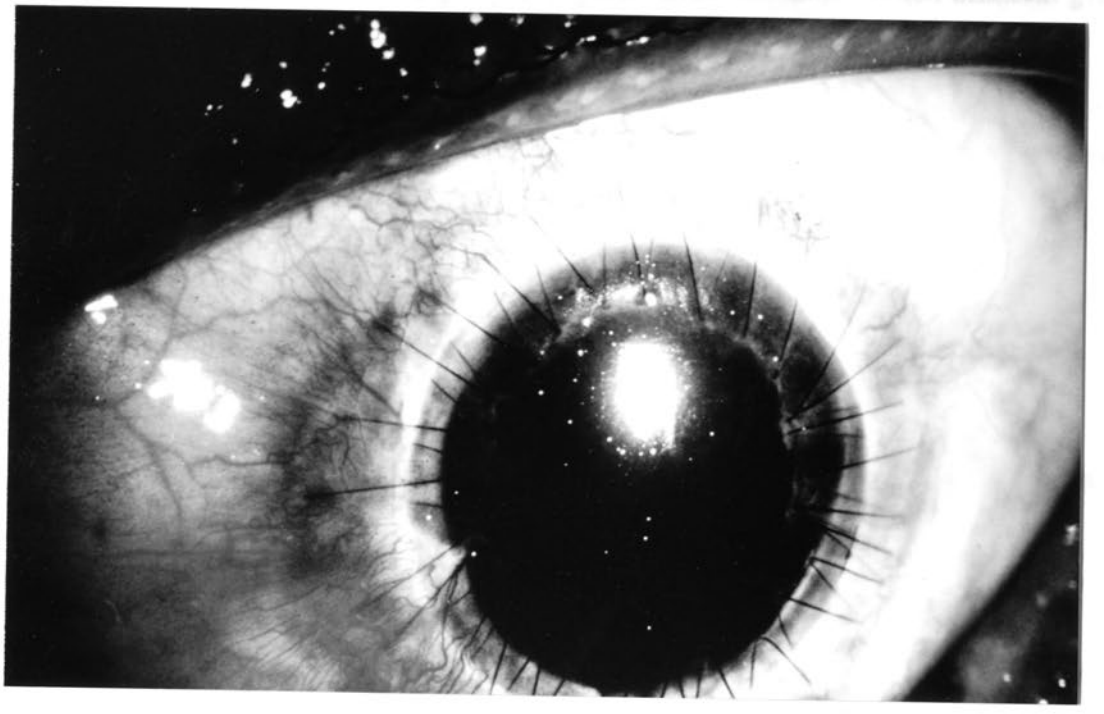


Fig.23(b). Case 1: postoperative photograph of left eye.

The technique of corneal grafting was similar in all the eyes. Donor corneas were preserved either as whole eyes in a moist chamber, with the corneoscleral disc being removed just prior to grafting, or as discs preserved in a culture medium (M.K. solution) at 4°C[392]. All donor grafts were between 7.5 and 8.5 mm and were 0.4mm larger than the recipient bed. A number of patients had simultaneous extracapsular cataract extraction as indicated above, in some cases with placement of posterior chamber intraocular lenses. All surgery was performed by myself. Tissue matching of donor and recipient was not undertaken. Sodium hyaluronate 1% (Healon) was used to protect donor endothelium and to dissect off any iris adhesions and a continuous 10/0 nylon suture was used to secure the grafts. The anterior chamber was reformed with balanced salt solution at completion of surgery and sub-conjunctival injections of gentamicin 20 mg and betamethasone (Celestone) 2 mg were given. Postoperatively all patients received topical dexamethasone 0.1% drops four times daily initially, the frequency was reduced over the ensuing months and at six months a twice daily dosage was continued. Topical antibiotics, anti-glaucoma therapy and cycloplegics were given when required. When bilateral grafts were performed an interval of one month between operations was allowed.

CSA was commenced at the time of operation, except for case two who had been on therapy for six weeks to control his Mooren's ulcer. A dose of 500mg twice daily was given on days 1 and 2, followed by 250mg twice daily on days 3 and 4. Thereafter the dose was regulated to maintain a trough serum CSA levels of between 200-400ng/ml: the dose necessary varied between 3-4mg/Kg/day. Prednisone 100mg daily was given for three days, followed by 25mg daily. This regimen was maintained for four months, thereafter CSA was reduced to 50mg twice daily (approximately 1.5mg/Kg/day) to maintain CSA trough levels of between 100-200ng/ml. At four months the prednisone was tailed off over a period of six weeks and then stopped. Case 1 continued on CSA 50mg twice daily and also prednisone 10mg daily after the four month period but stopped both at seven months

when he moved from Cape Town and follow up became less regular. Case 2 had both CSA and prednisone tailed off after three months for a similar reason. Details of CSA therapy are shown in figure 24 (page 156).

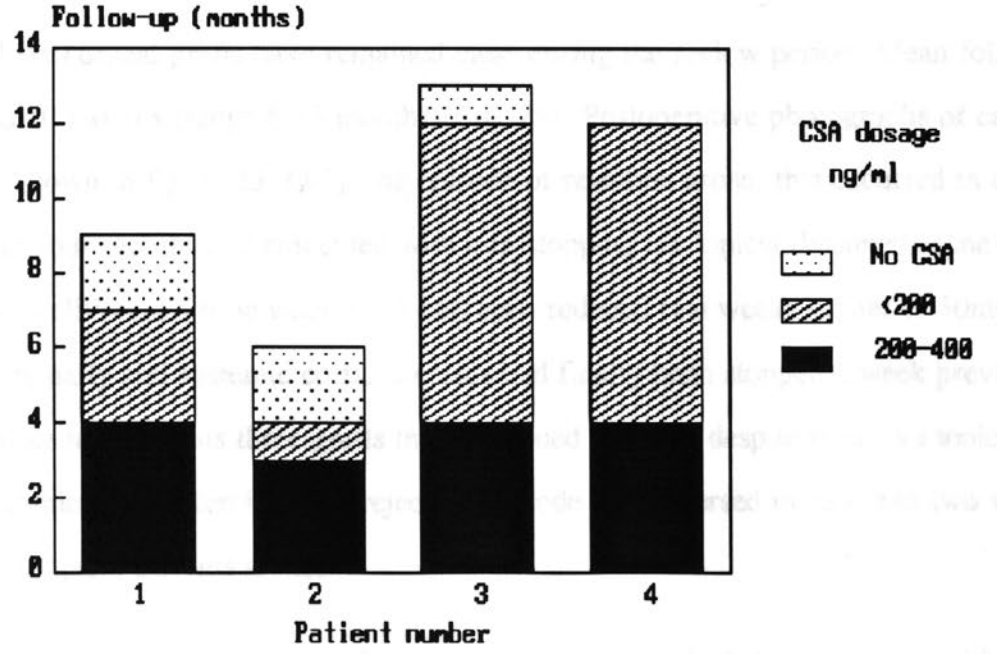


Fig. 24. Follow-up periods of the 4 patients and dosage of CSA expressed as trough serum levels (ng/ml).

Results.

All six corneal grafts have remained clear during the review period. Mean follow up was 10 months (range 6-13 months)(Fig. 24). Postoperative photographs of case 1 are shown in figure 23. Only one episode of rejection arose, this occurred in case 4 after four months and coincided with him stopping his topical dexamethasone of his own volition at a time when CSA had been reduced two weeks earlier to 50mg twice daily and systemic corticosteroids had finally been stopped a week previously. Unlike his previous three grafts that continued to reject despite intensive topical and systemic corticosteroids, this rejection episode was reversed in less than two weeks with topical steroids alone.

A rise in serum creatinine and urea occurred in all patients but remained within normal limits, except in case 1 where creatinine rose to 125 mmol/ml: this responded rapidly to lowering CSA dosage. Liver function tests, blood counts and urinalysis remained normal throughout and no other side-effects of CSA, such as gum hypertrophy, neuraesthesia, tremor or lymphoma were encountered.

Discussion.

All the corneas in this series were at high-risk of rejection because of the high degree of vascularization and history of previous graft rejection. Four eyes had a history of alkali burns. These are notorious for the damage inflicted on the anterior segment of the eye; which results in opaque vascularized corneas, glaucoma and cataracts. After repeated grafting long term results show a success rate of only 30% in these eyes[315].

All the grafts treated in this series had two or more of these poor prognostic factors, and therefore represented high-risk patients in respect to graft prognosis. In high-risk patients the incidence of rejection is increased, with rates of 60-70% being reported despite maximal conventional therapy with systemic and topical

corticosteroids[191,199,234]. Although corneal grafts can reject after more than 20 years, most rejection occurs in the first nine months[234] and especially during the first three months[4]. It is significant that all six grafts in this study remained clear with only one rejection episode having occurred, and this was easily reversed. Six previous grafts in our patients (3 grafts in case 4) had rejected and ultimately failed despite maximal topical and systemic steroids.

The greatly reduced incidence and severity of the rejection response in this series may be attributed to the use of CSA rather than to the use of systemic corticosteroids which were stopped in all, but case 1, by four months after surgery. This small study does not indicate the dose of CSA necessary to prevent graft rejection nor the duration of treatment required. The study suggests that after four months the dose can be reduced to lower levels (1.5 mg/Kg/day), however this lowers the serum concentration to sub-therapeutic levels for other forms of transplantation. Perhaps the drug can be stopped altogether at this stage, a conclusion suggested by cases 1 and 2 who remained clear for two months despite stopping CSA. The dosage of oral corticosteroids required is also not known; the large loading doses and long duration of treatment given in case 1 is probably unnecessary as smaller initial doses, with cessation of corticosteroid therapy at four months, did not adversely affect graft outcome in the other cases. However, caution must be maintained because the only rejection episode encountered occurred shortly after CSA was reduced and systemic steroids were withdrawn. Periocular and intraocular inflammation are potent precipitating factors for rejection, and must be controlled in severely damaged eyes: possibly topical steroids alone are sufficient to do this.

It was hoped that effective immunosuppression, given during the initial healing stage, would allow an avascular scar to be formed between donor and recipient. This would hopefully act as a barrier between the donor cornea and the host's immunological system, thus re-establishing a degree of immunological privilege.

Interestingly all eyes in this series developed blood vessels transgressing this interface (Fig. 23). Characteristically the single rejection episode seen (case 4) started at this point, and expanded forming a typical Khodadoust rejection line[191] in the stroma and endothelial layers. Therefore an avenue for recognition and subsequent rejection still exists in our patients. Possibly topical steroids alone will be sufficient to suppress rejection once the patient is through the critical early postoperative period, as occurred in case 4.

In our high-risk patients, CSA appears to have had a favourable effect in preventing corneal graft rejection. However, the number of patients reported is small and only a well designed prospective study will prove whether CSA has a use in corneal transplantation. Its potentially serious side-effects still provide cause for concern, especially in patients who are fit and healthy apart from their eye complaint. In our patients small doses of CSA combined with careful monitoring prevented significant complications. Recently we have maintained stricter control by monitoring creatinine clearance, as well as serum creatinine and trough serum CSA levels. In patients blind from corneal disease, to whom little hope of a successful corneal graft can be given, the chance of vision outweighs the small risks involved in the use of CSA. Thus encouraged by the results of this small preliminary trial, a prospective study was instigated into the use of systemic CSA in high-risk keratoplasty.

5.4 The Use Of Systemic Cyclosporine In High-risk Keratoplasty: A Prospective Trial.

Introduction

In laboratory studies CSA administered either locally or systemically has been shown to be of benefit in preventing graft rejection[280-283,286]. Our studies using a rabbit model for high risk keratoplasty demonstrated an improvement in graft survival using topical CSA but the results using systemic CSA were significantly better[314]. In this study CSA was administered for 100 days, treatment was then stopped and the animals were observed for a further 100 days. Eight of eleven grafts, in rabbits receiving systemic CSA, were clear at the end of the treatment period. These all remained clear during the observation period; indicating that CSA may confer long term benefit, possibly by the re-establishment of a degree of immunological privilege. In a preliminary report in human high risk keratoplasty patients, discussed in the previous section, I reported the use of systemic CSA given prophylactically in conjunction with systemic corticosteroids[393]. In this section of the thesis I present the results of a prospective study in which patients undergoing high-risk keratoplasty were treated with systemic corticosteroids alone or in combination with systemic CSA. The results are compared to historical controls; these were similar high-risk keratoplasty patients operated upon using identical techniques and treated with topical corticosteroids alone.

Patients and methods

All patients included in the study were high-risk keratoplasty patients. Many of them had had previously rejected corneal grafts and all had visual acuities of less than 6/60 (20/200) in the affected eye. A high-risk cornea was defined as one in which all 4 quadrants were vascularised, both superficially and deeply, and in which the total number of vessels reaching the proposed donor site exceeded sixteen.

The following three groups were studied:

A. Patients receiving topical corticosteroids only.

B. Patients receiving topical and systemic corticosteroids.

C. Patients receiving topical and systemic corticosteroids together with systemic cyclosporin.

At the commencement of the trial all high-risk keratoplasty patients undergoing surgery were invited to participate in the trial providing they fulfilled the criteria outlined above. All patients who agreed to participate gave informed consent and were randomly allocated to either Group B or C. The only exception were patients originally allocated to Group B and who then rejected their grafts despite being given systemic corticosteroids. These patients when they re-entered the trial were allocated to Group C and received CSA. Patients in Groups B and C were therefore studied prospectively; twenty subjects were allocated to each group. A comparable control group of patients treated with topical corticosteroids alone was obtained historically. At the commencement of the trial the previous twenty patients who had undergone corneal grafting and who fulfilled the criteria of high risk keratoplasty were studied and formed Group A. Patients in all groups were healthy with no serious systemic disease; those receiving systemic medication in Groups B and C had a full blood and metabolic work-up with particular attention given to the detection of renal and hepatic disease. The original corneal pathology of the patients in the three groups is shown in Table VII (page162).

Table VII

Indications For Keratoplasty

Diagnosis	Group A	Group B	Group C
Alkaline burns	4	3	5
Herpes simplex	1	2	1
Keratoconus (regraft)	2	5	4
Previous bacterial keratitis	6	4	3
Fuch's (regraft)	2	3	2
Measles keratitis	2	0	2
I.K. (regraft)	2	1	2
Trauma	1	2	1
Total	**20**	**20**	**20**

All donor grafts were between 7.5 and 8.5 mm and were 0.5 mm larger than the recipient bed. Donor corneas were preserved as whole eyes, with the corneoscleral disc being removed just prior to grafting, or as discs preserved in culture medium (M.K. or KSol solution) at 4°C. The keratoplasty techniques were identical in all patients; a number of patients had simultaneous extracapsular cataract extraction, in some cases with placement of posterior chamber intraocular lenses. All surgery was performed by myself. Tissue matching of donor and recipient was not undertaken. Sodium hyaluronate 1% (Healon) was used to protect donor endothelium and to dissect any iris adhesions. Eight buried interrupted 10/0 nylon sutures and a continuous 10/0 nylon suture were used to secure the graft. The anterior chamber was reformed with balanced salt solution at completion of surgery and subconjunctival injections of gentamicin 20 mg and betamethasone (Celestone) 1.5 mg were given.

Postoperatively all patients received topical dexamethasone 0.1% drops four times daily initially, the frequency was reduced over the ensuing months and was stopped at six months unless persistent inflammation required extended therapy. Topical antibiotics and cycloplegics were given in the early postoperative period.

Patients in group B were given systemic corticosteroids in addition to topical therapy. Methylprednisolone 125 mg intravenously was given at completion of surgery. The following day oral prednisone 25mg daily was commenced. After one month the dose was reduced to 10 mg daily and this was continued for three months; the drug was then tapered off and stopped four months after surgery.

Patients in group C were given both topical and systemic corticosteroids according to the above regimens. In addition CSA was given to maintain therapeutic whole blood levels of between 250 and 400 ng/ml. CSA was administered twice daily by mouth, except the first dose that was given intravenously at the time of surgery. An initial loading dose of 15 mg\Kg\day was given for two days followed by 7.5

mg\Kg\day for two days, thereafter the dose was adjusted to maintain therapeutic blood levels. The usual dose given was 3-4 mg\Kg\day. During the early postoperative period blood CSA levels, together with serum chemistry, were estimated twice weekly; creatinine clearance tests were performed weekly. When the blood CSA levels stabilised, usually at three or four weeks, the frequency of the tests was reduced to weekly and eventually to fortnightly. CSA was continued for one year in the early period of the trial (11 patients). The marked improvement in prognosis, apparent in these patients, made us consider that this length of therapy was possibly unnecessarily long and CSA treatment was reduced to four months. By this time it was hoped that the immunological privilege of the cornea would be re-established.

Rejection was diagnosed when a previously clear cornea became significantly thickened, when a typical Khodadoust line developed or when unexplained intraocular inflammation or stromal infiltration occurred. The treatment of rejection consisted of hourly dexamethasone 0.1% drops, a subconjunctival injection of betamethasone (Celestone) 1.5 mg alternate days and oral prednisone 60 mg daily. Treatment was continued until the inflammation subsided and was then gradually withdrawn. The time to the commencement of the rejection episode was noted.

The results were analyzed by survival analysis methods using generalized Wilcoxon[290] and Kruskal-Wallis[291] tests.

Results

Group A

This group consisted of 20 patients undergoing keratoplasty (20 eyes). One patient was excluded from the trial because of primary graft failure. The mean age was 33.6 years (range 3 - 68), ten eyes had had unsuccessful grafts previously (Table VIII, p.167). Of the 19 grafts only two survived giving an overall success rate of 10.5% (Table IX, p.168). The median time to rejection was four months, in two grafts the initial rejection episode was reversed but the grafts subsequently rejected. The follow up of the two clear grafts was 31 and 38 months respectively. Sixteen grafts started to reject while still on the topical corticosteroid treatment.

Group B

This group consisted of 20 patients (20 eyes), there were no exclusions from the trial. The mean age was 33 years (range 8 - 67), 16 eyes had been grafted in the past (Table VIII, p.167). Only one graft (5%) survived, median interval to rejection was four months (Table IX, p. 168). Follow up of the remaining clear graft was 18 months. Eleven grafts rejected while the patient was receiving systemic prednisone, all of these rejected during the period when the patient received only 10 mg daily. An initial rejection episode was reversed in three patients, all these grafts subsequently rejected.

Group C

This group consisted of 20 patients (22 eyes). Three patients (4 eyes) were excluded from the trial for the following reasons:

One primary graft failure

One graft failure resulting from dry eye (alkali burn)

One eye lost from trauma.

One graft lost from intractable severe glaucoma.

The mean age was 30.1 years (range 4 - 59) and 15 eyes had been previously grafted (Table VIII, p.167). Of the 18 grafts in the trial only two rejected. In one rejection occurred at 13 months, one month after CSA was stopped. The other patient developed a drug induced skin rash, CSA was stopped after one month and the graft subsequently rejected three months postoperatively. The results of this patient are included in the statistical analysis as a failure because the outcome was treatment related. A further three grafts developed rejection episodes which were successfully treated, two of these episodes occurred while CSA treatment was being used (at two and four months). The remaining episode occurred at seven months, the CSA having been stopped three months earlier. Of the 18 grafts in the trial two rejected giving an overall success rate of 88.9%. Mean follow up was 21.6 months (range 12 - 49) (Table IX, p.168).

Table Nomenclature

Group A : Topical corticosteroids only.

Group B: Topical and systemic corticosteroids.

Group C: Topical and systemic corticosteroids and CSA.

Table VIII

Systemic CSA Study: Patient Data

Group	No. Patients (Eyes)	Age (Yrs) Mean Range	No. Previous Grafts 0	1-2	3+
A	19 (19)	33.6 (3-68)	9	8	2
B	20 (20)	33.0 (8-67)	4	14	2
C	17 (18)	30.1 (4-59)	3	12	3

Table IX

Systemic CSA Study: Graft Outcome Results

Group	No. (%) Grafts Rejected	Median Survival Time (Months)
A	17 (89.5%)	4
B	19 (95.0%)	4
C	2 (11.1%)	>11

An overall statistical comparison was made between graft survival in the three groups using the Kruskal-Wallis test, a significant difference exists (p= 0.0001) (Table X). Multiple comparisons of graft survival were made between the three groups using the Wilcoxon test. No significant difference exists between group A (topical steroids) and group B (topical and systemic steroids) (p= 0.8095). However, a significant difference exists between group C (steroids and CSA) and group A (p= 0.0001) and group B (p= 0.0001). The survival estimates for the three groups are shown in figure 25 (page 170).

Table X

Comparison Of Treatment Regimens

GROUPS	**A vs B**	No significant improvement	p = 0.8095
GROUPS	**C vs A**	Significant improvement	p = 0.0001
GROUPS	**C vs B**	Significant improvement	p = 0.0001

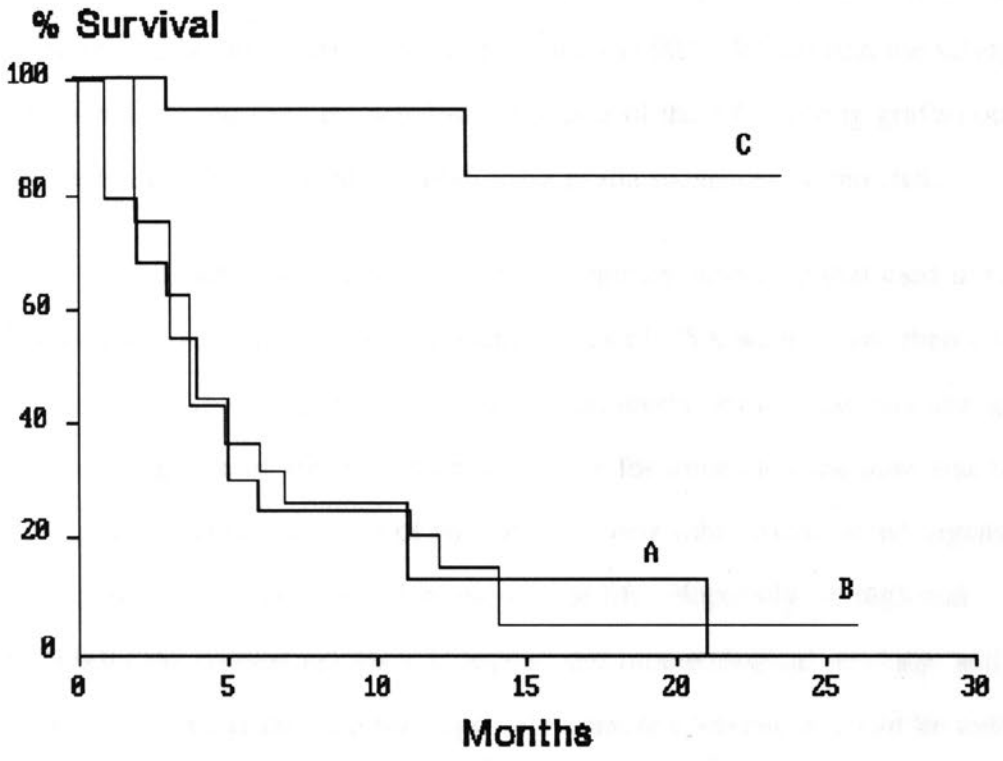


Fig. 25. Systemic CSA study. Survival estimates for the three treatment groups.

Discussion

The success rate of grafting highly vascularised corneas is low with failure rates of between 60 and 90% having been reported[191,199,225,234,315]. The poor success rate is confirmed by the patients in this study who received topical corticosteroids alone, the failure rate in these very high risk patients was 90%. In addition the salvage rate of grafts that started to reject was low; in only 2 of the 17 rejecting grafts could the rejection episode be reversed and both these grafts subsequently rejected.

When I first started using CSA in 1984[393], a regimen similar to that used in renal transplantation was implemented. Loading doses of CSA were given, thereafter the dose was adjusted to maintain therapeutic blood levels. Prednisone was also given, after a loading dose of 100 mg prednisone daily for three days the dose was reduced to 25 mg daily. In the majority of patients receiving other transplanted organs, systemic immunosuppression is maintained for life. Hopefully, in high-risk keratoplasty the corneal vessels will regress and immunological privilege will be re-established. If this is the case then systemic immunosuppression could be withdrawn after a period of time. In our preliminary study 25mg prednisone was given for four months and CSA was given for one year. Using this regimen a success rate of 93% was achieved. It was possible that much of the benefit of this regimen was derived from the use of systemic steroids: this present study was designed to test this hypothesis. The results show that there is no significant benefit to graft survival by using, in addition to topical corticosteroids, systemic steroids in the doses given in this study ($p = 0.8095$).

The use of systemic CSA is associated with a significant improvement in graft survival ($p = 0.0001$). After eleven patients it was felt that a year of CSA treatment was possibly excessive and the period of medication was reduced to four months. There was no difference in graft survival between these two treatment periods. The only graft that rejected after receiving a course of CSA, did so at thirteen months -

one month after a twelve month course of CSA was stopped. Two grafts in the twelve month group, and one in the four month group, had rejection episodes reversed. Low graft numbers and short follow-up prevent meaningful statistical analysis, but clinically the shorter course of CSA appears to be as beneficial as the longer twelve month course. The potential dangers and cost of CSA makes a four month regimen of this drug more attractive.

One patient had CSA withdrawn after one month because of an intractable itchy skin rash: the graft rejected at three months. The rash resolved completely shortly after cessation of therapy. This graft was regarded as a failure of treatment in the statistical analysis even though the patient only received CSA for one month. Inclusion of this patient in the results and the fact that three patients with rejected grafts in group B were subsequently re-allocated to group C will tend to bias the results of this group. This bias causes an under-estimation of the treatment effect of group C.

No patient suffered permanent damage from CSA therapy. One patient developed a rise in serum creatinine above the upper limit of normal (120 mmol/l), this responded to lowering the dose of CSA while still maintaining blood levels within the therapeutic range. One female patient complained of hirsutism but this resolved on cessation of therapy. Many patients requiring corneal grafts are healthy, without the systemic diseases seen in patients undergoing other types of transplantation. CSA, if carefully monitored to detect early hepatic and renal damage that is reversible, is an effective and relatively safe drug. The concomitant use of systemic corticosteroids is probably unnecessary, although they may potentiate the effect of CSA. Further studies using CSA alone for four months are presently underway.

5.5 Systemic Cyclosporine In High-risk Keratoplasty: Short Versus Long Term Therapy.

Introduction

In high-risk keratoplasty the rejection rate is high, with failure rates from rejection of 60% to 90% being reported depending on the criteria for defining high risk[190,199,225,234,315]. Corneal vascularization leads to breakdown of the "immunological privilege" of the cornea by allowing access to immunologically competent cells. Previous graft rejection, another important risk factor, may cause corneal vascularization and may result in sensitization to the transplanted antigens.

Corticosteroids remain the mainstay for treating corneal graft rejection. They are used topically for both prophylaxis and in the management of a rejection episode. In severe endothelial rejection, systemic therapy may be added either as oral medication or as an intravenous pulse[394,395]. The prolonged use of corticosteroids is associated with serious side-effects which includes raised intraocular pressure, delayed wound healing and cataract formation[396]. Despite the use of steroids in high doses, rejection in high-risk keratoplasty is not reliably prevented.

In other fields of transplantation advances have been made using other immunosuppressive agents most notably CSA. In a preliminary report on human high risk keratoplasty patients detailed earlier in this manuscript, I used systemic CSA given prophylactically in conjunction with systemic corticosteroids[393]. A subsequent prospective study[397] indicated that the addition of systemic corticosteroids offered no benefit over the use of CSA alone. The success of the twelve month regimen of CSA led me to shorten the time of treatment to between four and six months, to reduce the potential side-effects and cost: by this time it was hoped that immunological privilege had been re-established. My initial results suggested that the shorter course of CSA was equally effective in preventing graft

rejection, this finding was supported by other workers[398]. This present study re-examines the effectiveness of a shorter course of treatment of CSA in preventing graft rejection, using additional patient data and longer follow-up.

Patients And Methods

All patients included in this prospective study were high-risk keratoplasty patients defined as having three or four quadrants of superficial and deep corneal vascularization with the total number of vessels reaching the proposed donor site exceeding sixteen. Many of them had had previously rejected corneal grafts (Table XI, page 175) and all had visual acuities of less than 20/200 in the affected eye.

Patients were allocated to a treatment group according to the vision in the other eye. CSA was offered to healthy patients who were either uniocular or had bilateral corneal disease, except for three patients who had a seeing contralateral eye but required binocular vision for their employment. Patients who had a good contralateral eye were not offered cyclosporine because of the cost of the drug and monitoring, but were offered a graft with standard treatment. At the beginning of the study CSA was given for twelve months but because of our initial success it was felt that the length of treatment could be reduced to economise and prevent potential side-effects. Consequently the remaining patients were treated with CSA for between four and six months.

TABLE XI

Short Versus Long CSA: Patient Details

Group	N.	Age Mean (Range)	Previous Grafts Mean (Range)	Follow-up* Mean (Range)
Control	37	33.6 (4-69)	0.57 (0-3)	29.6 (6-63)
Short CSA	29	35.3 (4-66)	1.62 (0-4)	26.7 (7-49)
Long CSA	14	24.5 (7-42)	1.86 (0-5)	52.8 (14-82)

*** Follow-up refers to clear grafts only, in months.**

Forty three patients were given CSA; fourteen patients received CSA for twelve months (long CSA group) and 29 patients received the drug for four to six months (short CSA group). In both groups the dose of CSA was administered twice daily by mouth, except the first dose that was given intravenously at the time of surgery. An initial loading dose of 15 mg\Kg\day was given for two days followed by 7.5 mg\Kg\day for two days, thereafter the dose was adjusted to maintain therapeutic blood levels. During this study the method used by our laboratory to estimate CSA blood levels has changed a number of times, initially serum levels were measured but at present whole blood values are preferred. The current method used is the specific whole blood method and the therapeutic range of our laboratory is a trough level of 130-250 μg/L. The usual dose given was in the range of 3-4 mg/Kg/day; we attempt to keep blood levels at the lower end of the therapeutic range, at present a value of between 130-170 μg/L is aimed for. During the early postoperative period blood CSA levels, together with serum chemistry, were estimated twice weekly. When the blood CSA levels stabilised, usually at three or four weeks, the frequency of the tests was reduced.

A control group of 37 patients, who had similar high-risk corneas but who had unilateral disease only, received topical corticosteroids alone.

Patients in all groups were healthy with no serious systemic disease; those receiving CSA had a full blood and metabolic work-up with particular attention given to the detection of renal and hepatic disease. The original corneal pathology of the patients in the three groups is shown in Table XII (page 177).

Table XII

Short Versus Long CSA: Original Corneal Pathology

Diagnosis	Short CSA	Long CSA	Control
Herpetic keratitis	2	1	3
Chemical burn	6	4	8
Keratoconus	3	4	2
Bullous keratopathy	3	0	3
Trachoma	2	0	3
Interstitial keratitis	4	0	10
Scarring post keratitis	7	5	5
Other	2	0	3

All donor grafts were between 7.5 and 8.5 mm and were 0.5 mm larger than the recipient bed. Donor corneas were preserved as corneoscleral discs in culture medium (M.K. or KSol solution) at 4°C. The keratoplasty techniques were identical in all patients; a number of patients had simultaneous extracapsular cataract extraction with placement of posterior chamber intraocular lenses. All surgery was performed by myself. Tissue matching of donor and recipient was not undertaken. A viscoelastic substance was used to protect donor endothelium and to dissect off any iris adhesions. A 10/0 nylon suture was used to secure the graft using interrupted or continuous sutures or a combination of both. The anterior chamber

was reformed with balanced salt solution and sub-conjunctival injections of gentamicin 20 mg and betamethasone (Celestone) 1.5 mg were given at completion of surgery.

Postoperatively all patients received topical dexamethasone 0.1% drops four times daily and betamethasone 0.1% ointment at night. In the presence of inflammation the frequency of the corticosteroid drops was increased as required, but normally the drops were reduced slowly over the ensuing months and stopped at six months unless persistent inflammation required extended therapy. Topical antibiotics and cycloplegics were given in the early postoperative period.

A rejection episode was diagnosed when a previously clear cornea became significantly thickened with or without a typical Khodadoust line, associated with intraocular inflammation or stromal infiltration. The treatment of rejection consisted of hourly dexamethasone 0.1% drops and a subconjunctival injection of 1.5 mg betamethasone (Celestone). In severe cases of endothelial rejection oral prednisone 60 mg daily was added. Treatment was continued until the inflammation subsided and was then gradually withdrawn. Later in the series a 500mg intravenous pulse of methylprednisolone was substituted for the subconjunctival and oral corticosteroids in the severe cases. A graft was deemed irreversibly rejected when it remained thickened and oedematous with severe visual loss despite maximal medical therapy. In all three groups a number of grafts failed for reasons other than graft rejection. Non-rejection graft failure was diagnosed when a disease process affected the graft without the signs of an immunological reaction outlined above. Graft failure was deemed to have occurred when the visual acuity dropped to below 20/60 and this time was taken as the follow-up end-point for the purposes of this study.

Graft survival was analysed using the Cox regression method[246], and the Cox proportional hazard model. The end-point for survival analysis was the commencement of a rejection episode in a graft that became irreversibly rejected.

Results

The mean number of previous grafts in the affected eyes for the three groups is shown in Table XI (page 175). It can be seen that the control group had received fewer previous grafts (0.57) than the two CSA groups: 1.62 and 1.86 respectively. Follow-up refers to clear grafts and indicates the interval until the last visit. In the long CSA group follow-up was longer because this was the first CSA regimen to be used.

The incidence of irreversible graft rejection is shown in Table XIII (page 180). Twenty-three grafts (62.2%) in the control group were lost from rejection at a mean time of 11.2 months and a median time of 8 months postoperatively. The irreversible rejection rate in the two CSA groups was lower: in the short CSA group nine grafts (31%) rejected and in the long CSA group one graft (7.1%) rejected. Only one graft irreversibly rejected while the patient was taking CSA, this occurred one month postoperative in the short CSA group. Treatment with CSA resulted in a significant improvement in survival compared to no treatment ($p = 0.0005$). However, if the duration of CSA treatment is taken into account as a variable in the Cox regression method, then the model fitted better and significance increased ($p = 0.00008$). Actuarial survival curves for the three treatment groups are shown in figure 26 (page 181), the end-point is irreversible graft failure. Using the Cox proportional hazard model; the hazard of graft failure from rejection at any point in time for the short CSA group is only 34.4% that for a patient on no treatment. For the long CSA group the risk is only 11.9%.

TABLE XIII

Short Versus Long CSA: Grafts Irreversibly Lost From Rejection

Group	Grafts Rejected		Interval To Rejection	
	No.	(%)	Mean (Range)	Median
Control	23	(62.2%)	11.2 (1-40)	8
Short CSA	9	(31.0%)	13.1 (1-31)	12
Long CSA	1	(7.1%)	14	14

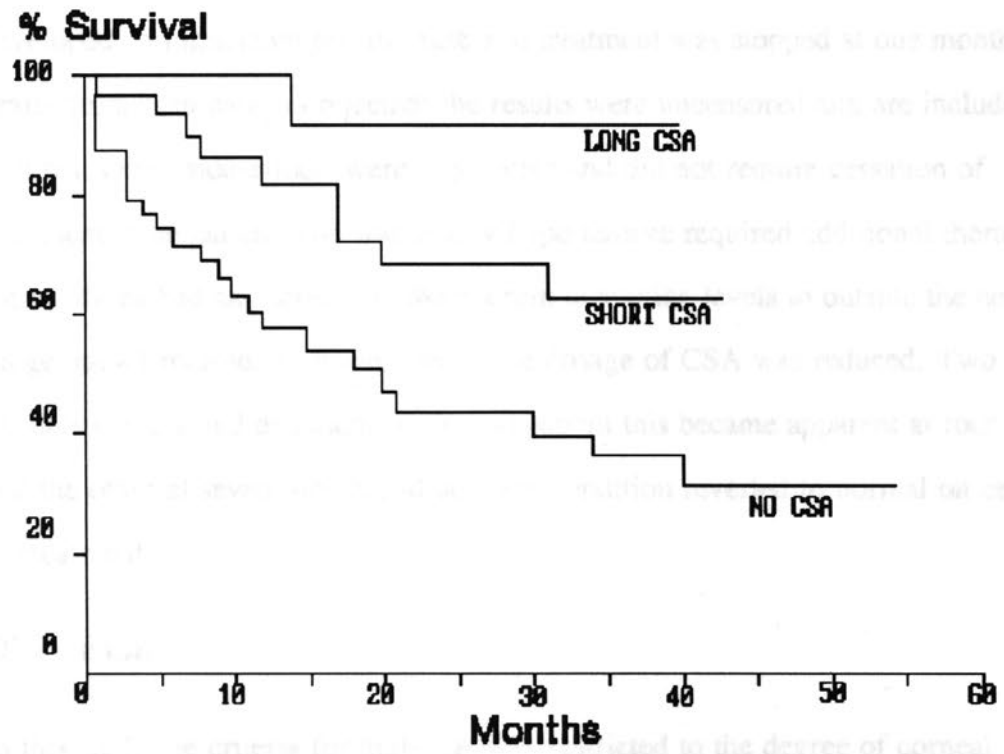


Fig. 26. Short versus long CSA: Survival estimates for the three treatment groups.

One patient in the short CSA group developed a TB abscess in the neck, presumably from a reactivation of an undiagnosed inactive lesion; specific TB therapy was commenced and CSA stopped at two months. Another patient in the same group developed an intractable pruritic rash and treatment was stopped at one month. The grafts from both patients rejected: the results were uncensored and are included as failures. Other side-effects were less severe and did not require cessation of treatment. One patient who was mildly hypertensive required additional therapy and four patients had an increase in their serum creatinine levels to outside the normal range but all returned to normal when the dosage of CSA was reduced. Two female patients complained of hirsutism, in one patient this became apparent at four months and the other at seven months, in both the condition reverted to normal on cessation of treatment.

Discussion

In this study the criteria for high-risk was restricted to the degree of corneal vascularization, namely three or four quadrants involved with at least sixteen vessels passing over the proposed graft site. The number of previous grafts is also an important factor, the group treated with topical corticosteroids alone had less previous grafts than the two CSA groups and this group was possibly at less risk. The failure rate in this control group was high (62.2%) but compares to failure rates of between 60 and 90% in other studies of high risk keratoplasty[190,199,225,234,315]. The definition of high-risk has not been universally agreed, in some studies a previously rejected graft constitutes high-risk even without corneal vessels. The degree of vascularization necessary to classify a cornea high-risk also varies with many studies accepting involvement of two quadrants. In high-risk patients, blind from corneal disease, these high failure rates do not offer much hope for the prospect of vision.

In this study the dosage of topical corticosteroids is arguably low. However, the dexamethasone 0.1% drops four times daily with betamethasone 0.1% ointment at night was sufficient to suppress local inflammation in the majority of patients. Persistent inflammation was treated by more frequent application of topical corticosteroids. In corneal graft rejection the precise site this reaction is initiated is not known. If it occurs in the environs of the eye, then higher dosages of topical corticosteroids or topical CSA could suppress the reaction by preventing the afferent arc of the rejection cycle. If the recognition and initiation of the rejection process occurs outside the eye, perhaps at a local lymph node, then these topical preparations can only work on the efferent arc and suppress the activated T cells. High dose topical steroids have not been shown to significantly improve the prognosis in high-risk keratoplasty but can lead to a greater complication rate. In this study a dose adequate to effectively suppress inflammation and prevent the occurrence of inflammatory cells was used. Topical CSA has been shown to improve graft survival[386,389,390] but our animal model studies[314] indicate that the improvement in graft survival was less than with systemic CSA. A recently concluded multicentre trial of topical CSA showed no benefit in high-risk keratoplasty and other similar trials have been stopped (personal communication, Sandoz Pharmaceutical). Apart from the use of topical corticosteroids and topical CSA there is little other treatment that can be offered to high-risk keratoplasty patients. Tissue matching or crossmatching to detect the presence of preformed antibodies should in theory be beneficial and a number of reports have confirmed this clinically[199,234] but a recent collaborative study indicates that it offers little advantage[108]. The role of tissue matching therefore needs to be reappraised in the light of these conflicting reports. Moreover tissue typing is only available at a few centres and obtaining a good tissue match may require a considerable wait. Since 1985 we have used CSA systemically in high-risk keratoplasty patients who are blind from their disease and in whom no systemic contra-indications exist. In high-risk keratoplasty the immunological privilege of the cornea is broken down resulting

in a high incidence of rejection. This is particularly apparent during the early postoperative period: of the 23 rejected grafts in the untreated group, eleven rejected during the first six months and sixteen during the first year. It would be logical to use an agent that prevented rejection during this crucial period in the hope that immunological privilege is re-established by the time the drug is withdrawn. CSA is an effective drug in preventing graft rejection as can be seen in this study. Only one patient rejected while on the drug. This patient, an 18 year old female, had had three previous grafts and was known to have circulating antibodies to a number of HLA antigens. She rejected her graft acutely within three weeks and although tissue typing of the donor was not done, the graft possibly carried antigens against which she had been previously sensitized.

This was not a masked trial and therefore a patient's treatment group was known to the observers. However, it is highly unlikely that bias in assessing graft clarity was introduced as the end-points were clearly evident. Grafts that irreversibly rejected invariably developed severe rejection episodes resulting in significant endothelial loss and final corneal thickness measurements in excess of 700 microns centrally. In our original regimen CSA was continued for a year. Because of the success of this regimen the treatment period was shortened to between four and six months to reduce potential side-effects and the cost of therapy and blood monitoring. The survival curves show that the shorter regimen improves the survival of high-risk corneal grafts but is not so effective as the longer CSA regimen. Statistical analysis indicates that time of treatment with CSA is an important factor in preventing rejection and for improving long-term survival. From this study one can conclude that CSA is effective in improving graft survival in high-risk keratoplasty but for maximal benefit a twelve month period of treatment is probably necessary.

After what period of time immunological privilege returns has not been established by this study, but it is unlikely to be at a circumscribed time but more likely a gradual re-establishment occurs. Certainly after twelve months of CSA treatment,

the prognosis for survival is good and remains good after CSA is stopped. A shorter course of CSA confers an improved prognosis compared to the control group, but on withdrawal of CSA survival is worse than the long CSA group indicating that immunological privilege has only been partially re-established in the short CSA group.

It should be stressed that careful pre-operative systemic work-up and postoperative monitoring are essential to minimize the potential dangers of using this drug. Nephrotoxicity[376,377], hepatotoxicity[379] and hypertension[378] are known complications of CSA use. In addition the suppression of T cell function may lead to infections or, as in our patient, to re-activation of an existing infection. Apart from this patient the complication rate in this study was low; possibly because of the good general health of the patients, but also because the dosage of CSA used was kept to a minimum by keeping the blood CSA levels at the lower end of the therapeutic range. Apart from one case of hirsutism all the complications occurred within the first few months of treatment and settled once the dosage was adjusted. The longer period of treatment in the long CSA group did not give rise to additional side-effects and after six months, monitoring was reduced to monthly intervals. For high-risk keratoplasty patients who are blind from their disease, systemic CSA offers some hope of a successful graft and vision.

5.6 Systemic Cyclosporine In High Risk Keratoplasty: Long Term Results.

Introduction

In previous sections of this manuscript I have reported on the use of systemic CSA in high-risk keratoplasty and have shown that it improves graft survival by reducing the incidence of irreversible graft rejection. If rejection can be prevented during the early postoperative period, a time when rejection is more likely to occur, then later some degree of immunological privilege may be re-established allowing CSA to be safely withdrawn. The studies have shown that a twelve month course of systemic CSA is more effective in this regard than the shorter four to six month course of therapy. CSA is a potent immunosuppressant agent with a high degree of specificity for T lymphocytes. It suppresses cell-mediated reactions and in transplantation it is thought to prevent the early stages of antigenic sensitization and the resulting proliferation of immuno-competant T cells[305]. Therefore CSA should prevent rejection episodes from occurring. My previous studies reported that while on CSA treatment, only one patient had an irreversible graft rejection but a number of grafts developed reversible rejection episodes. In high-risk keratoplasty, it is not known whether systemic CSA reduces the incidence of rejection episodes, or whether the incidence remains high but CSA influences the outcome by making the treatment of rejection episodes more successful. This present study investigates these aspects of CSA therapy, and examines graft survival to determine whether the beneficial effects of CSA continue after therapy is stopped.

Although rejection remains the commonest cause of graft failure in high-risk keratoplasty, associated ocular damage may lead to graft failure from other causes such as glaucoma, tear film abnormalities or recurrent scarring. If CSA prevents failure from rejection there is the possibility that failure from other causes will become more of a problem. This present study investigates these aspects by

analysing graft failure from all causes and presenting the final visual acuity in a group of high-risk keratoplasty patients given systemic CSA.

Patients And Methods

The patients in this prospective study are the same cohort as those reported in my previous study. All were high-risk keratoplasty patients defined as having 3 or 4 quadrants of superficial and deep corneal vascularization with the total number of vessels reaching the proposed donor site exceeding sixteen. Many of them had had previously rejected corneal grafts and all had visual acuities of less than 20/200 in the affected eye.

Forty three patients were given CSA; fourteen patients received CSA for twelve months (long CSA group) and 29 patients received the drug for between four and six months (short CSA group). The CSA dosage, monitoring regimens and operation details have been detailed previously. A control group of 37 patients, who had similar high-risk corneas but who had unilateral disease only, received topical corticosteroids alone. The keratoplasty techniques were identical in all patients, tissue matching of donor and recipient was not undertaken. Postoperatively all patients received topical dexamethasone 0.1% drops four times daily and betamethasone 0.1% ointment at night, normally the drops were reduced slowly over the ensuing months and stopped at six months unless persistent inflammation required extended therapy.

A rejection episode was diagnosed when a previously clear cornea became significantly thickened with or without a typical Khodadoust line, associated with intraocular inflammation or stromal infiltration. The treatment of rejection consisted of hourly dexamethasone 0.1% drops and a subconjunctival injection of 1.5 mg betamethasone (Celestone). In severe cases of endothelial rejection, oral prednisone 60 mg daily was added. Treatment was continued until the inflammation subsided and was then gradually withdrawn. Later in the series a 500mg intravenous pulse of

methylprednisolone was substituted for the subconjunctival and oral corticosteroids in the severe cases. A graft was deemed irreversibly rejected when it remained thickened and oedematous with severe visual loss despite maximal medical therapy. In the first part of this study the incidence of graft rejection is studied regardless of whether the reaction was subsequently reversed. Statistical analysis was performed using the log-rank method[248] with the end-point being the commencement of any graft rejection episode. The number of rejection episodes that were reversed by treatment in the three groups was analysed using chi-square or Fisher's tests.

In the second part of the study graft failure from any cause was studied. The end-point in the survival analysis was the commencement of a rejection episode in a graft that became irreversibly rejected or failure from any other cause. Failure from another cause was defined as a graft complication causing a loss of vision to an acuity of 20/60 or less, the end-point in the statistical analysis was the time when vision dropped to this level. Graft survival was compared by log-rank analysis[247-8] and the Cox proportional hazard model[246].

Results

The number of grafts that had one or more rejection episodes is shown in Table XIV (page 190). In the control group 27 of the 37 grafts (73.0%) started to reject, compared to 21 of the 43 grafts (48.8%) in the combined CSA group. These differences are significant ($p = <0.025$). When the total CSA group is subdivided into short CSA and long CSA groups, the number of grafts that started to reject was 15 (51.7%) and 6 (42.6%) respectively. There is no statistical difference (log-rank) between the two CSA groups. When comparing survival to the control group, neither the short CSA group ($p = 0.099$) nor the long CSA group ($p = 0.055$) reaches a level of statistical significance. Both the mean and median time to the first rejection episode were shorter in the control group. Figure 27 (page 191) shows actuarial survival curves of the three treatment groups, the end-point of survival in these graphs is the commencement of the first rejection episode. In respect of developing a rejection episode, it can be seen that the long CSA group shows the best survival, followed by the short CSA group.

Table XIV

Number Of Grafts With Rejection Episodes

Group	N	Grafts Rejecting No. (%)	Time To Rejection* (Months) Mean:	Median
CONTROL	37	27 (73.0%)	9.1	6
SHORT CSA	29	15 (51.7%)	11.3	8
LONG CSA	14	6 (42.6%)	12.0	10
TOTAL CSA	43	21 (48.8%) **	11.5	8

* time until first rejection episode if more than one

** this result is significant (P = <0.025) compared to control group.

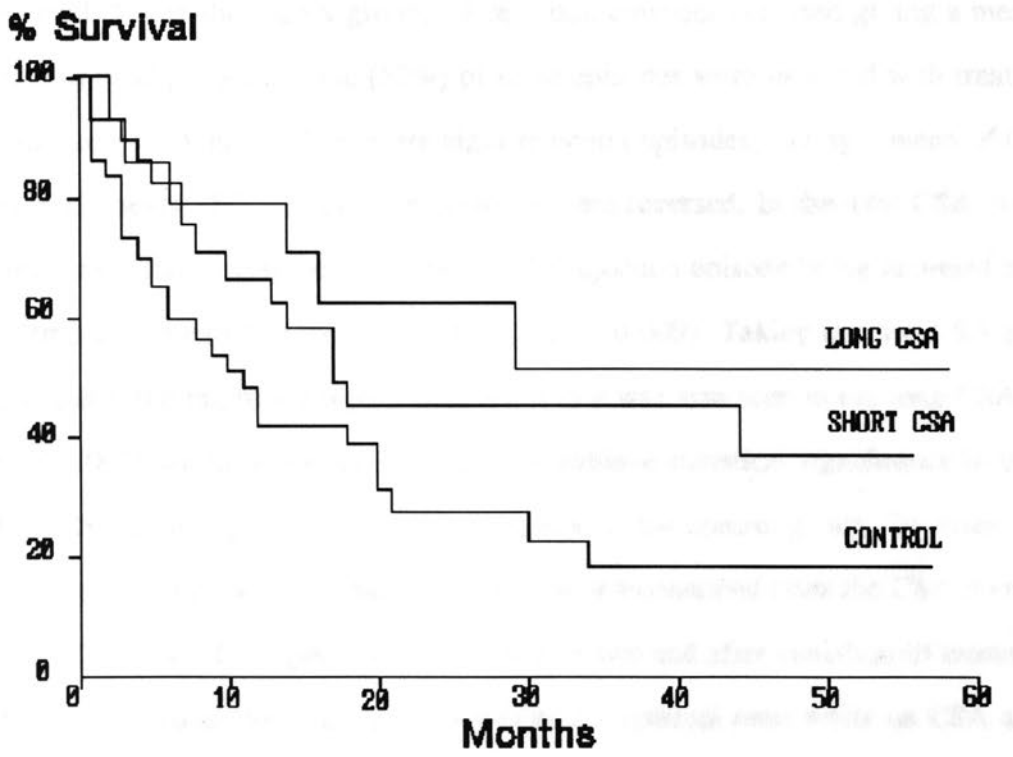


Fig. 27. Survival estimates for the 3 treatment groups, end-point is the commencement of a rejection episode.

The **total** numbers of rejection episodes in the three groups are shown in Table XV (page 193). In the control group a total of 30 rejection episodes occurred, giving a mean number of 0.81 per graft; of these 30 episodes only seven (23.3%) were reversed. In the short CSA group, 18 rejection episodes occurred giving a mean value of 0.62 per graft; nine (50%) of these episodes were reversed with treatment. In the long CSA group there were eight rejection episodes, giving a mean of 0.57 per graft; seven (87.5%) of these episodes were reversed. In the total CSA group there was a statistically better chance of the rejection episode being reversed by treatment, compared to the control group ($p = 0.009$). Taking the two CSA groups separately, the improved rejection reversal rate was also seen in the long CSA group ($p = 0.002$) but the improvement failed to achieve statistical significance in the short CSA group ($p = 0.06$), when compared to the control group. To assess whether this improved rejection reversal rate is maintained after the CSA therapy is stopped, the two CSA groups are analysed before and after cessation of treatment (Table XVI, page 194). It can be seen that the reversal rates while on CSA are high; 75% and 100% for the short and high CSA groups respectively. After treatment is stopped the reversal rates drop to 42.9% and 80% respectively, but both are better than in the control group (23.3%). However, only the results of the long CSA group achieve statistical significance compared to the control group ($p = 0.03$).

Table XV

Total Number Of Rejection Episodes

Group	N	No. Episodes	Mean	No. Reversed (%)
CONTROL	37	30	0.81	7 (23.3%)
SHORT CSA	29	18	0.62	9 (50%) *p=0.06
LONG CSA	14	8	0.57	7 (87.5%) *p=0.002
TOTAL CSA	43	26	0.60	16 (61.5%) *p=0.009

* = significance compared to control group

Table XVI

Number Of Rejection Episodes While On CSA

Group	No. Episodes	No. Reversed (%)
Control	30	7 (23.3%)
Short CSA		
On CSA	4	3 (75%)
Off CSA	14	6 (42.9%) *P=0.16
Long CSA		
On CSA	3	3 (100%)
Off CSA	5	4 (80%) *P= 0.03
Total CSA		
On CSA	7	6 (85.7%)
Off CSA	19	10 (52.6%) *P=0.07

* = significance compared to control group

Although irreversible rejection was the commonest cause of graft failure, a number of grafts failed from other causes (Table XVII, page 196): failure being defined as a final visual acuity of 20/60 or worse; in the table the follow-up refers to clear grafts only. The number of grafts that failed for reasons other than rejection are as follows: control group five (13.5%), short CSA group three (8.1%) and long CSA group four (28.6%). Figure 28 (page 197) shows survival curves of the three groups, the end-point being irreversible graft failure from rejection or failure from any other cause. There was a significantly improved survival of grafts in the short CSA group ($p = < 0.02$), long CSA group ($p = < 0.006$) and combined CSA group ($p = < 0.004$) when compared to the controls. There was no difference between the two CSA groups in terms of survival ($p = 0.43$). Using the Cox proportional hazards model a difference in survival between the CSA groups exists: at any point in time the hazard of a graft in the short CSA group becoming a failure is only 50.2% that of a graft in the control group, in the long CSA group the value is only 25.2% compared to the control group.

Table XVII

Graft Failure From Other Causes

Group	Clear	Rejected	Other Failures		Follow-up
	No.	No.	No.	Cause	Months (Range)
Control	9	23	5	HSV	29.6 (6-63)
				HSV	
				Glaucoma	
				Bact.scar	
				Epith. Defect	
Short CSA	17	9	3	HSV	26.7 (7-49)
				Bact. Scar	
				Epith. Defect	
Long CSA	9	1	4	Bact. Scar	52.8 (14-82)
				Epith. Defect	
				Epith. Defect	
				Late Decomp.	

HSV = Recurrent herpetic keratitis leading to scarring.

Bact. Scar = Bacterial keratitis leading to scarring.

Epith. Defect = Persistent epithelial defect leading to scarring and/or vascularization in patients with previous chemical burns.

Glaucoma = Graft damage resulting from uncontrolled raised intraocular pressure.

Late Decomp. = Late graft decompensation without inflammation.

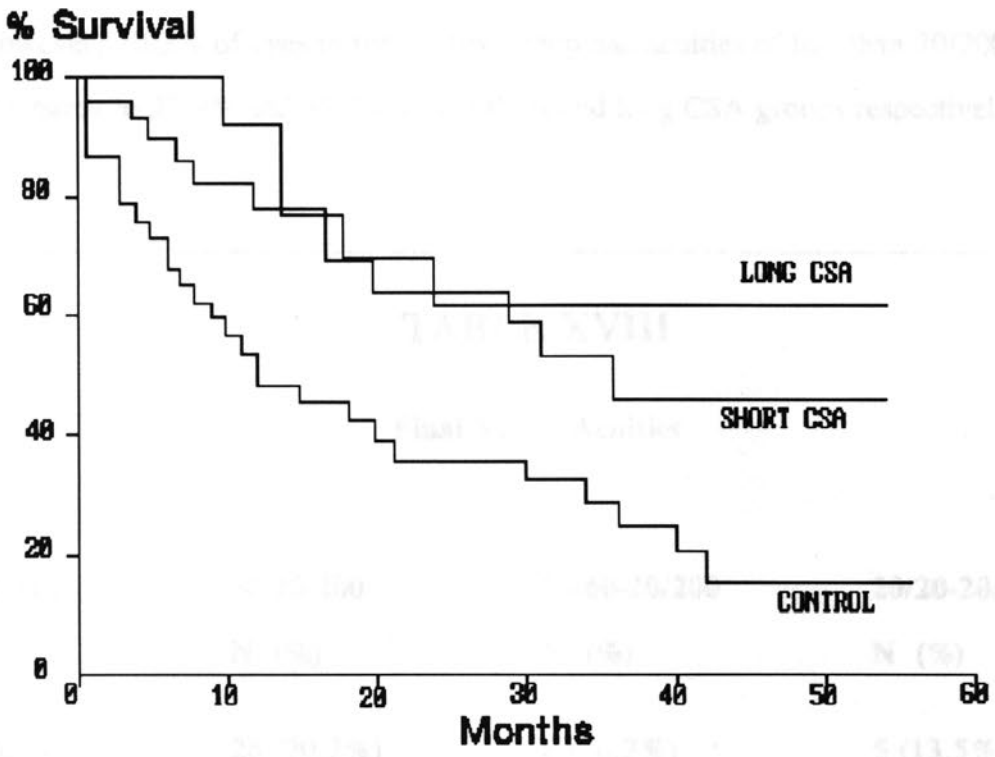


Fig. 28. Survival estimates for the 3 treatment groups, end-point is graft failure from any cause.

The final visual acuities of the eyes in the three groups is shown in Table XVIII below and diagrammatically in figure 29 (page 199). Acuities of 20/40 or better were achieved by 44.8% and 50% of eyes in the short and long CSA groups respectively. These compared to only 13.5% of eyes in the control group. The number of eyes achieving 20/60 to 20/200 acuity was similar in the three groups. However, 70.3% of eyes in the control group had acuities of less than 20/200 compared to 37.9% and 35.7% in the short and long CSA groups respectively.

TABLE XVIII

Final Visual Acuities

Group	**< 20/200**	**20/60-20/200**	**20/20-20/40**
	N (%)	**N (%)**	**N (%)**
Control	26 (70.3%)	6 (16.2%)	5 (13.5%)
Short CSA	11 (37.9%)	5 (17.2%)	13 (44.8%)
Long CSA	5 (35.7%)	2 (14.3%)	7 (50%)

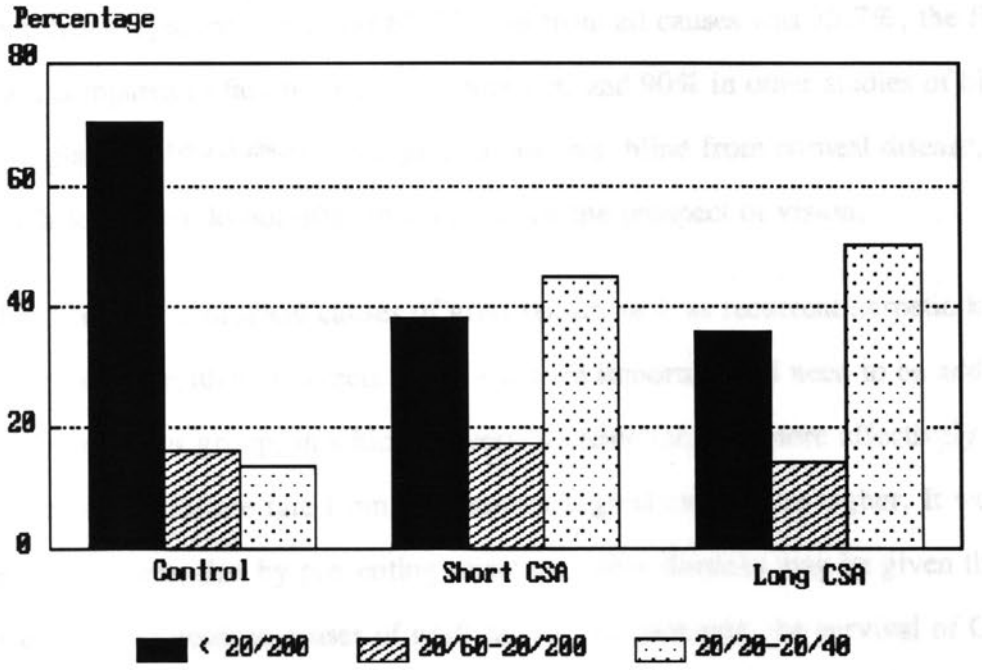


Fig. 29. Final visual acuities in each group, shown as a percentage.

Discussion

The definition of high-risk in this study, namely three or four quadrants involved with at least sixteen vessels passing over the proposed graft site, is stricter than other studies[108] that define high-risk as two or more quadrants of vascularization and/or a previously rejected graft in the affected eye. The failure rate in the control group from rejection alone was 62.2% and from all causes was 75.7%, the final figure compares to failure rates of between 60 and 90% in other studies of high-risk keratoplasty[190,199,225,234,315]. In high-risk patients, blind from corneal disease, these high failure rates do not offer much hope for the prospect of vision.

Other non-immunological causes of graft failure such as recurrent herpetic keratitis and persistent epithelial defects in dry eyes are important and need to be addressed. In the long CSA group, in which irreversible rejection was more effectively prevented, the failure rate from non-immunological causes was higher. It would seem, therefore, that by preventing rejection, other diseases may be given the opportunity to become causes of graft failure. Despite this, the survival of CSA treated grafts and especially those in the long CSA group, remained statistically better than the controls, taking failure from any cause as the end-point. This improved survival was reflected by the improved final visual acuity results found in the two CSA groups, both of which were substantially better than those of the control group. It should be noted that the follow-up of the long CSA group was longer than for the other two groups, theoretically giving more time for late complications to arise.

Without CSA the major cause of graft failure in high-risk keratoplasty remains rejection. In my previous study it was shown that systemic CSA effectively prevents irreversible graft rejection, especially if given for twelve months. In this present study it is apparent that CSA achieves this effect by two actions. Firstly CSA reduces the number of rejection episodes, as shown by the improved survival of CSA treated grafts when any rejection episode was used as an end-point. Secondly

in CSA treated grafts the rejection episodes were more effectively reversed compared to the controls. In both respects the outcome of grafts treated with CSA for twelve months was better. It could be expected that while on CSA treatment any rejection episode is more easily reversed because the patient is taking two immunosuppressant drugs (CSA and corticosteroids) whereas the control group is only being given one (corticosteroids). However, even after CSA is stopped the rejection reversal rates remain higher in the CSA treated patients; possibly because of small numbers, statistical significance was only demonstrated in the long CSA group. The improved survival of grafts, that is maintained after cessation of CSA treatment, indicates that some degree of immunological privilege has been re-established. This study confirms the findings of the previous study, when only irreversible rejection was analysed, that the longer course of CSA is more beneficial than the shorter one. Even when graft failure from any cause is taken as the end-point, graft survival is significantly better in both the CSA groups; with the long CSA group again having a statistical advantage.

The trial was not masked and therefore a patient's treatment group was known to the observers. However, it is highly unlikely that bias in assessing graft clarity was introduced as the end-points were clearly evident. Grafts that irreversibly rejected invariably developed severe rejection episodes resulting in significant endothelial loss and final corneal thickness measurements in excess of 700 microns centrally. In the patients with non-immunological failure the end-point was clearly defined as a best visual acuity of 20/60 or worse. The diagnosis was made clinically and in those grafts that had associated ocular inflammation, no other evidence of rejection, such as a rejection line, keratic precipitates or graft edema from endothelial loss was present.

As stressed previously, careful pre-operative systemic work-up and postoperative monitoring are essential to minimize the potential dangers of using this drug. The complication rate in these studies was low; possibly because of the good general

health of the patients selected but also because the dosage of CSA used was kept to a minimum by keeping the blood CSA levels at the lower end of the therapeutic range. The risks of using systemic CSA are therefore justified in high-risk keratoplasty patients who are blind from their disease, who are healthy, and who are also willing to comply with treatment and monitoring regimens.

process. Although the shorter period of CSA treatment (four to six months) had a statistical advantage over no treatment, best graft survival was found in the grafts treated with CSA for the longer period (12 months). For maximum advantage it would seem that patients who warrant CSA therapy should be treated for this longer period.

Patients with high-risk corneas often have associated ocular diseases that can lead to graft failure from other causes. One concern was, that if failure from rejection was prevented, these diseases would become more apparent and the overall failure rate would remain the same, albeit from different causes. In the long CSA group the grafts lost from non-immunological causes did increase compared to the other groups. However, in the survival analysis considering graft failure from any cause, the beneficial effect of CSA therapy was maintained and again the longer period of CSA treatment showed a statistically better survival compared to the shorter period of treatment. The final visual acuity results of both the CSA groups were similar. The percentage of eyes in the short CSA (44.8%) and long CSA (50%) groups achieving an acuity of 20/40 or better were superior to the control group, in which only 13.5% of eyes achieved this level of acuity. The use of CSA therapy therefore improves the outcome of all aspects of high-risk keratoplasty, thereby offering this group of patients some increased hope for a successful graft and visual outcome.

Section Six: The Use Of Intravenous Corticosteroid Pulse Therapy In Corneal Graft Rejection.

6.1 Introduction.

6.2 Preliminary report on the use of pulse therapy in corneal graft rejection.

6.3 Oral versus pulse therapy in corneal graft rejection.

6.4 Single versus double pulse therapy.

6.5 Conclusion.

6.1 Introduction

Corneal graft rejection is now the commonest cause of graft failure after the immediate postoperative period[190,191]. Khodadoust and Silverstein[99] have shown that all three layers of the cornea can be rejected, either alone or in combination. The endothelium is the most important layer to be affected by rejection because of its role in maintaining graft clarity. Endothelial cells are lost at the time of surgery and for the first two to three years as cells migrate to replace cells lost at the edge of the graft during surgery[399]. A rejection episode results in large numbers of endothelial cells being destroyed[183] and even if the rejection process is reversed sufficient cells may not survive to maintain graft clarity[184]. Rejection episodes should therefore be reversed as quickly as possible to preserve the maximal number of endothelial cells[185].

Corticosteroids remain the mainstay in the treatment of graft rejection. Although some authors use only topical corticosteroid drops[160,208], others treat the more severe rejection episodes involving the endothelium with systemic steroids and/or subconjunctival steroids[88,184,190]. Allograft reactions have been defined as definite, probable and possible depending on the clinical findings[167]. We have found that the use of topical corticosteroids alone in cases of definite and probable rejection has a low success rate (39%) in reversing graft rejection (unpublished data). Very little data exists regarding the success of specific regimens for graft rejection; studies that used different routes of corticosteroids, depending on the severity of the immune reaction, quote success rates of between 50 - 76% in reversing the rejection process[78,88,158,191,235]. In a recent study Boisjoly et al[160] reported that 17 of 23 grafts (73.9%) that developed a single episode of endothelial rejection failed when only topical corticosteroids were used. Our poor results using topical corticosteroids alone led us to modify our treatment regimen for cases of definite and probable rejection: oral prednisone 60 - 80mg daily (depending on body weight) was given in addition to hourly prednisolone acetate 1% drops. Medication was tapered off after

two weeks or before if the graft began to recover, the systemic prednisone usually being stopped by 6 to 8 weeks. Grafts with epithelial rejection or stromal infiltrates with no graft swelling continue to be treated with topical therapy alone.

The use of pulsed corticosteroid therapy is gaining acceptance in other fields of medicine and has been used in the management of ocular inflammation[400,401] and in renal transplant rejection[402]. A single intravenous dose of 125 mg methylprednisolone sodium has been advocated in the management of severe graft rejection[394]. Currently larger doses of corticosteroid are being used in single dose pulse therapy for other conditions[400-402], and we considered that similar therapy could have a place in corneal graft rejection to alleviate the need for prolonged oral doses of systemic corticosteroids.

6.2 Preliminary Report On The Use Of Pulse Therapy In Corneal Graft Rejection.

A preliminary clinical study was undertaken to assess the efficacy of intravenous corticosteroid pulse therapy in corneal graft rejection.

Patients and methods

Ten consecutive adult patients presenting with endothelial rejection were entered into the trial; all gave informed consent prior to participation. Endothelial rejection was diagnosed when an eye with a previously clear, thin graft became inflamed with: flare and cells in the anterior chamber, keratic precipitates limited to the donor endothelium, and thickening of the graft either diffusely (probable rejection)[167] or in the form of an advancing rejection line (definite rejection)[167]. All patients were treated with hourly prednisolone acetate 1% drops while awake and a single intravenous injection of methylprednisolone 500mg. The clinical details are shown in Table XIX (page 210); five males and five females were included in the study, with a mean age of 51.4 years (range 18 - 68). Of the six patients with previously failed grafts, two had had three previous grafts and one patient four previous grafts. Seven patients had vascularization of the host cornea prior to grafting. For preoperative clinical details of the corneas, see Table XX (page 211). The interval from the onset of rejection to treatment ranged from four days to three weeks. Three patients had a definite endothelial rejection line and the remaining seven had diffuse endothelial rejection. The effects of the treatment were monitored by assessing the improvement in clinical signs and graft clarity and by the measurement of central graft thickness by ultrasonic pachymetry. A full blood and differential count was performed prior to treatment and repeated daily for three days.

Results

In eight (80%) of the ten grafts the rejection episode reversed and the graft became clear. The thickness of the central cornea during the rejection episode and the value two months later are recorded, together with the pre-rejection measurements, in Table XXI (page 212). Both of the patients that rejected, and whose grafts remained hazy, had very oedematous corneas (963 and >1000 microns) on presentation and had delayed in seeking medical help. All the grafts in which the rejection episode was reversed regained a central graft thickness of less than 600 microns. No complications were encountered with the corticosteroid pulse therapy.

The total white cell count rose during the first two to three days: this was predominantly the result of an increased number of neutrophils. The mean lymphocyte count was decreased on the day following therapy but this had recovered by the second day (Fig. 30, page 213).

Table XIX

Clinical Details I

Patient No.	Sex	Age	Diagnosis
1	M	59	old bacterial scarring
2	F	64	Fuch's dystrophy
3	F	18	Herpetic scarring
4	F	67	Keratoconus
5	F	68	Interstitial keratitis
6	M	37	Pellucid degeneration
7	M	62	Fuch's dystrophy
8	F	24	Keratoconus
9	M	48	old bacterial scarring
10	M	67	old bacterial scarring

Table XX

Clinical Details II

No.	Preoperative vascularization	Previous grafts	Treatment delay	Outcome
1	++	3	1 week	Clear
2	-	0	4 days	Clear
3	++	3	8 days	Clear
4	-	0	1 week	Clear
5	+	0	2 weeks	Clear
6	++	1	2 weeks	Rejected
7	+	1	3 weeks	Rejected
8	-	0	10 days	Clear
9	++	4	3 weeks	Clear
10	++	2	3 weeks	Clear

Vascularization

- = Nil;

\+ = Mild (< 10 vessels);

++ = Severe (10+ vessels).

Table XXI

Corneal Thickness Measurements (Microns)

No.	Pre-rejection	Rejection	Post-rejection+
1	454	771	474
2	537	620*	480
3	563	>1000	592
4	560	650*	531
5	554	799	510
6	557	963	690**
7	554	>1000	>1000**
8	462	>1000	553
9	436	530*	413
10	438	890	510

\+ measurements were taken 2 months after start of rejection.

* Corneal rejection line.

** Graft failed to clear.

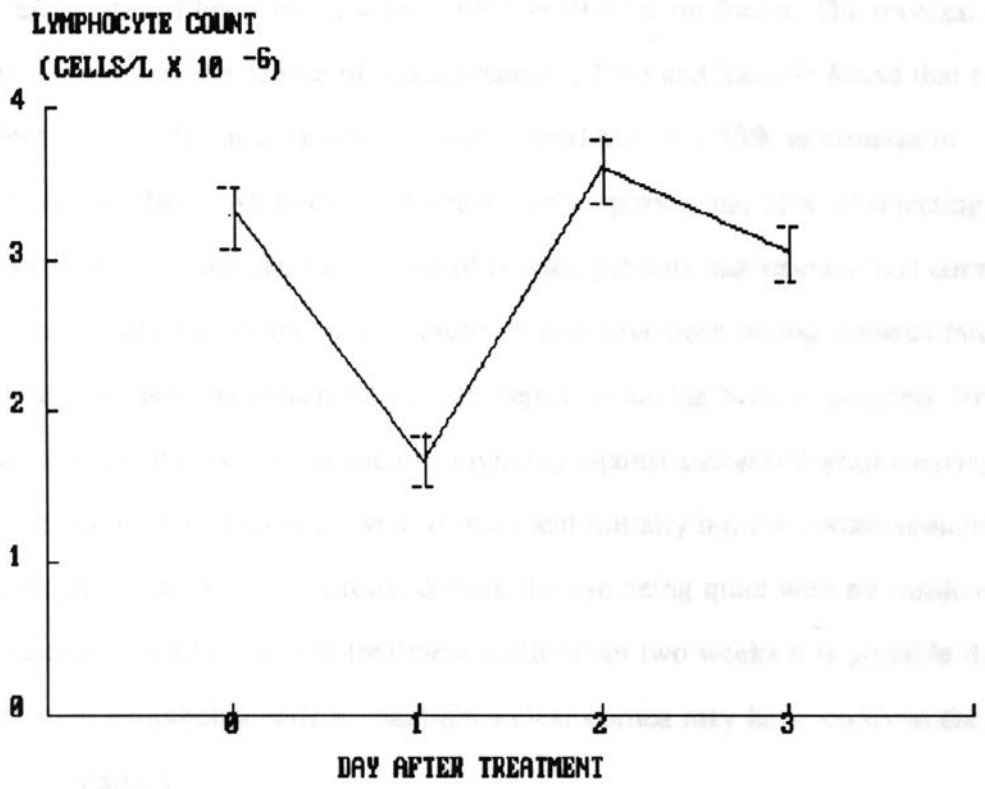


Fig. 30. Mean number of lymphocytes in circulating blood following a single intravenous pulse of 500mg methylprednisolone.

Discussion

The rejection reversal rate of 80% compares favourably with other studies in which reversal rates of between 50% and 76%[78,88,158,235] were found. The reversal rate also depends on the degree of vascularization: Fine and Stein[225] found that 66% of rejection episodes in avascular corneas cleared but only 50% in corneas in vascularized beds. Alldredge and Krachmer[88] reported that 65% of rejecting grafts in vascularized beds cleared. Seven of our ten patients had vascularised corneas preoperatively and therefore the results should have been biased towards failure. In addition six patients presented with the rejection having been in progress for more than a week, this is another factor mitigating against successful graft clearing[88]. Patient number 6 responded well to treatment initially but the cornea remained hazy with a thickness of 690 microns, despite the eye being quiet with no residual signs of rejection. Had he sought treatment earlier than two weeks it is possible that sufficient endothelial cells to maintain a clear cornea may have survived the rejection attack.

The response of the circulating white blood cells to the pulse of corticosteroid is similar to that reported by authors working in other fields[403]. The exact mechanism by which intravenous pulse therapy operates is not well understood but may follow the course of events suggested by Meyer et al[401]. Studies have shown that a transient lymphopenia occurs which is maximal at four to six hours with T lymphocytes being affected to a greater extent than B cells, and with a relatively greater depletion of the helper/inducer subpopulation[403]. The concentration of lymphocytes returns to normal by 48 hours, a similar pattern was found in our studies. It is thought that the decreased lymphocyte count is due to a change in distribution rather than to cell lysis[404,405] although local lymphocyte lysis has been demonstrated when topical corticosteroids are used in corneal graft rejection[406]. After a single pulse of corticosteroid, delayed hypersensitivity skin tests and primary and secondary antibody responses return to normal by 48 hours[407]: at the same time that circulating

lymphocytes are restored. However, the anti-inflammatory effect lasts four to seven days and it is possible that the clinical effect of pulse therapy is anti-inflammatory and not immunolytic[403]. A pulse of corticosteroid appears able to "reset" an aberrant immune response by the simultaneous occurrence of inhibition of the proliferating clone, the temporary removal of recirculating T lymphocytes from the blood and eye, and the profound suppression of peripheral inflammation[401]. This immunological manipulation has been shown to induce long term remissions in destructive corneal and scleral disease[401]. From this small preliminary trial it would appeared that pulsed corticosteroid therapy was beneficial in the management of corneal graft rejection and further studies were undertaken to compare the efficacy to other treatment regimens.

6.3 Oral Versus Pulse Therapy In Corneal Graft Rejection

This study compares the regimen using a single pulse of intravenous methylprednisolone 500mg with our original regimen using oral corticosteroids. In both regimens hourly topical corticosteroid drops were used.

Patients and methods

Forty-eight adult patients who presented consecutively with endothelial rejection were entered into the trial, all gave informed consent prior to participation. Endothelial rejection was diagnosed when an eye with a previously clear, thin graft became inflamed with: flare and cells in the anterior chamber, keratic precipitates limited to the donor endothelium, and thickening of the graft either diffusely (probable rejection)[167] or in the form of an advancing rejection line (definite rejection)[167]. In the probable rejection cases only those with the central corneal thickness exceeding 620 microns, measured by ultrasound pachymetry, were included in this study. Eyes with a history of herpes simplex keratitis were also treated with topical antiviral medication.

The patients were assigned to one of two treatment groups by random selection:

One group was treated with hourly prednisolone acetate 1% drops and a single intravenous injection of methylprednisolone 500mg (pulse group).

The other group was treated with hourly prednisolone acetate 1% drops and oral prednisone 60 - 80mg daily, depending on the mass of the patient (oral group).

The oral medication and the drops in both groups were tapered off after two weeks or earlier if rejection episode showed signs of reversing. The oral prednisone was completely withdrawn by six to eight weeks.

The response to treatment was monitored by assessing the improvement in clinical signs, the return of graft clarity and restoration of visual acuity. The central corneal thickness was measured by ultrasound pachymetry at the time of rejection and at intervals during treatment. After two months the thickness had usually stabilized and this figure was taken as the final value. Because our machine can only measure up to 1000 microns, thicknesses greater than this value are recorded as being >1000, however in the statistical analysis the values are taken as being 1000 microns. Statistical comparison of the results from the two groups was performed using Fisher's exact test. Subsequent rejection episodes in the surviving grafts were noted: survival curves were calculated using the Kaplan-Meier survival analysis method[244], statistical comparisons were made using generalized Wilcoxon[290] and Kruskal-Wallis[291] tests.

Results

The clinical details are shown in Table XXII (page 219). Both groups were well matched with respect to mean age, sex distribution and original diagnosis. The mean interval between grafting and the rejection episode was similar in the two groups although the median interval was a little shorter in the oral group. Sixteen corneas in the pulse group and fifteen in the oral group were vascularised preoperatively; more cases with severe vascularization (two quadrants of the cornea involved and >10 vessels crossing the proposed graft-host junction) were present in the pulse group. More patients in the pulse group than in the oral group had had previous grafts that had failed in the affected eye.

The delay between the onset of rejection and the time the patient sought treatment was similar for the two groups and ranged between three days and three weeks (Table XXII). The median delay for the pulse group was 8 days and for the oral group 8.5 days. Considering the two groups as a whole, 19 (79.2%) of the grafts in the pulse group survived compared to 15 (62.5%) in the oral group (Table XXIII,

page 220): there is no statistical difference between the results of the two groups ($p = 0.17$).

The longer the rejection process has been in progress the worse the prognosis becomes because of irreversible damage to the endothelial layer which is then unable to maintain a clear graft[21]. To study this aspect further the two groups were sub-divided into patients that presented within the median time (8 days) and those who delayed seeking treatment (over 8 days); the results are shown in Tables XXIV and XXV (pages 221 and 222) respectively. Graft survival in the pulse group patients who presented early (92.3%) was statistically better ($p = < 0.05$) compared to graft survival in the oral group patients who presented early (54.5%). In the patients who presented late, graft survival was similar between the two groups: 63.6% and 69.2% respectively. The time of eight days was chosen arbitrarily because it was the median time of presentation. However significance remained at the 5% level, between the early and late presenters, for any division from eight to eleven days.

The number of grafts that cleared and subsequently had a further rejection episode is shown in Table XXIII. Only 26.3% of the successfully treated grafts in the pulse group had a subsequent rejection episode compared to 67% in the oral group: the difference is significant ($p = < 0.025$). Similar values for the incidence of further rejection episodes are found when the early and late presenting groups are analysed separately (Tables XXIV and XXV). Survival analysis of the time from treatment to a subsequent rejection episode for the two groups is shown in figure 31 (page 223).

Central corneal thickness measurements of both groups are shown in Table XXVI (page 224). Mean values pre-rejection, at the time of rejection and two month post-rejection are recorded. No complications were encountered with either regimen and no patients were withdrawn from the trial.

Table XXII

Clinical Details

	Pulse Group	Oral Group
N	24	24
Sex (M:F)	10:14	9:15
Mean Age (Range)	44 (18-81)	47.8 (18-86)
Vascularization		
Nil	8	9
Mild (<10 Vessels)	11	12
Severe (=>10 " ")	5	3
Graft-rejection Interval		
(Months) Median	11.5	9
Mean (Range)	16.9 (1-100)	16.2 (1-132)
Previous Failed Grafts		
Nil	7	15
1+	17	9
Original Diagnosis		
Keratoconus	6	4
HSV Scarring	5	4
Other Scarring	4	8
Aphakic Bull. Ker.	0	1
Fuchs Dystrophy	4	3
I.K.	3	2
Trauma	2	2

Table XXIII

Graft Outcome (Total Group)

	Pulse Group	Oral Group
Median no. of days of rejection-treatment interval (Range)	8 (4 - 21)	8.5 (3 - 21)
No. of graft survivals (%)	19 (79.2%) **(p = 0.17)**	15 (62.5%)
No. of further rejection episodes in surviving grafts (%)	5 (26.3%) **(p = <0.025)**	10 (66.7%)

Table XXIV

Outcome In Patients Presenting Early*

	Pulse Group (N =13)	Oral Group (N = 11)
No. Of Graft Survivals (%)	12 (92.3%)	6 (54.5%)
	P = <0.05	
No. Of Further Rejection Episodes In Surviving Grafts (%)	3 (25%)	4 (66.7%)

* Eight days or less.

Table XXV

Outcome In Patients Presenting Late**

	Pulse Group (N=11)	Oral Group (N=13)
No. Of Graft Survivals (%)	7 (63.6%)	9 (69.2%)
No. Of Further Rejection Episodes In Surviving Grafts (%)	2 (28.6%)	6 (66.7%)

** Over eight days.

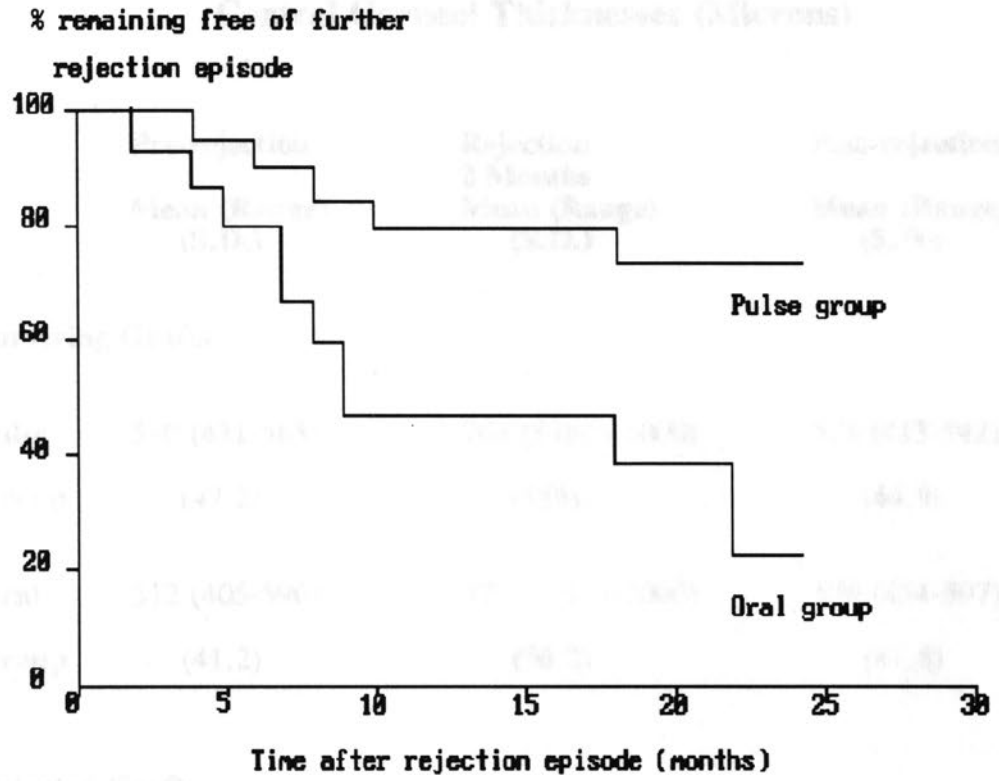


Fig. 31. Survival graphs showing the risk of a subsequent rejection episode following successful graft rejection treatment.

Table XXVI

Central Corneal Thicknesses (Microns)

	Pre-rejection Mean (Range) (S.D.)	Rejection 2 Months Mean (Range) (S.D.)	Post-rejection Mean (Range) (S.D.)
Surviving Grafts			
Pulse	510 (431-565)	763 (530- > 1000)	525 (413-592)
Group	(47.2)	(155)	(44.9)
Oral	512 (405-590)	772 (551- > 1000)	536 (454-597)
Group	(41.2)	(90.2)	(41.8)
Rejected Grafts			
Pulse	518 (469-557)	883 (670- > 1000)	805 (662- > 1000
Group	(38.7)	(128.4)	(136.8)
Oral	506 (440-560)	800 (570-980)	841 (643- > 1000
Group	(43.6)	(147.7)	(134.0)

Discussion

The rejection reversal rates of 79.2% and 62.5% in the pulse and oral groups respectively compare favourably with other studies in which reversal rates of between 50% and 76% were found[78,88,158,191,235]. The reversal rate also depends on the degree of corneal vascularization: Fine and Stein[225] found that 66% of rejection episodes in avascular corneas cleared but only 50% in vascularised corneas. Sixteen of the pulse group grafts and fifteen in the oral group had vascularised corneas preoperatively. Many of these corneas were heavily vascularised and therefore the results should have been biased towards failure. Half of the patients had delayed seeking treatment until the rejection episode had been in progress for over 8 days: delay in initiating rejection treatment results in a lower success rate[88]. The resultant destruction of endothelial cells may leave insufficient cells to maintain graft clarity despite the rejection episode having been successfully reversed. The results in the patients who presented early showed a significantly higher survival rate ($p = < 0.05$) with pulsed therapy (92.3%) compared to oral therapy (54.5%). Grafts treated with pulsed therapy fared statistically better than those in the oral group if they presented up to 11 days after symptoms developed. Thereafter no difference was found between the two treatment groups.

An interesting finding was that grafts treated with pulsed therapy were statistically less likely to have a rejection episode in the future, compared to those treated with oral steroids ($p = < 0.025$). This may be an important finding as multiple rejection episodes are associated with a significantly decreased survival of grafts, especially in higher risk keratoplasties[408].

The mechanism by which pulse therapy works is not known. Studies have shown that a transient lymphopenia occurs which is maximal at four to six hours with T lymphocytes being affected to a greater extent than B cells, and with a relatively greater depletion of the helper/inducer subpopulation[403]. The concentration of

lymphocytes returns to normal by 48 hours. A similar pattern was found in our preliminary studies using pulse therapy in corneal graft rejection. It is thought that the decreased lymphocyte count is due to a change in distribution rather than to cell lysis[404,405], although local lymphocyte lysis has been demonstrated when topical corticosteroids are used in corneal graft rejection[406]. After a single pulse of steroid, delayed hypersensitivity skin tests and primary and secondary antibody responses return to normal by 48 hours[407]: at the same time that circulating lymphocytes are restored. However, the anti-inflammatory effect lasts four to seven days and it is possible that the clinical effect of pulse therapy is anti-inflammatory and not immunolytic[403]. A pulse of corticosteroid appears able to "reset" an aberrant immune response by the simultaneous occurrence of inhibition of the proliferating clone, the temporary removal of recirculating T lymphocytes from the blood and eye, and the profound suppression of peripheral inflammation[401]. This immunological manipulation has been shown to induce long term remissions in destructive corneal and scleral disease[401]. A similar explanation probably accounts for the decreased incidence of subsequent rejection episodes found in the patients treated with pulse therapy in this study.

We had hoped that pulsed therapy would reverse the rejection episode quicker and preserve more endothelial cells. Endothelial cell counts were not performed but cell density may be reflected by corneal thickness. Considering the grafts that survived the mean pre-rejection central corneal thicknesses of the two groups were similar: 510 and 512 microns respectively. The post-rejection measurements of the two groups showed the value in the pulse group (514 microns) to be less than that for the oral group (536 microns). Further work, including studies measuring endothelial cell densities, will need to be performed before pulse therapy is shown to be of benefit in this respect.

Although there was no overall significant improvement in graft survival when using pulse therapy, the improvement in cases presenting early, the simplicity of the

regimen and the avoidance of prolonged oral administration makes pulse therapy attractive. The decreased incidence of subsequent rejection episodes may be an important advantage, especially in high-risk cases. No complications of pulse therapy were encountered in this series, but careful monitoring is essential. Electrolyte and psychological disturbances are usually transient[401] but rare cases of unexplained death have been reported following pulse therapy[409,410].

6.4 Single versus double pulse therapy.

After a preliminary study[411] demonstrated that pulse therapy may have a role in treating severe endothelial rejection, a study comparing oral versus a single pulse of 500 mg of methylprednisolone was performed[395]. Graft survival was 79.2% in the group that received pulse therapy compared to 62.5% in the oral group: the difference was not significant (p=0.17). However, in patients who sought treatment early ($\leq$ 8 days) the survival rates for the groups became 92.3% and 54.5% respectively: values indicating a significant advantage for pulse therapy (p= $<$0.05). Pulse therapy also appeared to help prevent subsequent rejection episodes. Five (26.3%) of the nineteen surviving grafts in the pulse group had a further rejection episode compared to ten (66.7%) of the surviving fifteen grafts in the oral group: the difference is significant (p= $<$0.025).

Studies have shown that following a single pulse of steroid there is a profound drop in circulating lymphocytes maximal at four to six hours with recovery by 48 hours[403,411]. It was thought that if a second pulse of corticosteroid was given, a more sustained drop in the mean lymphocyte count would be achieved with possible improvement in graft prognosis. In this study patients with endothelial rejection were treated with either a single pulse or two pulses of methylprednisolone, the second being given after an interval of either 24 or 48 hours after the first.

Patients And Methods

Adult patients who presented with endothelial rejection were entered into the trial providing that symptoms of rejection had been present for less than three weeks. All gave informed consent prior to participation. Endothelial rejection was diagnosed when an eye with a previously clear, thin graft became inflamed with: flare and cells in the anterior chamber, keratic precipitates limited to the donor endothelium, and thickening of the graft either diffusely (probable rejection[167]) or in the form of

an advancing rejection line (definite rejection[167]). In the probable rejection cases only those with the central corneal thickness exceeding 620 microns, measured by ultrasound pachymetry, were included in this study. Eyes with a history of herpes simplex keratitis were also treated with topical antiviral medication.

The patients were assigned to one of three treatment groups as follows. The first 30 patients were treated with a single intravenous injection of methylprednisolone 500mg (single pulse group), this included the cohort of 24 patients assigned randomly to the single pulse group in our previous study comparing a single pulse to oral steroids[395]. The next 60 patients who presented consecutively with endothelial graft rejection were assigned randomly to two treatment groups. Both groups received 500 mg of methylprednisolone intravenously on admission. One group then received a second steroid pulse approximately 24 hours later (double 1 group) and the other group were given a second pulse at approximately 48 hours later (double 2 group). All three groups were also treated with hourly prednisolone acetate 1% drops during waking hours. The drops were tapered off after two weeks or earlier if the rejection episode showed signs of reversing. The response to treatment was monitored by assessing the improvement in clinical signs, the return of graft clarity and normal corneal thickness and restoration of visual acuity. Statistical comparison of the results from the three groups was performed using Chi square and Fisher's exact tests. Subsequent rejection episodes in the surviving grafts were noted: statistical comparisons were made using the log rank method[247-8] and survival curves were calculated using the Kaplan-Meier survival analysis method[244].

Results

The clinical details are shown in Tables XXVII and XXVIII (pages 231 and 232). The groups were reasonably well matched with respect to mean age and original diagnosis. The mean interval between grafting and the rejection episode was similar in the three groups but the median value was shorter in the single pulse group.

Preoperative corneal vascularization was classified by the number of quadrants containing stromal vessels crossing the proposed graft-host junction. Highly vascularised corneas were defined as having two or more quadrants involved. A high-risk cornea was defined as a cornea with two or more quadrants of stromal vascularization and/or previous graft failure from rejection. In this series there was a large number of high-risk corneas. There was an excess of heavily vascularised and high-risk corneas in the double 1 group.

The delay between the onset of rejection and the time the patient sought treatment was similar for the two groups and ranged between one day and three weeks (Table XXIX, page 233). The median delay in treatment for the three groups was 7 days. The number of grafts that survived was as follows: single pulse group 25 (83.3%), double 1 group 23 (74.2%) and double 2 group 23 (79.3%). There is no statistical difference between the number of grafts surviving the rejection episode in the three groups.

Table XXVII

Clinical Details

	Single	Double 1	Double 2
N	30	31	29
Sex (M:F)	16:14	20:11	17:12
Mean Age (Range)	37.7 (18-81)	46.1 (20-69)	43.7 (18-82)
Vascularization			
0-1 Quadrants	14	8	16
2+ Quadrants	16	23	13
Previous Grafts			
Nil	17	15	21
1+	13	16	8
High-risk			
No	10	7	15
Yes	20	24	14
Graft-rejection Interval (Months)			
Median	12	18	15
Mean (Range)	20.3 (1-114)	19.9 (1-59)	21.6 (1-90)

Table XXVIII

Original Diagnosis

	Single	Double 1	Double 2
Keratoconus	6	3	5
HSV Scarring	6	6	5
Other Scarring	4	3	3
Aphakic Bull. Ker.	0	2	3
Fuchs Dystrophy	4	2	2
I.K.	3	4	3
Trauma	3	4	4
Climatic Drop. Ker.	1	1	1
Acute Bact. Ulcer.	1	3	1
Other	2	3	2

Table XXIX

Outcome (Total Groups)

	Single	Double 1	Double 2
Median no. of days of rejection-treatment interval (Range)	7 (2-21)	7 (1-21)	7 (1-21)
Grafts surviving N (%)	25 (83.3%)	23 (74.2%)	23 (79.3%)
Surviving grafts having a further rejection episode N (%)	8 (32.0%)	8 (34.8%)	7 (30.4%)

The longer the rejection process has been in progress the worse the prognosis becomes because of irreversible damage to the endothelial layer which is then unable to maintain a clear graft[412]. The groups were sub-divided into patients that presented within the median time (7 days) and those who delayed seeking treatment (over 7 days); the results are shown in Table XXX (page 235). Graft survival in the patients who presented early was better in all three groups, but the differences did not reach statistical significance, possibly because of the small numbers in some of the groups. If the three groups are combined (Table XXX) then early presenters did show a statistically better survival compared to late presenters ($p = < 0.025$). The time of seven days was chosen arbitrarily because it was the median time of presentation. However significance remained at the 5% level, between the early and late presenters, for any division from seven to eleven days.

Table XXX

Graft Outcome In Early And Late Presenters

Group	N.	Graft Survival (%)
Single Pulse		
Early	15	14 (93.3%)
Late	15	11 (73.3%)
Double 1		
Early	17	15 (88.2%)
Late	14	8 (57.1%)
Double 2		
Early	15	13 (86.7%)
Late	14	10 (71.4%)
Total Group		
Early	47	42 (89.4%)
Late	43	29 (67.4%)
		(P = <0.025)

Patient presentation: **early ≤ 7 days, late ≥ 8 days.**

When the three groups were subdivided into high-risk and low-risk corneas (assessed at the time of the keratoplasty) the low-risk corneas fared better (Table XXXI, page 237) but there was no statistically significant difference in survival. Similar studies using previous graft rejection or vascularization as risk factors again showed no statistical significance.

The number of grafts that cleared and subsequently had a further rejection episode is shown in Table XXIX (page 233). The numbers are as follows: single pulse eight of twenty-five (32%), double 1 group eight of twenty-three (34.8%) and double 2 group seven of twenty-three (30.4%). The differences between the groups are not significant, survival analysis graphs of the time from treatment to a subsequent rejection episode is shown in figure 32 (page 238).

Table XXXI

Graft Outcome In High And Low Risk Corneas

Group	N.	Graft Survival (%)
Single Pulse		
Low	10	9 (90.0%)
High	20	16 (80.0%)
Double 1		
Low	7	6 (85.7%)
High	24	17 (70.8%)
Double 2		
Low	15	12 (80.0%)
High	14	11 (78.6%)
Total Group		
Low	32	27 (84.4%)
High	58	44 (75.9%)
		(P = 0.25)

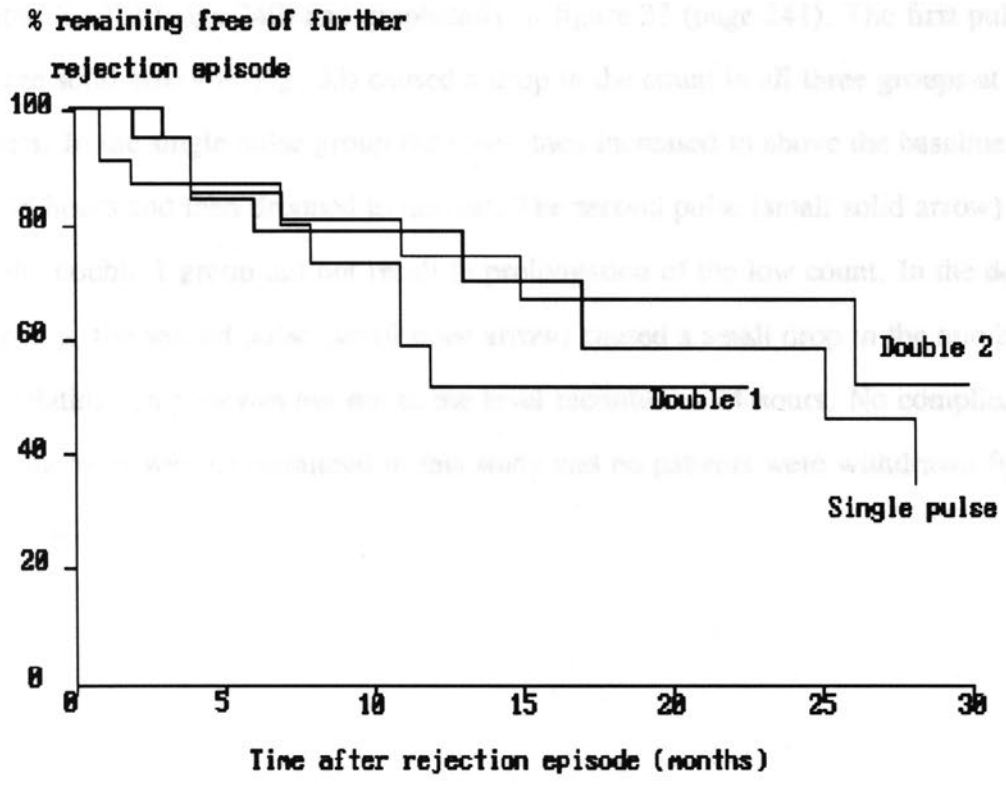


Fig. 32. Survival curves showing the risk of a subsequent rejection episode following successful graft rejection treatment.

The mean circulating lymphocyte counts are shown with standard deviations in Table XXXII (page 240) and graphically in figure 33 (page 241). The first pulse (large solid arrow in Fig. 33) caused a drop in the count in all three groups at 24 hours. In the single pulse group the count then increased to above the baseline level at 48 hours and then dropped to normal. The second pulse (small solid arrow) given to the double 1 group did not result in prolongation of the low count. In the double 2 group, the second pulse (small open arrow) caused a small drop in the number of circulating lymphocytes but not to the level recorded at 24 hours. No complications of treatment were encountered in this study and no patients were withdrawn from the trial.

Table XXXII

Circulating Lymphocyte Counts Following Pulse Therapy

Lymphocyte Count/l X 10^{-6} (S.D.)

	Baseline	24 Hours	48 Hours	72 Hours
Single	3.2 (0.45)	1.8 (0.31)	3.6 (0.42)	3.4 (0.30)
Double 1	2.9 (0.17)	2.0 (0.48)	3.0 (0.59)	3.5 (0.54)
Double 2	2.7 (0.30)	1.6 (0.41)	2.9 (0.39)	2.4 (0.26)

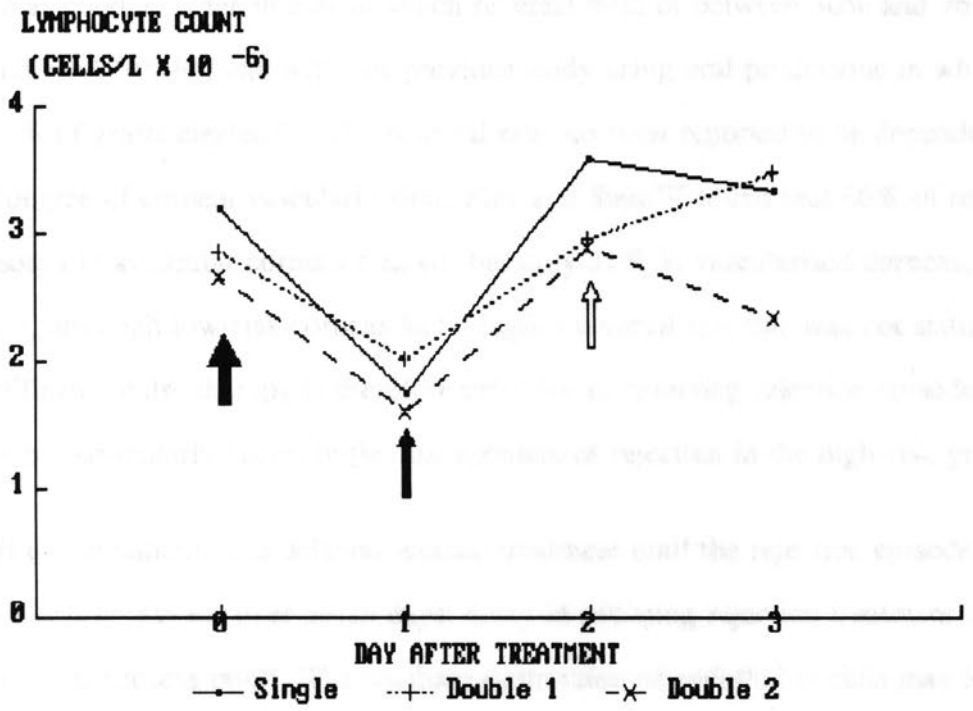


Fig. 33. The number of lymphocytes in circulating blood following pulse therapy.

Large solid arrow = initial pulse in all 3 groups.

Small solid arrow = second pulse in double 1 group.

Small open arrow = second pulse in double 2 group.

Discussion

The rejection reversal rates in the three groups of 83.3%, 74.2% and 79.3% are at least as good as other studies in which reversal rates of between 50% and 76% were found[78,88,158,191,235], and with our previous study using oral prednisone in which 62.5% of grafts cleared[395]. The reversal rate has been reported to be dependent on the degree of corneal vascularization: Fine and Stein[225] found that 66% of rejection episodes in avascular corneas cleared, but only 50% in vascularised corneas. In this study, although low-risk corneas had a higher reversal rate this was not statistically significant. Pulse therapy is therefore effective in reversing rejection episodes but may be particularly useful in the management of rejection in the high-risk groups.

Half of the patients had delayed seeking treatment until the rejection episode had been in progress for over seven days: delay in initiating rejection treatment results in a lower success rate[88]. The resultant destruction of endothelial cells may leave insufficient cells to maintain graft clarity despite the rejection episode having been successfully reversed. The results in the patients who presented early showed higher survival rates compared to late presenters in all groups, this reached statistical significance when the three groups were combined ($p = < 0.025$). This improved graft survival remained statistically significant for patients presenting up to eleven days after the start of the rejection episode.

An interesting finding in our previous report was that grafts treated with pulsed therapy were statistically less likely to have a rejection episode in the future, compared to those treated with oral steroids ($p = < 0.025$)[395]. In this study all the pulse groups were similar in having a low recurrent rejection rate, compared to the rate found with oral steroid therapy. This may be an important finding as multiple rejection episodes are associated with a significantly decreased survival of grafts, especially in higher risk keratoplasties[408].

The mechanism by which pulse therapy works is not known and this has been discussed in the previous section. Studies have shown that a transient lymphopenia occurs which is maximal at four to six hours with T lymphocytes being affected to a greater extent than B cells, and with a relatively greater depletion of the helper/inducer subpopulation[403]. The concentration of lymphocytes returns to normal by 48 hours, a similar pattern was found in our studies with a single pulse. Giving a second pulse at 24 hours did not prolong the circulating lymphopenia nor did a second pulse at 48 hours induce a new lymphopenia. Likewise no improvement in clinical outcome was achieved by giving a second pulse. After a single pulse of steroid, delayed hypersensitivity skin tests and primary and secondary antibody responses return to normal by 48 hours[407]: at the same time that circulating lymphocytes are restored. However, the anti-inflammatory effect lasts four to seven days and it is possible that the clinical effect of pulse therapy is anti-inflammatory and not immunolytic[403]. This study does not indicate whether this lack of benefit from a second pulse was due to inability to induce a longer or new lymphopenia, or whether a single pulse produces a maximal anti-inflammmatory effect lasting four to seven days that cannot be enhanced by an additional pulse.

In our previous study a single 500mg pulse of intravenous methylprednisolone was shown to give statistically better rejection reversal rates in patients with endothelial rejection presenting early; compared to patients treated with oral prednisone. Also grafts that cleared in the pulse group were statistically less likely to undergo a further rejection episode. In this study we investigated whether a second pulse of methylprednisolone would improve the prognosis further. The results indicate that no benefit is derived from giving the second pulse. However, this report confirms that pulse therapy is effective in reversing graft rejection and indicates that it may have a particular role in high-risk corneas undergoing rejection. The simplicity of the regimen and the avoidance of prolonged oral administration makes pulse therapy attractive. No complications of pulse therapy were encountered in this series.

However, as we warned in the previous study, careful monitoring is essential. Electrolyte and psychological disturbances are usually transient[401] but rare cases of unexplained death have been reported following pulse therapy[409,410].

6.5 Conclusion.

These series of experiments have shown that intravenous pulse therapy is effective in reversing graft rejection. In cases of endothelial rejection in which topical corticosteroids alone may be insufficient to reverse an established reaction it is necessary to use systemic corticosteroids. A single intravenous pulse of 500mg methylprednisolone is at least as effective as oral prednisone (60 - 80mg daily) and is statistically better in reversing graft rejection in patients who present early (< 11 days). In our studies day 8 was chosen as the divide between early and late presenters, but the statistically improved survival was present with delays of treatment up to 11 days. With current treatment regimens for rejection, high-risk corneas fare worse than corneas at low-risk; with pulse therapy this discrepancy was not so apparent indicating that pulse therapy may have a particular role in high-risk corneas undergoing a rejection episode. Another interesting finding was that grafts which cleared with pulse therapy seem to be less likely to have a further graft rejection episode compared to grafts treated with oral medication. The reason for this is unknown but it seems that a corticosteroid pulse "resets" the immune response by inhibiting the proliferating clone, temporarily removing recirculating T lymphocytes from the blood and eye, and by suppressing peripheral inflammation. No complications from the use of pulse therapy were encountered. This form of treatment therefore offers the advantage of an improved prognosis and avoids a prolonged course of oral medication with all its potential complications. A second pulse of methylprednisolone was not shown to offer any advantage over a single dose and is therefore unnecessary in treating graft rejection.

Section Seven: Conclusions

Section Seven: Conclusions

The advent of improved microsurgical techniques and instruments, better understanding of corneal physiology and improved methods of corneal storage have all contributed to improved prognosis of corneal grafting. Corneal graft rejection remains the commonest cause of graft failure. Improved surgical techniques have also contributed to lowering the incidence of rejection by allowing better wound apposition and the prevention of anterior synechiae. Both of these factors are known to encourage corneal vascularization. These risk factors and methods to prevent them have been discussed in Section 2 of this thesis. The awareness of the risk factors in corneal grafting enables additional precautions to be taken to prevent rejection in high-risk cases. Tissue matching has been shown to be beneficial in other fields of transplantation. In keratoplasty the results of tissue matching studies have been inconclusive and in many cases contradictory. Our studies have not contributed anything positive, but appear to confirm the existing contradictions. There is the impression that presently unknown factors are playing a role in corneal transplantation and more research is required to elucidate these. More sophisticated tissue typing techniques, such as DNA typing, are becoming available and will allow more accurate typing of the major and minor histocompatibility antigens. Hopefully, this in turn will result in closer donor matching and improved graft survival.

This thesis has looked at other methods of preventing corneal graft rejection. Our animal model has shown that cyclosporine given both systemically and topically helps to prevent rejection. There have been no conclusive studies that show that topical cyclosporine improves corneal graft survival in humans. Possibly, better preparations will be developed that allow greater concentrations of the drug to reach the eye, hopefully this would then lead to improved graft survival. The studies in

this thesis have shown that systemic cyclosporine improves graft survival. Improved graft survival is the result of both a reduction in the incidence of rejection episodes and of an improved rejection reversal rate. These effects appear to continue after cyclosporine is withdrawn, indicating that some degree of immunological privilege has been re-established. It would appear that treatment should be given for a year to obtain optimal benefit. Because the drug is expensive and has potentially serious side effects, cyclosporine has been reserved for very high-risk patients who either have only one eye or who are blind in both eyes.

Another important avenue of research is the role of immunomodulation to reduce the antigenicity of transplanted tissue. In the animal model it has been shown that preoperative irradiation with ultraviolet B can reduce the number of Langerhan's cells and improve the survival of transplanted corneas. More research is necessary to ensure that irradiating the cornea from the epithelial side has no damaging effect on the corneal endothelium. Any damage may not become apparent in some species, such as the rabbit, which has the ability to regenerate their endothelium. It will be necessary to study the effect of ultraviolet B radiation on primates prior to using the technique in humans. Providing no damage is done to the donor cornea, this avenue of research may have an important role in human corneal transplantation.

Corticosteroids remain the mainstay in the treatment of corneal graft rejection. Studies in this thesis have shown that a single intravenous pulse of 500 mg. methylprednisolone is as effective as oral prednisolone, given for a number of weeks, in reversing the rejection episode. Providing that the patient presents early, pulse therapy appears to offer advantages by improved rejection reversal rates and by preventing further rejection episodes. Serum markers for rejection are being investigated in other fields of transplantation and if found may allow earlier detection and treatment of rejection. Giving a second pulse does not seem to confer any additional benefit and our standard treatment for severe corneal graft rejection is now a single pulse of corticosteroid together with topical treatment.

References

References

1. Thylefors B. Prevention of blindness: the current focus. WHO Chronical 1985; 39: 149-54.

2. Dieffenbach JF. Beiträge zur Verpflanzung der Hornhaut (cited in Mannis and Krachmer, ref.3). Festschr Ophthalmol (von Ammon) 1831; 1: 172-6.

3. Mannis MJ, Krachmer JH. Keratoplasty: a historical perspective. Surv Ophthalmol 1981; 25: 333-8.

4. Casey TA, Mayer DJ. Corneal grafting; principles and practice. Philadelphia: Saunders, 1984: 309-24.

5. Pellier de Quengsy G. Précis au cours d'operations sur la chirurgie des yeux. Paris: Didot, 1789

6. Reisinger F. Die Keratoplastik, ein Versuch zur Erweiterung der Angenheilkunst. Bayerische Annalen 1824; 1: 207-15.

7. Bigger S. An inquiry into the possibility of transplanting the cornea, with the view of relieving blindness (hitherto deemed incurable) caused by several diseases of that structure. Dublin J Med Sci 1837; 11: 408-17.

8. Kissam RS. Ceratoplastice in Man. NY J Med 1844; 2: 281-2.

9. Von Hippel A. Eine neue Methode der Hornhauttransplantation (cited in Mannis and Krachmer, ref.3). Archiv Für Ophthalmologie 1888; 34: 108-20.

10. Zirm E. Eine erfolgreiche totale Keratoplastik. Archiv für Ophthalmol 1906; 64: 580-93.

11. Filatov VP. My path in science (cited in Mannis and Krachmer, ref.3).Moscow: Foreign Languages Publishing House, 1957.

12. Maumenee AE. The influence of donor-recipient sensitization on corneal grafts. Am J Ophthalmol 1951; 34: 142-52.

13. Chandler JW. Immunologic considerations in corneal transplantation. In: Kaufman H, McDonald MB, Barron BA, Waltman SR, ed. The cornea. New York: Churchill Livingstone, 1988: 725-41.

14. Klein J. Evolution and function of the major histocompatibility system: facts and speculation. In: Gotze D, ed. The major histocompatibility system in man and animals. Berlin: Springer-Verlag, 1977: 339-51.

15. Owen MJ, Crumpton MJ. Biochemistry of major human histocompatibility antigens. Immunol Today 1980; 1: 117-24.

16. Daar AS, Fuggle SV, Fabre JW, et al. The detailed distribution of HLA-A,B,C antigens in normal human organs. Transplantation 1984; 38: 287-92.

17. deWaal RMW, Bogman MJJ, Maass CN, et al. Variable expression of Ia antigens on the vascular endothelium of mouse skin grafts. Nature 1983; 303: 426-9.

18. Roitt IV. Essential immunology. (7th ed.) Oxford: Blackwell Scientific, 1991

19. Katz SM, Liebert M, Gill TJ, et al. The relative roles of MHC and non-MHC genes in heart and skin allograft survival. Transplantation 1983; 36: 96-101.

20. Klempnauer J, Wonigeit K, Gunther E, Pichlmayr R. Pancreas whole organ transplant in the rat: evidence for a strong effect of non-MHC incompatibilities. Transplant Proc 1983; 15: 1649-51.

21. Rosenthal AS. Regulation of the immune response: Role of the macrophage. New Engl J Med 1980; 303: 1153-6.

22. Thorsby E, Albrechtsen O, Hirschberg N, et al. MLC-activating HLA-D determinants: identification, tissue distribution, and significance. Transplant Proc 1977; 9: 393-400.

23. Schmidt GM, Bross KJ, Blume KG. Demonstration of HLA antigens on human skin fibroblasts by the peroxidase-anti-peroxidase method. Transplantation 1980; 29: 344-7.

24. Greineder DK, Rosenthal AS. Macrophage activation of allogeneic lymphocyte proliferation in the guinea pig mixed leukocyte culture. J Immunol 1975; 114: 1541-7.

25. Sher SE, Hull BE, Rosen S, et al. Acceptance of allogeneic fibroblasts in skin equivalent transplants. Transplantation 1983; 36: 552-7.

26. Bretscher P, Cohn M. A theory of self-nonself discrimination. Science 1970; 169: 1042-9.

27. Lafferty KJ, Woolnough J. The origin and mechanisms of allograft rejection. Immunol Rev 1977; 35: 231-62.

28. Bach FH, Bach ML, Sondel PM. Differential functions of major histocompatibility complex antigens in T-lymphocyte activation. Nature 1976; 259: 273-81.

29. Silvers WK, Bartlett ST, Chen HD, et al. Major histocompatibility complex restriction and transplantation immunity: a possible solution to the allograft problem. Transplantation 1984; 37: 28-32.

30. Chandler JW, Ray-Keil L, Gillette TE. Experimental corneal allograft rejection: description of a murine model and a new hypothesis of immunopathogenesis. Curr Eye Res 1983; 2: 387-97.

31. Cantor H, Boyle EA. Lymphocytes as models for the study of mammalian cellular differentiation. Immunol Rev 1977; 33: 105-24.

32. Reinherz EL, Shlossman SF. The differentiation and function of human T-lymphocytes. Cell 1980; 19: 821-7.

33. Chen HD, Silvers WK. Influence of Langerhans cells on the survival of H-Y incompatible skin grafts in rats. J Invest Dermatol 1983; 81: 20-3.

34. Stark WJ, Opelz G, Newsome D, et al. Sensitization to human lymphocyte antigens by corneal transplantation. Invest Ophthalmol Vis Sci 1973; 12: 639-45.

35. Falcon MG. Editorial: expression of HLA antigens in the cornea. Br J Ophthalmol 1989; 73: 585.

36. Terasaki PI, Perdue ST, Sasaki N, et al. Improving success rates of corneal transplantation. JAMA 1983; 250: 1065-8.

37. Opelz G, Mickey MR, Terasaki PI. HL-A and kidney transplants: reexamination. Transplantation 1974; 17: 371-82.

38. Moen T, Albrechtsen D, Flatmark A, et al. Importance of HLA-DR matching in cadaveric renal transplantation: a prospective one-centre study of 170 transplants. N Engl J Med 1980; 303: 850-4.

39. Batchelor JR. Transplantation. In: Brostoff J, Scadding GK, Male D, Roitt IM, ed. Clinical immunology. Philadelphia: Lippincott, 1991:

40. Hennige M, Kohler CO, Opelz G. Multivariate prediction model of kidney transplant success rates. Transplantation 1986; 42: 491-3.

41. Opelz G. Effect of HLA matching in 10,000 cyclosporine-treated cadaver kidney transplants. Transplant Proc 1987; 19: 641-6.

42. Steinmuller D, Hart EA. Passenger leukocytes and induction of allograft immunity. Transplant Proc 1971; 3: 673-75.

43. Lafferty KJ, Bootes A, Dart G, Talmage DW. Effect of organ culture on the survival of thyroid allografts in mice. Transplantation 1976; 22: 138-49.

44. Parr EL, Lafferty KJ, Bowen KM, MacKenzie IF. H-2 complex and Ia antigens on cells dissociated from mouse thyroid glands and islets of Langerhans. Transplantation 1980; 30: 142-8.

45. Parr EL, Bowen KM, Lafferty KJ. Cellular changes in cultured mouse thyroid glands and islets of Langerhans. Transplantation 1980; 30: 135-41.

46. Talmage DW, Dart GA. Effect of oxygen pressure during culture on survival of mouse thyroid allografts. Science 1978; 200: 1066-7.

47. Bowen KM, Lafferty KJ. Reversal of diabetes by allogeneic islet transplantation without immunosuppression. Aust J Exp Biol Med Sci 1980; 58: 441-7.

48. Kedinger M, Haffen K, Grenier J, Elroy R. In vitro culture reduces immunogenicity of pancreatic endocrine islets. Nature 1977; 270: 736-8.

49. Rabinovitch A, Alejandro R, Noel J, et al. Tissue culture reduces Ia antigen-bearing cells in rat islets, and prolongs islet allograft survival. Diabetes 1982; 31 (Suppl 4): 48-54.

50. Hart DNJ, Newton MR, Reece-smith H, et al. Major histocompatibility complex antigens in the rat pancreas, isolated pancreatic islets, thyroid and adrenal. Transplantation 1983; 36: 431-5.

51. Lau H, Reemtsma K, Hardy MA. Prolongation of rat islet allograft survival by direct ultraviolet irradiation of the graft. Science 1984; 223: 607-9.

52. Bach FH, Solinger HW. Collaboration between in vivo responses to LD and SD antigen of major histocompatibility complex. Nature 1976; 249: 487-8.

53. Bach FH, Alter BJ. Alternative pathways of T lymphocyte activation. J Exp Med 1978; 148: 829-34.

54. Lafferty KJ. Immunogenicity of foreign tissues. Transplantation 1980; 29: 179-82.

55. Alter BJ, Bach FH. Speculations on alternative pathways of T-lymphocyte responses. Scand J Immunol 1979; 10: 87-93.

56. Bain B, Vas MR, Lowenstein L. The development of large immature mononuclear cells in mixed leukocyte culture. Blood 1964; 23: 108-16.

57. Hayry P, Defendi V. Mixed lymphocyte cultures produce effector cells: model in vitro for allograft rejection. Science 1970; 168: 133-5.

58. Klein J, Juretic A, Baxevanis N, Nagy ZA. The traditional and a new version of the mouse H-2 complex. Nature 1981; 291: 455-60.

59. Hodes RJ, Svedmys EAJ. Specific cytotoxicity of H-2 incompatible mature lymphocytes following mixed culture in vitro. Transplantation 1970; 9: 470-7.

60. Lightbody J, Benoco D, Miggiano VC, Ceppellini R. Cell mediated lympholysis in man after sensitization of effector lymphocytes through mixed leukocyte cultures. G Batteriol Virol Immunol 1971; 64: 243-54.

61. Solliday S, Bach FH. Cytotoxicity: specificity after in vitro sensitization. Science 1970; 170: 1406-9.

62. David CS. Role of Ia antigen in immune response. Transplant Proc 1979; 11: 677-82.

63. Despont J, Steiner F, Banderet E. Functional and biochemical characteristics of human "null" lymphoid cells. Transplantation 1981; 31: 251-6.

64. Davies P, Bonney RJ. Secretory products of mononuclear phagocytes: a brief review. J Reticuloendothelial Soc 1979; 26: 37-47.

65. Katz DH, Benacerraf B, (eds.). The role of the products of the histocompatibility complex in immune responses. New York: Academic Press, 1976.

66. Unanue ER. Cooperation between mononuclear phagocytes and lymphocytes in immunity. N Engl J Med 1980; 303: 977-85.

67. Barcinski MA, Rosenthal AS. Immune response gene control of determinant selection: 1, Intramolecular mapping of the immunogenic sites on insulin recognized by guinea pig T and B cells. J Exp Med 1977; 145: 726-42.

68. Chandler JW, Heise ER, Weiser RS. Induction of delayed-type sensitivity-like reactions in the eye by the injections of lymphokines. Invest Ophthalmol Vis Sci 1973; 12: 400-6.

69. Oppenheim JJ, Stadler BM, Siraganian RP, et al. Lymphokines: their role in lymphocyte responses, Properties of interleukin 1. Fed Proc 1982; 41: 257-62.

70. Loveland BE, McKenzie IFC. What T cells cause graft rejection? Transplantation 1982; 33: 217-21.

71. Dallman MJ, Mason DW. Role of thymus derived and thymus-independent cells in murine skin allograft rejection. Transplantation 1982; 33: 221-3.

72. Kim B, Rosenstein M, Weiland D, al e. Clonal analysis of the lymphoid cells mediating skin allograft rejection. Transplantation 1983; 36: 525-32.

73. Carpenter CB. The cellular basis of allograft rejection. Immunology Today 1981; 2: 50-4.

74. Strom TB, Tilney NL, Paradysz JM, et al. Cellular components of allograft rejection: identity, specificity, and cytotoxic functions of cells infiltrating acutely rejecting allografts. J Immunol 1977; 118: 2020-6.

75. McCluskey RT. Comments on targets in rejecting allografts. Transplant Proc 1980; 12 (3 (Suppl 1)): 22-5.

76. Medawar PB. Immunity to homologous grafted skin, III, the fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye. Br J Exp Pathol 1948; 29: 58-69.

77. Billingham RE, Boswell T. Studies on the problem of corneal homografts. Proc R Soc Lond Biol 1953; 141: 392-406.

78. Chandler JW, Kaufman HE. Graft reactions after keratoplasty for keratoconus. Am J Ophthalmol 1974; 77: 543-7.

79. Khodadoust AA, Silverstein AM. Studies on the nature of privilege enjoyed by corneal allografts. Invest Ophthalmol Vis Sci 1972; 11: 137-48.

80. Niederkorn JY, Peeler JS, Ross J, Callanan D. The immunogenic privilege of corneal allografts. Regional Immunol 1989; 2: 117-24.

81. Callanan D, Peeler J, Niederkorn JY. Characteristics of rejection of orthotopic corneal grafts in the rat. Transplantation 1988; 45: 437-43.

82. Treseler PA, Sanfilippo F. The expression of major histocompatibility complex and leukocyte antigens by cells in the rat cornea. Transplantation 1986; 41: 248-52.

83. Treseler PA, Sanfilippo F. The relative immunogenicity of corneal epithelium, stroma, and endothelium. Transplantation 1986; 41: 229-34.

84. Basu PK, Miller I, Ormsby HL. Sex chromatin as a biological cell marker in the study of the fate of corneal transplants. Am J Ophthalmol 1960; 49: 513-5.

85. Chi HH, Teng CC, Katxin HM. The fate of endothelial cells in corneal homografts. Ophthalmology 1965; 59: 186-91.

86. Hanna C, Irwin ES. Fate of cells in the corneal graft. Arch Ophthalmol 1962; 68: 810-7.

87. Polack FM, Smelser GK, Rose J. Long-term survival of isotopically labelled stromal and endothelial cells in corneal homografts. Am J Ophthalmol 1964; 57: 67-78.

88. Alldredge OC, Krachmer JH. Clinical types of corneal rejection: their manifestations, frequency, preoperative correlates, and treatment. Arch Ophthalmol 1981; 99: 599-604.

89. Streilein JW, Niederkorn JY. Induction of anterior chamber-associated immune deviation requires an intact, functional spleen. J Exp Med 1981; 153: 1058-67.

90. Niederkorn JY, Streilein JW. Alloantigens placed into the anterior chamber of the eye induce specific suppression of delayed-type hypersensitivity but normal cytotoxic T lymphocyte and helper T lymphocyte responses. J Immunol 1983; 131: 2670-4.

91. Kaplan HJ, Stevens TR. A reconsideration of immunological privilege within the anterior chamber of the eye. Transplantation 1975; 19: 302-9.

92. Kaplan HJ, Sreilein JW. Immune response to immunization via the anterior chamber of the eye. 1, F1lymphocyte-induced immune deviation. J Immunol 1977; 118: 809-14.

93. Head JR, Billingham RE. Immunologically privileged sites in transplantation immunology and oncology. Perspect Biol Med 1985; 29: 115-31.

94. Streilein JW. Immune regulation and the eye: a dangerous compromise. FASEB J 1987; 1: 199-208.

95. Streilein JW. Anterior chamber associated immune deviation: the privilege of immunity in the eye. Surv Ophthalmol 1990; 35: 67-73.

96. Sonoda Y, Streilein JW. Orthotopic corneal transplantation in mice: evidence that the immunogenetic rules of rejection do not apply. Transplantation 1992; 54: 694-704.

97. Paufique L, Sourdille GP, Offret G. Les greffes de la cornee.Paris: Masson et Cie, 1948

98. Maumenee AE. The immune concept: its relation to corneal homotransplantation. Ann NY Acad Sci 1955; 59: 453-61.

99. Khodadoust AA, Silverstein AM. Transplantation and rejection of individual cell layers of the cornea. Invest Ophthalmol Vis Sci 1969; 8: 180-95.

100. Khodadoust AA, Silverstein AM. The survival and rejection of epithelium in experimental corneal grafts. Invest Ophthalmol Vis Sci 1969; 8: 169-79.

101. Maumenee AE. Clinical aspects of the corneal homograft reaction. Invest Ophthalmol Vis Sci 1962; 1: 244-51.

102. Polack FM, Kanai A. Electron microscope studies of graft endothelium in corneal graft rejection. Am J Ophthalmol 1972; 73: 711-7.

103. Nelken E, Michaelson IC, Nelken D, Gurevitch J. ABO antigens in the human cornea. Nature 1956; 177: 840.

104. Herold W. Nachweis der Blutgruppenantigene A und B in der menschlichen Hornhaut mittels der Immunofluoreszenztechnik. Klin Monatbl Augenheilkd 1972; 161: 658-62.

105. Dua HS, Shidham VB. Application of specific red blood cell adherence test to the human cornea and conjunctiva. Am J Ophthalmol 1979; 88: 1067-71.

106. Salisbury JD, Gebhardt BM. Blood group antigens on human corneal cells demonstrated by immunoperoxidase staining. Am J Ophthalmol 1981; 91: 46-50.

107. Treseler PA, Foulks GN, Sanfilippo F. The expression of HLA antigens by cells in the human cornea. Am J Ophthalmol 1984; 98: 763-72.

108. The Collaborative Corneal Transplantation Studies Research Group. The Collaborative Corneal Transplantation Studies (CCTS). Effectiveness of histocompatibility matching in high-risk corneal transplantation. Arch Ophthalmol 1992; 110: 1392-403.

109. Ehlers N, Ahrons S. Corneal transplantation and histocompatibility. Acta Ophthalmol (Copenh) 1971; 49: 513-27.

110. Newsome DA, Takasugi M, Kenyon KR, et al. Human corneal cells in vitro: morphology and histocompatibility (HLA) antigen of pure cell populations. Invest Ophthalmol Vis Sci 1974; 13: 23-32.

111. Whitsett CF, Stulting RD. The distribution of HLA antigens on human corneal tissue. Invest Ophthalmol Vis Sci 1984; 25: 519-24.

112. Fujikawa LS, Calvin RB, Bhan AK, et al. Expression of HLA-A/B/C and -DR locus antigens on epithelial stromal and endothelial cells of the human cornea. Cornea 1982; 1: 213-22.

113. Mayer DJ, Daar AS, Casey TA, Fabre JW. Localization of HLA A B C and HLA DR antigens in the human cornea: practical significance for grafting technique and HLA typing. Transplant Proc 1983; 15: 126-9.

114. Gillette TE, Chandler JW, Greiner JV. Langerhans cells of the ocular surface. Ophthalmology 1982; 89: 700-11.

115. Young E, Stark WJ, Prendergast RA. Immunology of corneal allograft rejection: HLA-DR antigens on human corneal cells. Invest Ophthalmol Vis Sci 1985; 26: 571-4.

116. Mayer DJ. Tissue typing: present state of the art and results. In: Brightbill FS, ed. Corneal surgery: theory, technique and tissue. 2nd ed. St Louis: Mosby, 1993: 640-4.

117. El-Asrar AMA, van den Oord JJ, Billiau A, et al. Recombinant interferon-gamma induces HLA-DR expression on human corneal epithelial and endothelial cells in vitro: a preliminary report. Br J Ophthalmol 1989; 73: 587-90.

118. Pepose JS, Gardner KM, Nestor NS, et al. Detection of HLA class I and II antigens in rejected human corneal allografts. Ophthalmology 1985; 92: 1480-4.

119. Mayer DJ, Casey TA. Reducing the risk of corneal graft rejection. A comparison of different methods. Cornea 1987; 6: 261-8.

120. Treseler PA, Sanfilippo F. Relative contribution of major histocompatibility complex antigens to the immunogenicity of corneal allografts. Transplantation 1986; 41: 508-14.

121. Katami M, Madden PW, White DJ, et al. The extent of immunological privilege of orthotopic corneal grafts in the inbred rat. Transplantation 1989; 48: 371-6.

122. Langerhans P. Ueber die Nerven der menschlichen Haut. Virchows Arch Path Anat Physiol 1868; 44: 325-37.

123. Sugiura S, Matsuda H. Ultrastructure of Langerhans cell in human corneal limbus. Japan J Ophthalmol 1969; 13: 63-68.

124. Rodrigues MM, Rowden G, Hackett J, Bakos I. Langerhans cells in the normal conjunctiva and peripheral cornea of selected species. Invest Ophthalmol Vis Sci 1981; 21: 759-65.

125. Birbeck MS, Breathnach AS, Everall JD. An electrom microscope study of basal melanocytes and high-level clear cells (Langerhans cells) in vitiligo. J Invest Dermatol 1961; 37: 51-64.

126. Wolff K, Winkelmann RK. Ultrastructural localization of nucleoside triphosphatase in Langerhans cells. J Invest Derm 1967; 48: 50-54.

127. Mackenzie IC, Squier CA. Cytochemical identification of ATPase-positive Langerhans cells in EDTA-separated sheets of mouse epidermis. Br J Derm 1975; 92: 523-533.

128. Baker KW, Habowsky JE. EDTA separation and ATPase Langerhans cell staining in the mouse epidermis. J Invest Derm 1983; 80: 104-7.

129. Peeler JS, Niederkorn JY. Antigen presentation by Langerhans cells in vivo: donor-derived Ia+ Langerhans are required for induction of delayed-type hypersensitivity but not for cytotoxic T lymphocyte responses to alloantigens. J Immunol 1986; 136: 4362-71.

130. Harrist TJ, Muhlbauer JE, Murphy GF, et al. T6 is superior to Ia (HLA-DR) as a marker for Langerhans cells and indeterminate cells in normal epidermis: a monoclonal antibody study. J Invest Derm 1983; 80: 100-103.

131. Stingl G, Wolff-Schreiner EC, Pichler WJ, et al. Epidermal Langerhans cells bear Fc and C3 receptors. Nature 1977; 268: 245-246.

132. Rowden G, Lewis MG, Sullivan AK. Ia antigen expression on human epidermal Langerhans cells. Nature 1977; 268: 247-248.

133. Klareskog L, Tjernlund UM, Forsum U, Peterson PA. Epidermal Langerhans cells express Ia antigens. Nature 1977; 268: 248-250.

134. Katz SI, Tamaki K, Sachs DH. Epidermal Langerhans cells are derived from cells originating in the bone marrow. Nature 1979; 282: 324-326.

135. Streilein JW, Grammer SF. In vitro evidence that Langerhans cells can adopt two functionally distinct forms capable of antigen presentation to T lymphocytes. J Immunol 1989; 143: 3925-3933.

136. Shackelford DA, Kaufman JF, Korman AJ, Strominger JL. HLA-DR antigens: structure, separations of subpopulations, gene cloning and function. Immunol Rev 1982; 66: 133-87.

137. Williams KA, White MA, Ash JK, Coster DJ. Leukocytes in the graft bed associated with corneal graft failure. Analysis by immunohistology and actuarial graft survival. Ophthalmology 1989; 96: 38-44.

138. Snell GD. The homograft reaction. Ann Rev Microbiol 1957; 11: 439-58.

139. Lafferty KJ, Cooley MA, Woolnough J, Walker KZ. Thyroid allograft immunogenicity is reduced after a period in organ culture. Science 1968; 188: 259-62.

140. Faustman D, Hauptfeld V, Lacy P, Davie JM. Prolongation of murine islet allograft survival by pretreatment of islets with antibody directed to Ia determinants. Proc Natl Acad Sci USA 1981; 78: 5156-9.

141. Morison WL, Parrish JA, Bloch KJ, Krugler JI. In vivo effect of UV-B on lymphocyte function. Br J Dermatol 1979; 101: 513-9.

142. Hersey P, Haran G, Hasic E, Edwards A. Alteration of T cell subsets and induction of suppressor T cell activity in normal subjects after exposure to sunlight. J Immunol 1983; 131: 171-4.

143. Lindahl-Kiessling K, Safwenberg J. Inability of UV-irradiated lymphocytes to stimulate allogenic cells in mixed lymphocyte culture. Int Arch Allergy 1971; 41: 670.

144. Deeg HJ. Ultraviolet irradiation in transplantation biology. Transplantation 1988; 45: 845-51.

145. Chabot J, Weber C, Hardy MA, et al. Synergy of ALS and UV-B in prolongation of primate-to- mouse islet xenograft survival. Transplant Proc 1987; 19: 1160-5.

146. Ray-Keil L, Chandler JW. Reduction in the incidence of rejection of hetertopic murine corneal transplants by pretreatment with ultraviolet irradiation. Transplantation 1986; 42: 403-6.

147. Ray-Keil L, Chandler JW. Rejection of murine heterotopic corneal transplants. Transplantation 1985; 39: 473-7.

148. Dana MR, Olkowski ST, Ahmadian H, et al. Low-dose ultraviolet-B irradiation of donor corneal endothelium and graft survival. Invest Ophthalmol Vis Sci 1990; 31: 2261-8.

149. Klareskog L, Forsum U, Tjerlund UM, et al. Expression of Ia-like molecules on cells in the corneal epithelium. Invest Ophthalmol Vis Sci 1979; 18: 310-3.

150. Streilein JW, Bergstresser PR. Ia antigens and epidermal Langerhans' cells. Transplantation 1980; 30: 319-23.

151. Aberer W, Stingl G, Stingl-Gazze LA, Wolff K. Langerhans cells as stimulator cells in the murine primary epidermal cell-lymphocyte reaction: alterations by UV-B irradiation. J Invest Dermatol 1982; 79: 129-35.

152. Noonan FP, Kripke ML, Pedersen JM, Greene MI. Suppression of contact hypersensitivity in mice by ultraviolet radiation is associated with defective antigen presentation. Immunology 1981; 43: 527-33.

153. Noonan FP, Bucana C, Sauder DN, DeFabo FC. Effect of ultraviolet radiation on epidermal Langerhans' cell morphology and function: a dichotomy. J Invest Dermatol 1982; 78: 345.

154. Austad J, Braather LR. Effect of UVB on alloactivating and antigen-presenting capacity of human epidermal Langerhans cells. Scand J Immunol 1985; 21: 417-23.

155. Baadsgaard O, Fox DA, Cooper KA. Human epidermal cells from ultraviolet light-exposed skin preferentially activate autoreactive CD4+ 2H4+ suppressor-inducer lymphocytes and CD8+ suppressor/cytotoxic lymphocytes. J Immunol 1988; 140: 1738-44.

156. Kripe ML. T cell-suppressors in ultraviolet carcinogenesis. Transplant Proc 1984; 16: 474-5.

157. Katami M. Corneal transplantation - immunologically privileged status. Eye 1991; 5: 528-48.

158. Gibbs DC, Batchelor JR, Werb A, et al. The influence of tissue-type compatibility on the fate of full thickness corneal grafts. Trans Ophthalmol Soc UK 1974; 94: 101-26.

159. Tuberville AW, Foster CS, Wood TO. The effect of donor corneal epithelium removal on the incidence of allograft rejection reactions. Ophthalmology 1983; 90: 1351-6.

160. Boisjoly HM, Bernard P, Dube I, et al. Effect of factors unrelated to tissue matching on corneal transplant endothelial rejection. Am J Ophthalmol 1989; 107: 647-54.

161. Sanfilippo F, MacQueen JM, Vaughn WK, Foulks GN. Reduced graft rejection with good HLA A and B matching in high risk corneal transplantation. New Engl J Med 1986; 315: 29-35.

162. Völker-Dieben HJM, D'Amaro J, Kok-van Alphen CC. Hierarchy of prognostic factors for corneal allograft survival. Aust NZ J Ophthalmol 1987; 15: 11-8.

163. Völker-Dieben HJM, Kok-van Alphen CC, Lansbergen Q, Persijn GG. Different influences on corneal graft survival in 539 transplants. Acta Ophthalmol (Copenh) 1982; 60: 198-202.

164. Engelstein JM, Herberman RB, Waltman SR. Protection of penetrating corneal allografts from immune rejection. Am J Ophthalmol 1972; 74: 311-6.

165. Jones BR. Summing up: present knowledge and problems of corneal graft failure. In: Porter R, Knight J, ed. Corneal Graft Failure. (Ciba Found Symp 15 new ser). Amsterdam: Elsevier, 1973: 349-54.

166. Sundmacher R. The role of donor epithelium in perforating keratoplasty. Dev Ophthalmol 1985; 11: 61-7.

167. Stulting RD, Waring GO, Bridges WZ, Cavanagh HD. Effect of donor epithelium on corneal transplant survival. Ophthalmology 1988; 95: 803-12.

168. Holland EJ, DeRuyter DN, Doughman DJ. Langerhans cells in organ-cultured corneas. Arch Ophthalmol 1987; 105: 542-5.

169. Pels E, van der Gaag R. HLA-A,B,C, and HLA-DR antigens and dendritic cells in fresh and organ culture preserved corneas. Cornea 1984/5; 3: 231-9.

170. Khodadoust AA, Silverstein AM. Induction of corneal graft rejection by passive cell transfer. Invest Ophthalmol Vis Sci 1976; 15: 89-95.

171. Khodadoust AA, Silverstein AM. Local graft versus host reactions within the anterior chamber of the eye: the formation of corneal endothelial pocks. Invest Ophthalmol Vis Sci 1975; 14: 640-7.

172. Grönemeyer U, Pülhorn G, Müller-Ruchholtz W. Allogeneic corneal grafting in inbred strains of rats: histology of graft reaction. Albrecht von Graefes Arch Klin Exp Ophthalmol 1978; 208: 247.

173. Polack FM. Histopathological and histochemical alterations in the early stages of graft rejection. J Exp Med 1962; 116: 709.

174. Polack FM, Gonzales CE. The response of lymphoid tissue to corneal heterografts. Arch Ophthalmol 1968; 80: 321.

175. MacDonald AL, Basu PK. Systemic sensitization of corneal allograft recipients before the clinical onset of graft reaction. Can J Ophthalmol 1977; 12: 60-2.

176. Sher NA, Doughman DJ, Mindrup E, et al. Macrophage migration inhibition factor activity in the aqueous humor during experimental corneal xenograft and allograft rejection. Am J Ophthalmol 1976; 82: 858-65.

177. Steinmuller D. Which T cells mediate allograft rejection? Transplantation 1985; 40: 229-33.

178. Matoba AY, Peeler JS, Niederkorn JY. T cell subsets in the immune rejection of murine hetertopic corneal allografts. Invest Ophthalmol Vis Sci 1986; 27: 1244-54.

179. Ehlers N, Olsen T, Johnson HE. Corneal graft rejection probably mediated by antibodies. Acta Ophthalmol (Copenh) 1981; 59: 119-25.

180. Wilson SE, Kaufman HE. Graft failure after penetrating keratoplasty. Surv Ophthalmol 1990; 34: 325-56.

181. Shapiro MB, Mandel MR, Krachmer JH. Rejection: clinical forms, diagnosis, and treatment. In: Brightbill FS, ed. Corneal surgery: theory, technique and tissue. 2nd ed. St Louis: Mosby, 1993: 254-60.

182. Meuller H, Maumenee AE. Consideration sur la maladie du greffon. Arch Ophthalmol (Paris) 1951; 11: 146-54.

183. Watson AP, Simcock PR, Ridgway AEA. Endothelial cell loss due to repeated traumatic wound dehiscence after penetrating keratoplasty. Cornea 1987; 6: 216-8.

184. Smolin G, Goodman D. Corneal graft reaction. Int Ophthalmol Clin 1988; 28: 30-6.

185. Brooks AMV, Grant G, Gillies WE. Assessment of the corneal endothelium following keratoplasty. Aust NZ J Ophthalmol 1989; 17: 379-85.

186. Donshik PC, Cavanagh HD, Boruchoff SA, Dohlman CH. Effect of bilateral and unilateral grafts on the incidence of rejections in keratoconus. Am J Ophthalmol 1979; 87: 823-6.

187. Meyer RF. Corneal allograft rejection in bilateral penetrating keratoplasty: clinical and laboratory studies. Trans Am Ophthalmol Soc 1986; 84: 664-742.

188. Foulks GN, Sanfilippo FP, Locascio JA, et al. Histocompatibility testing for keratoplasty in high-risk patients. Ophthalmology 1983; 90: 239-44.

189. Boisjoly HM, Roy R, Dubé I, et al. HLA-A, B and DR matching in corneal transplantation. Ophthalmology 1986; 93: 1290-7.

190. Arentsen JJ. Corneal transplant allograft reaction: possible predisposing factors. Trans Am Ophthalmol Soc 1983; 81: 361-402.

191. Khodadoust AA. The allograft rejection reaction: the leading cause of late graft failure of clinical corneal grafts. In: Porter R, Knight J, ed. Corneal Graft Failure. (Ciba Found Symp 15 new ser). Amsterdam: Elsevier, 1973: 151-63.

192. Stark WJ. Transplantation immunology of penetrating keratoplasty. Trans Am Ophthalmol Soc 1980; 78: 1079-117.

193. Maumenee AE, ed. Clinical patterns of corneal graft failure. Amsterdam: Amsterdam, 1973:5-15. (Porter R, Knight J, ed. Corneal Graft Failure. (Ciba Found Symp 15 new ser).

194. Krachmer JH, Alldredge OC. Subepithelial infiltrates. A probable sign of corneal transplant rejection. Arch ophthalmol 1978; 96: 2234-7.

195. Smolin G, Biswell R. Corneal graft rejection associated with anterior iris adhesion: case report. Ann Ophthalmol 1978; 10: 1603-4.

196. Polack FM. Scanning electron microscopy of the host-graft endothelial junction in corneal graft reaction. Am J Ophthalmol 1972; 73: 704-11.

197. Offret G, Pouliquen Y, Guyot D. Aspects cliniques des réactions immunitaires après kératoplasties transfixiantes chez l'homme. Arch Ophthalmol (Paris) 1970; 30: 209-21.

198. Polack FM. Corneal transplantation.New York: Grune & Stratton, 1977:201-34.

199. Batchelor JR, Casey TA, Gibbs DC, et al. HLA matching and corneal grafting. Lancet 1976; 1: 551-4.

200. Cobo LM, Coster DJ, Rice NSC, Jones BR. Prognosis and management of corneal transplantation for herpes keratitis. Arch Ophthalmol 1980; 98: 1755-9.

201. Völker-Dieben HJM, Kok-van Alphen CC, D'Amaro J, de Lange P. The effect of prospective HLA-A and -B matching in 288 penetrating keratoplasties for herpes simplex keratitis. Acta Ophthalmol (Copenh) 1984; 62: 513-23.

202. Cherry PMH, Pashby RC, Tadros ML, et al. An analysis of corneal transplantation. 1. Graft clarity. Ann Ophthalmol 1979; 11: 461-9.

203. Smolin G, Hyndiuk RA. Lymphatic drainage from vascularized rabbit cornea. Am J Ophthalmol 1971; 72: 147-51.

204. Collin HB. Corneal lymphatics in alloxan vascularized rabbit eyes. Invest Ophthalmol Vis Sci 1966; 5: 1-13.

205. Collin HB. Lymphatic drainage of 131 I-albumin from the vascularized cornea. Invest Ophthalmol Vis Sci 1970; 9: 146-55.

206. Paque J, Poirier RH. Corneal allograft reaction and its relationship to suture site neovascularization. Ophthalmic Surg 1977; 8: 71-4.

207. Shaw EL. Suture removal. In: Brightbill FS, ed. Corneal surgery. Theory, technique, and tissue. St Louis: C V Mosby, 1986: 286-91.

208. Ficker LA, Kirkness CM, Rice NSC, Steele ADMG. The changing management and improved prognosis for corneal grafting in herpes simplex keratitis. Ophthalmology 1989; 96: 1587-96.

209. Cavanagh HD, Leveille AS. Extended-wear contact lenses in patients with corneal grafts and aphakia. Ophthalmology 1982; 89: 643-50.

210. Lemp MA. The effect of extended-wear aphakic hydrophilic contact lenses after penetrating keratoplasty. Am J Ophthalmol 1980; 90: 331-5.

211. Mackman G, Polack FM, Sidrys L. Fluorescein angiography of soft lens induced vascularization in penetrating keratoplasty. Ophthalmic Surg 1985; 16: 157-61.

212. Weinberg RJ. Deep corneal vascularization caused by aphakic soft contact lens wear. Am J Ophthalmol 1977; 83: 121-2.

213. Narankari VS, Baer JC. Corneal argon laser photocoagulation for neovascularization in penetrating keratoplasty. Ophthalmology 1986; 93: 1304-9.

214. Cherry PMH, Faulknew JD, Shaver RP. Argon laser treatment of corneal neovascularization. Ann Ophthalmol 1973; 5: 911-20.

215. D'Amaro J, Völker-Dieben HJ, Kruit PJ, et al. Influence of pretransplant sensitization on the survival of corneal allografts. Transplant Proc 1991; 23: 368-72.

216. Stark WJ, Taylor HR, Datiles M, et al. Transplantation antigens and keratoplasty. Aust J Ophthalmol 1983; 11: 333-9.

217. Allansmith MR, Fine M, Payne R. Histocompatibility typing and corneal transplantation. Trans Am Acad Ophthalmol Otolaryngol 1974; 78: 445-60.

218. Ruedemann AD. Clinical course of keratoconus. Trans Am Acad Ophthalmol Otolarygol 1970; 74: 384-98.

219. Khodadoust AA, Karnema Y. Corneal grafts in the second eye. Cornea 1984; 3: 17-20.

220. Musch DC, Meyer RF. Risk of endothelial rejection after bilateral penetrating keratoplasty. Ophthalmology 1989; 96: 1139-43.

221. Dale DC, Fauci AS, Wolff SM. Alternate-day prednisone. N Engl J Med 1974; 291: 1154-9.

222. Rinne JR, Stulting RD. Current practices in the prevention and treatment of corneal graft rejection. Cornea 1992; 11: 326-8.

223. Hwang DG, Stern WH, Hwang PH, MacGowan-smith LA. Collagen shield enhancement of topical dexamethasone penetration. Arch Ophthalmol 1989; 107: 1375-80.

224. Brown SI, Tragakis MP, Pearce DB. Corneal transplantation for severe alkali burns. Trans Am Acad Ophthalmol Otolaryngol 1972; 76: 1266-74.

225. Fine M, Stein M. The role of corneal vascularization in human corneal graft reactions. In: Porter R, Knight J, eds. Corneal Graft Failure. (Ciba Found Symp 15 new ser). Amsterdam: Elsevier, 1973: 193-204.

226. Maumenee AE. The role of steroids in the prevention of corneal graft failure. In: Porter R, Knight J, eds. Corneal Graft Failure. (Ciba Found Symp 15 new ser). Amsterdam: Elsevier, 1973: 241-4.

227. Hughes WF, Kallmeyer J. Aetiology and treatment of the corneal homograft reaction including azathioprine (imuran). S Afr Med J 1967; 41: 548-51.

228. Mackay IR, Bigwell JL, Smith PH, Crawford BA. Prevention of corneal-graft failure with the immunosuppressive drug azathioprine. Lancet 1967; 2: 479-82.

229. Polack FM. Effect of azathioprine (imuran) on corneal graft reaction. Am J Ophthalmol 1967; 64: 233-44.

230. McCulloch C. Immunosuppression and keratoplasty. Can J Ophthalmol 1971; 6: 161-9.

231. Philipsen WMJG. The treatment of corneal transplantations with Imuran. Ophthalmologica 1972; 165: 529-32.

232. Barraquer J. Immunosuppressive agents in penetrating keratoplasty. Am J Ophthalmol 1985; 100: 61-4.

233. Hughes WF. Keratoplasty for corneal dystrophies. Am J Ophthalmol 1960; 50: 1100-14.

234. Foulks GN, Sanfilippo F. Beneficial effects of histocompatibility in high-risk corneal transplantation. Am J Ophthalmol 1982; 94: 622-9.

235. Williams KA, Sawyer MA, White MA, et al. Report from the Australian corneal graft registry. Transplant Proc 1989; 21: 3142-4.

236. Terasaki PI, McClelland JD. Microdroplet assay of human serum cytotoxins. Nature 1964; 204: 998-1009.

237. Treseler PA, Foulks GN, Sanfilippo F. Contribution of major histocompatibility complex antigens to the immunogenicity of corneal allografts. Transplantation 1986; 41: 508-14.

238. Kissmeyer-Nielsen F, Olsen S, Posborg-Petersen V, et al. Hyperacute rejection of kidney allografts associated with pre-existing humoral antibodies against donor cells. Lancet 1966; 2: 662-5.

239. Amos DB, Bashir H, Boyle W, et al. A simple microcytotoxicity test. Transplantation 1969; 7: 220-3.

240. Nelken E, Nelken D. Serological studies in keratoplasty. Br J Ophthalmol 1965; 49: 159-62.

241. Polack FM. Clinical and pathological aspects of the corneal graft reaction. Trans Amer Acad Ophthalmol & Otolarygol 1973; 77: 418-31.

242. Stark WJ, Taylor HR, Bias WB, Maumenee AE. Histocompatibility (HLA) antigens and keratoplasty. Am J Ophthalmol 1978; 86: 595-604.

243. Siegel D. Analysis of follow-up. Arch Ophthalmol 1985; 103: 647-8.

244. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. J Am Statist Assoc 1958; 53: 457-81.

245. Mantel N. Evaluation of survival data and two new rank order statistics arising in its considerations. Cancer Chemother Rep 1966; 50: 163-70.

246. Cox DR. Regression models and life-tables. J R Statist Soc 1972; 34: 187-202.

247. Peto R, Pike MC, Armitage P, et al. Design and analysis of randomized clinical trials requiring prolonged observation of each patient. I. Introduction and design. Br J Cancer 1976; 34: 585-612.

248. Peto R, Pike MC, Armitage P, et al. Design and analysis of randomized clinical trials requiring prolonged observation of each patient. II. Analysis and examples. Br J Cancer 1977; 35: 1-39.

249. Kissmeyer-Nielsen F, Ehlers N. Cornea transplantation and matching for HLA-A and B. Scand J Urol Nephrol 1977; 42 (Suppl): 44-5.

250. Vannas S, Karjalalainen K, Ruusuvaara P, Tilikainen A. HLA-compatible donor cornea for prevention of allograft reaction. Albrecht von Graefe's Arch Klin Exp Ophthalmol 1976; 198: 217-222.

251. Völker-Dieben HJM, Kok-van Alphen CC, Kruit PJ. Advances and disappointments, indications and restrictions regarding HLA-matched corneal grafts in high risk cases. Doc Ophthalmol 1979; 46: 219-26.

252. Ehlers N, Kissmeyer-Nielsen F. Corneal transplantation and HLA histocompatiblity. Acta Ophthalmol (Copenh) 1979; 57: 738-41.

253. Völker-Dieben HJM, Kok-van Alphen CC, Lansbergen Q, Persijn GG. The effect of prospective HLA-A and -B matching on corneal graft survival. Acta Ophthalmol (Copenh) 1982; 60: 203-12.

254. Grönemeyer U, Westphal E, Böke W. Prognostic value of histocompatiblity testing in corneal grafting. A prospective study. Dev Ophthalmol 1985; 11: 56-60.

255. Özdemir Ö. A prospective study of histocompatability testing for keratoplasty in high-risk patients. Br J Ophthalmol 1986; 70: 183-6.

256. Hoffmann F, von Keyserlingk HJ, Wiederholt M. Die Bedeutung der HLA-typisierung bei Hornhauttransplantaten mit ungüunstiger Prognose unter Cyclosporin-A-therapie. Fortschr Ophthalmol 1986; 83: 542-6.

257. Hoffmann F, von Keyserlingk HJ, Wiederholt M. Importance of HLA DR matching for corneal transplantation in high-risk cases. Cornea 1986; 5: 139-43.

258. Busson M, Raffouv C, Bouteiller AM, et al. Influence of HLA A, B, and DR matching on the outcome of kidney transplant survival in pre-immunized patients. Transplantation 1984; 38: 227-30.

259. Fine RN, Terasaki PL, Ettenger RB, et al. Renal transplantation update. Ann Intern Med 1984; 100: 246-57.

260. Beekhuis WH, van Rij G, Renardel de Lavalette JG, et al. Corneal graft survival in HLA-A and HLA-B matched transplantation in high-risk cases with retrospective review of HLA-DR compatibility. Cornea 1991; 10: 9-12.

261. Hoffmann F, Pahlitzsch T. Predisposing factors in corneal graft rejection. Cornea 1989; 8: 215-9.

262. Völker-Dieben HJM. The interactions of HLA-DR, donor graft size and corneal vascularization of the recipient on graft survival. In: The effect of immunological and non-immunological factors on corneal graft survival. 1st ed. Dordrecht: Dr W Junk, 1984: 69-95.

263. Walford RL, Carter PK, Anderson RE. Leukocyte antibodies following skin homografting in the human. Transplant Bull 1962; 29: 16-8.

264. Opelz G, Terasaki PI. Histocompatibility matching utilizing responsiveness as a new dimension. Transplant Proc 1972; 4: 433-7.

265. Morris PJ, Williams GM, Hume DM. Serotyping for homotransplantation. 12. Occurrence of cytotoxic antibodies following kidney transplantation in man. Transplantation 1968; 6: 392-9.

266. Boisjoly HM, Roy R, Bernard PM, et al. Association between corneal allograft reactions and HLA compatibility. Ophthalmology 1990; 97: 1689-98.

267. Nelken E, Nelken D, Michaelson IC, Gurevitch J. Late clouding of experimental corneal grafts. Arch Ophthalmol 1961; 65: 584-90.

268. Mehri P, Becker B, Oglesby R. Corneal transplants and blood groups: a clinical study. Am J Ophthalmol 1959; 47: 48-53.

269. Mailäth L, Stenszky V, Alberth B, Aszödi L. Die Rolle der Blutgruppen-kompatibilität bei Keratoplastik. Klin Monatsbl Augenheilkd 1972; 160: 550-3.

270. Allansmith MR, Drell DW, Kajiyama AB, Fine M. ABO blood groups and corneal transplantation. Am J Ophthalmol 1975; 79: 493-501.

271. Völker-Dieben HJM. Different influences on corneal graft survival in 539 transplants. In: The effect of immunological and non-immunological factors on corneal graft survival. Dordrecht: Dr W Junk, 1984: 43-56.

272. Foulks GN, Sanfillippo F. The role of corneal antigens in graft success or failure. In: Brightbill FS, ed. Corneal surgery: theory, technique and tissue. 2nd ed. St Louis: Mosby, 1993: 644-50.

273. Meyer HJ. Spielen Blutfaktoren eine Rolle bei der Hornhauttransplantation? Dtsch Med Wochenschr 1971; 96: 1259-60.

274. The Collaborative Corneal Transplantation Studies Research Group. Design and methods of the Collaborative Corneal Transplantation Studies. Cornea 1993; 12: 93-103.

275. Tiercy JM, Morel C, Freidel AC, al e. Selection of unrelated donors for bone marrow transplantation is improved by HLA class II genotyping with oligonucleotide hybridization. Proc Natl Acad Sci USA 1991; 88: 7121-5.

276. Khodadoust AA, Abizadeh A. The fate of corneal regrafts after previous rejection reaction.New York: Masson, 1979:167-73. (Silverstein AM, O'Connor GR, ed. Immunology and immunopathy of the eye

277. Greaves DP. Experimental penetrating keratoplasty. Trans Ophthalmol UK 1954; 54: 135-42.

278. Khodadoust AA. Penetrating keratoplasty in the rabbit. Am J Ophthalmol 1968; 66: 899-905.

279. McEntyre JM. Experimental penetrating keratoplasty in the dog. Arch Ophthalmol 1968; 80: 372-6.

280. Coster DJ, Shepherd WFI, Fook CT, Rice NSC. Prolonged survival of corneal allografts in rabbits treated with cyclosporin A. Lancet 1979; 2: 688-9.

281. Shepherd WFI, Coster DJ, Fook TC, et al. Effect of cyclosporin A on the survival of corneal grafts in rabbits. Br J Ophthalmol 1980; 64: 148-53.

282. Bell TAG, Easty DL, McCullagh KG. A placebo-controlled double blind trial of cyclosporin-A in prevention of corneal graft rejection in rabbits. Br J Ophthalmol 1982; 66: 303-8.

283. Salisbury JD, Gebhardt BM. Suppression of corneal allograft rejection by cyclosporin A. Arch Ophthalmol 1981; 99: 1640-3.

284. Lorenzetti DWC, Kaufman HE. Experimental production of graft reactions with suppression by topical steroids. Arch Ophthalmol 1966; 76: 274-81.

285. Basu PK. Studies on the mechanism of corneal graft reaction using simplified systems.Oxford: Perganon, 1967:163. 2nd International Corneoplastic Conference

286. Hunter PA, Garner A, Wilhelmus KR, et al. Corneal graft rejection: a new rabbit model and cyclosporin-A. Br J Ophthalmol 1982; 66: 292-302.

287. Streilein JW, Toews GB, Bergstresser PR. Langerhans cells functional aspects revealed by in vivo grafting studies. J Invest Derm 1980; 75: 17-21.

288. Williams KA, Grutzmacher RD, Roussel TJ, Coster DJ. A comparison of the effects of topical cyclosporine and topical steroid on rabbit corneal allograft rejection Transplantation. 1985; 39: 242-4.

289. Donatsch P, Abisch E, Homberger M, et al. A radioimmune assay to measure cyclosporin A in plasma and serum samples. J Immunoassay 1981; 2: 19-22.

290. Gehan EA. A generalized Wilcoxon test for comparing arbitarily singly-censored samples. Biometrika 1965; 52: 202-3.

291. Breslow NE. A generalized Kruskal-Wallis test for comparing K samples subject to unequal patterns of censorship. Biometrika 1970; 57: 579-94.

292. Hunter PA, Rice NSC, Jones BR. Prolonged corneal graft survival using topically applied cyclosporin A. Trans Ophthalmol Soc UK 1982; 102: 19-20.

293. Hunter PA. Potential role of cyclosporin A in corneal grafting. J Roy Soc Med 1981; 74: 810-813.

294. Chen YF, Gebhardt BM, Reidy JJ, Kaufman HE. Cyclosporine-containing collagen shields suppress corneal allograft rejection. Am J Ophthalmol 1990; 109: 132-7.

295. Foets B, Missotten L, Van der veeren P, Goossens W. Prolonged survival of allogeneic corneal grafts in rabbits treated with topically applied cyclosporin A: systemic absorption and local immunosuppressive effect. Br J Ophthalmol 1985; 69: 600-603.

296. Morris PJ. Cyclosporin A: an overview. Transplantation 1981; 32: 349-54.

297. White DJG. Cyclosporin A. Amsterdam: Elsevier Biomedical, 1982

298. Gratwohl A, Riederer I, Graf E, Speck B. Cyclosporin toxicity in rabbits. Lab Anim 1986; 20: 213-20.

299. Lancki DW, Tissot RG, Cohen C. Histocompatibility in the rabbit. Transplantation 1979; 27: 79-86.

300. Bahn CF, MacCullum DK, Lovett EJ, et al. Corneal transplantation in the cat: cellular immune mechanisms in graft survival and rejection. Cornea 1983; 3: 263-75.

301. Chai CK, Lerner CP. Asymmetrical mixed lymphocyte reaction between inbred strains of rabbit. Immunogenetics 1975; 2: 363-8.

302. Cohen C, Tissot RG. The effect of the RL-A locus and MLC locus on graft survival in the rabbit. Transplantation 1974; 18: 150-4.

303. Tait BD, d'Apice AJF, Cove LY. The correlation of cadaver renal donor response in the mixed lymphocyte reaction with recipient renal allograft survival. Transplant Proc 1979; 11: 1248-9.

304. Jeffery JR, Cheung K, Masniuk J, Taylor D. Mixed lymphocyte culture responses: lack of correlation with cadaveric renal allograft survival and blood transfusions. Transplantation 1984; 38: 42-5.

305. Hess AD, Esa AH, Colombani PM. Mechanism of action of cyclosporine: effects on cells of the immune system and on subcellular events in T cell activation. Transplant Proc 1988; 20(2 (suppl 2)): 29-40.

306. Roussel TJ, Osato MS, Wilhelmus KR. Cyclosporine and experimental corneal transplantation. Transplant Proc 1983; 15: 3081-3.

307. Williams KA, Ash JK, Coster DJ. Histocompatibility antigen and passenger cell content of normal and diseased human cornea. Transplantation 1985; 39: 265-9.

308. Williams KA, Mann TS, Lewis M, Coster DJ. The role of resident accessory cells in corneal allograft rejection in the rabbit. Transplantation 1986; 42: 667-71.

309. Manca F, Fenoglio D, Kunkl A, et al. Effect of cyclosporine on the antigen-presenting function of human and murine accessory cells. Transplantation 1988; 46 Suppl: 40-3S.

310. Knight SC, Roberts M, Macatonia SE, Edwards AJ. Blocking of acquisition and presentation of antigen by dendritic cells with cyclosporine. Transplantation 1988; 46 Suppl: 48-53S.

311. Yoshimura N, Kahan BD, Matsui S, et al. Cyclosporine effect on immunoregulatory cells in kidney transplant recipients: suppression of gamma-interferon and interleukin production. Transplant Proc 1988; 20(2 Suppl 2): 69-74.

312. Mosteller MW, Gebhardt BM, Hamilton AM, Kaufman HE. Penetration of topical cyclosporine into the rabbit cornea, aqueous humor and serum. Arch Ophthalmol 1985; 103: 101-102.

313. Hardy MA, Lau H, Reemtsma K. Prolongation of rat islet allografts with the use of ultraviolet irradiation, without immunosuppression. Transplant Proc 1984; 16: 865-9.

314. Hill JC, Maske R. An animal model for corneal graft rejection in high-risk keratoplasty. Transplantation 1988; 46: 26-30.

315. Brown SI, Bloomfield SE, Pearce DB. A follow-up report on transplantation of the alkali-burned cornea. Am J Ophthalmol 1974; 77: 538-44.

316. Khodadoust AA, Abizadeh A. The fate of corneal regrafts after previous graft reactions.New York: Masson, 1977:167-73. (Silverstein L, O'Connor G, ed. Immunology and immunopathologyof the eye

317. Nussenblatt RB, Rook AH, Wacker WB, et al. Treatment of intraocular inflammatory disease with cyclosporin A. Lancet 1983; 2: 235-8.

318. Nussenblatt RB, Palestine AG. Cyclosporine: immunology, pharmacology and therapeutic uses. Surv Ophthalmol 1986; 31: 159-69.

319. Borel JF, Stähelin H. Cyclosporin A - The history and significance of its discovery. Sandorama 1983; 11: 5-9.

320. Borel JF. The history of cyclosporine A and its significance. In: White DJG, ed. Cyclosporine A. Amsterdam: Elsevier Biomedical Press, 1982: 5-8.

321. Borel JF, Feurer C, Gubler HU, Stähelin H. Biological effects of cyclosporin A: a new antilymphocytic agent. Agents Actions 1976; 6: 468-75.

322. Petcher TJ, Weber HP, Ruegger A. Crystals and molecular structure of an Iodo-derivative of the cyclic undecapeptide cyclosporin A. Helv Chem Acta 1976; 59: 1480-9.

323. Ruegger A, Kuhn M, Lichti H, et al. Cyclosporin A ein immunsuppressiv wirksamer Peptidmetabolit aus Trichoderma Polysporum (Link ex Pers) Rifai. Helv Chem Acta 1976; 59: 1075-92.

324. Beveridge T. Pharmacokinetics and metabolism of Cyclosporine A. In: White DIG, ed. Cyclosporine A. New York: Elsevier Biomedical Press, 1982: 35-44.

325. Keown PA, Stiller CR, Sinclair NR, et al. The clinical relevance of cyclosporine blood levels as measured by radioimmunoassay. Transplant Proc 1983; 15(4 Suppl): 2438-41.

326. Beveridge T, Gratwohl A, Michot F, et al. Cyclosporine A: pharmacokinetics after a single dose in man and serum levels after multiple dosing in recipients of allogeneic bone-marrow grafts. Curr Ther Res 1981; 30: 5-18.

327. Lemaire M, Tillement JP. Role of lipoproteins and erythrocytes in the in vitro binding and distribution of cyclosporin A in the blood. J Pharm Pharmacol 1982; 34: 715-8.

328. Niederberger W, Lemaire M, Maurer G, et al. Distribution and binding of cyclosporine in blood and tissues. In: Kahan BD, ed. Cyclosporine. Orlando: Grune and Stratton, 1984: 203-5.

329. Atkinson K, Boland J, Britton K, et al. Blood and tissue distribution of cyclosporine in humans and mice. In: Kahan BD, ed. Cyclosporine. Orlando: Grune and Stratton, 1984: 214-7.

330. Ried M, Gibbons S, Kwod D, et al. Cyclosporine levels in the human tissues of patients treated for one week to one year. In: Kahan BD, ed. Cyclosporine. Orlando: Grune and Stratton, 1984: 218-21.

331. Ptachcinski RJ, Burckart GJ, Venkataramanan R. Cyclosporine. Drug Intell Clin Pharm 1985; 19: 90-100.

332. Ferguson RM, Sommer BG. Cyclosporine (CsA) in renal transplantation: a single institution experience. Am J Kidney Dis 1985; 5: 296-306.

333. Venkataramanan R, Starzl TE, Yang S, et al. Biliary excretion of cyclosporine in liver transplant patients. Transplant Proc 1985; 17: 286-9.

334. Uchida K, Yamada N, Orihara A, al e. Minimal low dosage of cyclosporine therapy in renal transplantation by careful monitoring of high-performance liquid chromatography whole blood trough levels. Transplant Proc 1988; 20(2 (suppl 2)): 394-401.

335. Burckart GJ, Venkataramanan R, Griffith B, et al. Cyclosporine A blood concentrations in a child after intravenous and oral therapy (abstracted). Drug Intell Clin Pharm 1983; 17: 446.

336. Keown PA, Stiller CR, Laupacis AL, et al. The effects and side effects of cyclosporine: relationship to drug pharmacokinetics. Transplant Proc 1982; 14(4): 659-61.

337. Dieperink H, Moller J. Ketoconazole and cyclosporine (letter). Lancet 1982; 2: 1217.

338. de Smet MD, Rubin BI, Whitcup SM, et al. Combined use of cyclosporine and ketoconazole in treatment of endogenous uveitis. Am J Ophthalmol 1992; 113: 687-90.

339. Kennedy MS, Deeg HJ, Siegel M, et al. Acute renal toxicity with combined use of amphotericin B and cyclosporine after marrow transplantation. Transplantation 1983; 35: 211-5.

340. Thompson JF, Chalmers DHK, Hunnisett AGW, et al. Nephrotoxicity of trimethoprim and cotrimoxazole in renal allograft recipients treated with cyclosporine. Transplantation 1983; 36: 204-6.

341. White DJG, Plumb AM, Pawelec G, Brons G. Cyclosporin A: an immunosuppressant agent preferentially active against proliferating T-cells. Transplantation 1979; 27: 55-8.

342. Janco RL, English D. Cyclosporin and human neutrophil function. Transplantation 1983; 35: 501-3.

343. Granelli-Piperno A, Keane M, Steinman R. Evidence that cyclosporine inhibits cell-mediated immunity primarily at the level of the T lymphocyte rather than the accessory cell. Transplantation 1988; 46 suppl: 53s-60s.

344. Granelli-Piperno A, Keane M. Effects of cyclosporin A on T lymphocytes and accessory cells from human blood. Transplant Proc 1988; 20((No 2 suppl 2)): 136-42.

345. Furue M, Katz SI. Cyclosporin A inhibits accessory cell and antigen-presenting cell functions of epidermal Langerhans cells. Transplant Proc 1988; 20((No 2 suppl 2)): 87-91.

346. Bunjes D, Hardt C, Rollinghoff M, Wagner H. Cyclosporin A mediates immunosuppression of primary cytotoxic T-cell responses by impairing the release of Interleukin 1 and interleukin 2. Eur J Immunol 1981; 11: 657-61.

347. Keown PA, Essery-Rice G, Hellstrom A, et al. Inhibition of human in vivo cytotoxic T-lymphocyte generation following organ transplantation. Transplantation 1985; 40: 45-9.

348. Lafferty KJ, Andrus L, Prowse SJ. Role of lymphokine and antigen in the control of specific T-cell responses. Immunol Rev 1980; 51: 279-314.

349. Larsson EL. Cyclosporin A and dexamethasone suppress T-cell responses by selectively acting at distinct sites of the triggering mechanism. J Immunol 1980; 124: 2828-33.

350. Kaufman Y, Chang AE, Robb RJ, et al. Mechanism of action of cyclosporine A: inhibition of lymphokine secretion studied with antigen-stimulated T-cell hybridomas. J Immunol 1984; 133: 3107-11.

351. Bendtzen K, Dinarello CA. Mechanism of action of Cyclosporine A. Scand J Immunol 1984; 20: 43-51.

352. DosReis GA, Shevach EM. Effect of cyclosporine A on T-cell functions in vitro: the mechanism of suppression of T-cell proliferation depends on the nature of the T-cell stimulus as well as the differentiation state of the responding T-cell. J Immunol 1982; 129: 2360-7.

353. Borel JF, Lafferty KJ. Cyclosporine: speculation about its mechanism of action. Transplant Proc 1983; 15: 1881-5.

354. Koponen M, Greider A, Loor F. The effects of cyclosporines on the cell cycle of T-lymphoid cell lines. Exp Cell Res 1982; 140: 237-50.

355. Keown PA, Stiller CR, Carruthers G, et al. Cyclosporine: mechanisn of action, measurement and clinical use. In: Jacobs P, Macpherson C, ed. Perspectives in immunosuppression. Cape Town: University of Cape Town, 1985: 49-63.

356. Lillehoj HS, Malek TR, Shevach EM. Differential effect of cyclosporin A on the expression of T and B lymphocyte activation antigens. J Immunol 1984; 133: 244-50.

357. Chan CC, Palestine AG, Nussenblatt RB. Cyclosporine-induced alterations of humoral response in experimental autoimmune uveitis. Invest Ophthalmol Vis Sci 1984; 25: 867-70.

358. Wang BS, Heacock EH, Collins KH, et al. Suppressive effects of cyclosporin A on the induction of alloreactivity in vitro and in vivo. J Immunol 1981; 127: 89-93.

359. Leapman SB, Filo RS, Smith EJ, et al. In vitro effects of cyclosporin A on lymphocyte subpopulations. Transplantation 1980; 30: 404-8.

360. Wang BS, Heacock EH, Chang-Xue Z, et al. Evidence for the presence of suppressor T lymphocytes in animals treated with cyclosporin A. J Immunol 1982; 128: 1382-5.

361. Quesniaux VFJ, Schreier MH, Wenger RM, et al. Cyclosporin-cyclophilin interaction. Transplant Proc 1988; 20((No 2 suppl 2)): 58-62.

362. Handschumacher RE, Harding MW, et al. Cyclophilin: a specific cytosolic binding protein for cyclosporin A. Science 1984; 226: 544-7.

363. Calne RY, White DJG, Thiru S, et al. Cyclosporin A in patients receiving renal allografts from cadaver donors. Lancet 1978; 2: 1323-7.

364. Starzl TE. New approaches in the use of cyclosporine: with particular reference to the liver. Transplant Proc 1988; 20(No 3 (suppl 3)): 356-60.

365. Gluckman E, Devergie A, Lokiec F. Use of cyclosporine for prevention of graft-versus-host disease after allogeneic bone marrow transplantation. Transplant Proc 1988; 20(3 (suppl 3)): 461-9.

366. BenEzra D, Cohen E, Rakotomalala M, et al. Treatment of endogenous uveitis with cyclosporin A. Transplant Proc 1988; 20(3 suppl 4)): 122-7.

367. Brown MD, Gupta AK, Ellis CN, et al. Cyclosporine for the treatment of psoriasis. Transplant Proc 1988; 20(3 (suppl 4)): 26-31.

368. Goulon M, Elkharrat D, Lokiec F, Gajdos P. Results of a one-year open trial of cyclosporine in ten patients with severe myasthenia gravis. Transplant Proc 1988; 20(3 (suppl 4)): 211-7.

369. Amor B, Dougados M. Cyclosporine: therapeutic effects in rheumatoid diseases. Transplant Proc 1988; 20(3 (suppl 4)): 218-23.

370. Dupré J, Stiller GR, Gent M, et al. Effects of immunosuppression with cyclosporine in insulin-dependent diabetes mellitus of recent onset: the Canadian open study at 44 months. Transplant Proc 1988; 20(3 (suppl 4)): 184-92.

371. Weetman AP, Ludgate M, Mills PV, et al. Cyclosporin improves Graves' ophthalmopathy. Lancet 1983; 2: 486-9.

372. Utech C, Wulle KG, Panitz N, Kiefer H. Immunosuppressive treatment of Graves' ophthalmopathy with cyclosporin A. Transplant Proc 1988; 20(3 (suppl 4)): 173-7.

373. Masuda K, Nakajima A. A double-masked study of cyclosporine treatment in Behcets disease. In: Schindler R, ed. Ciclosporin in autoimmune disease: 1st international symposium. Basle: Springer-Verlag, 1985: 162-4.

374. Kriut PJ, Van Balen ATM, Stilma JS. Cyclosporin A treatment in two cases of corneal peripheral melting syndrome. Doc Ophthalmol 1985; 59: 33-39.

375. Hill JC, Potter P. Treatment of Mooren's ulcer with cyclosporin A: report of three cases. Br J Ophthalmol 1986; 71: 11-5.

376. Mihatsch MJ, Thiel G, Ryffel. Histopathology of cyclosporine neprotoxicity. Transplant Proc 1988; 20(No 3, Suppl 3): 759-71.

377. Palestine AG, Austin HA, Balow JE, et al. Renal histopathological alterations in patients treated with cyclosporine for uveitis. N Engl J Med 1986; 314: 1293-8.

378. Munoz SJ, Vlasses PH, Boullata JI, et al. Elevated arterial blood pressure in survivors of liver transplantation treated with cyclosporine and corticosteroids. Transplant Proc 1988; 20(No 3, Suppl 3): 623-7.

379. Vine W, Billiar T, Simmons R, Bowers LD. Cyclosporine-induced hepatotoxicity: a microassay by hepatocytes in tissue culture. Transplant Proc 1988; 20(No 3, Suppl 3): 859-62.

380. Kana JS, Hoffmann F, Buchen R, et al. Rabbit corneal allograft survival following topical administration of cyclosporin A. Invest Ophthalmol Vis Sci 1982; 22: 686-90.

381. Hunter PA, Wilhelmus KR, Rice NSC, Jones BR. Cyclosporin A applied topically to the recipient eye inhibits corneal graft rejection. Clin Exp Immunol 1981; 45: 173-177.

382. Foets B, Vanderveeren P, Missotten L. Inhibition of the allograft reaction in corneal transplantation by cyclosporin A: an experimental study with rabbits. Bull Soc Belge Ophthalmol 1983; 206: 21-6.

383. Bell TAG, Hunnisett. Cyclosporin A: tissue levels following topical and systemic administration to rabbits. Br J Ophthalmol 1986; 70: 852-5.

384. Diaz-Llopis M, Menezo JL. Penetration of 2% cyclosporin eyedrops into human aqueous humour. Br J Ophthalmol 1989; 73: 600-603.

385. Mahlberg K, Uusitalo RJ, Gebhardt B, Kaufman HE. Prevention of experimental corneal allograft rejection in rabbits using cyclosporin-collagen shields. Graefe's Archives for clinical and experimental Ophthalmology 1991; 229: 69-74.

386. Hoffmann F, Wiederholt M. Lokale Behandlung des Hornhauttransplantates beim Menschen mit Cyclosporin A. Klin Monatsbl Augenheikd 1985; 187: 92-6.

387. Kieselbach GF, Göttinger W. Doppelblindstudie mit Cyclosporin-A-Augentrofen an gesunden Probanden. Klin Monatsbl Augenheikd 1986; 189: 44-5.

388. Wiebking HJ, Mehlfeld T. Lokale Behandlung von Hornhautulcera und Skleromalazien mit Cyclosporin A. Fortschr Ophthalmol 1986; 83: 345-7.

389. Goichot-Bonnat L, Chemla P, Pouliquen Y. Cyclosporine A collyre dans la prévention du rejet de greffe de cornée à haut risque. II. Résultats cliniques post-opératoires. J Fr Ophtalmol 1987; 10: 213-7.

390. Belin MW, Bouchard CS, Frantz S, Chmielinska J. Topical cyclosporine in high-risk corneal transplants. Ophthalmology 1989; 96: 1144-50.

391. Data on file. Cyclosporine 3H: Ocular absorption and penetration following topical application of a 2% ointment prepared with a solution of cyclosporine in corn oil. In:Sandos Pharmaceuticals, 1988. (cited in proforma of topical cyclosporine collaborative study).

392. McCarey BE, Kaufman HE. Improved corneal storage. Invest Ophthalmol Vis Sci 1974; 13: 165-73.

393. Hill JC. The use of systemic cyclosporin in human corneal transplantation: a preliminary report. Doc Ophthalmol 1986; 62: 337-44.

394. Braude LS, Chandler JW. Corneal allograft rejection. The role of the major histocompatibility complex. Surv Ophthalmol 1983; 27: 290-305.

395. Hill JC, Maske RM, Watson P. Corticosteroids in corneal graft rejection. Oral versus single pulse therapy. Ophthalmology 1991; 98: 329-33.

396. Leibowitz HM. Ocular toxicity of corticosteroids.New York: John Wiley and Sons,, 1979:108-9. (Leopold IH, Burns RP, ed. Symposium on ocular therapy; vol II).

397. Hill JC. The use of cyclosporine in high-risk keratoplasty. Am J Ophthalmol 1989; 107: 506-10.

398. Miller K, Huber C, Niederwieser D, Göttinger W. Successful engraftment of high risk corneal allografts with short term immunosuppresssion with cyclosporine. Transplantation 1988; 45: 651-3.

399. Abbott RL, Fine M, Guillet E. Long term changes in corneal endothelium following penetrating keratoplasty: a specular microscope study. Ophthalmology 1983; 90: 676-85.

400. Wakefield D, McCluskey P, Penny P. Intravenous pulse methylprednisolone therapy in severe inflammatory eye disease. Arch Ophthalmol 1986; 104: 847-51.

401. Meyer PA, Watson PG, Franks W, Dubord P. "Pulsed" immunosuppressive therapy in the treatment of immunologically induced corneal and scleral disease. Eye 1987; 1: 487-95.

402. Orta-Sibu N, Chantler C, Bewick M, Haycock G. Comparison of high-dose intravenous methylprednisolone with low-dose oral prednisolone in acute renal allograft rejection in children. Br Med J (Clin Res) 1982; 285: 258-60.

403. Silverman ED, Myones BL, Miller JJ. Lymphocyte subpopulation alterations induced by intravenous megadose pulse methylprednisolone. J Rheumatol 1984; 11: 287-290.

404. Haynes BF, Fauci AS. The differential effect of in vivo hydrocortisone on the kinetics of subpopulations of human peripheral blood thymus-derived lymphocytes. J Clin Invest 1974; 53: 565-71.

405. Stevenson HC, Fauci AS. Effects of corticosteroids on the function and distribution of human lymphocytes. In: Franklin EC, ed. Clinical immunology update. New York: Elsevier North Holland Inc, 1981: 337-50.

406. Polack FM. Lymphocyte destruction during corneal homograft reaction: a scanning electron microscope study. Arch Ophthalmol 1973; 89: 413-6.

407. Fan PT, Yu DTY, Clements PJ, et al. Effects of corticosteroids on human immune responses: comparison of one and three daily 1 gram intravenous pulses of methylprednisolone. J Lab Clin 1978; 91: 625-34.

408. Epstein RJ, Seedor JA, Dreizen NG, et al. Penetrating keratoplasty for herpes simplex keratitis and keratoconus: allograft rejection and survival. Ophthalmology 1987; 94: 935-44.

409. Freedman MD, Schocket AL, Chapel N, Gerber JG. Anaphylaxis after intravenous methylprednisolone administration. J Am Med Ass 1981; 245: 607-8.

410. Thompson JF, Chalmers DH, Wood RF, et al. Sudden death following high-dose intravenous methylprednisolone. Transplantation 1983; 36: 594-6.

411. Hill JC, Maske R, Watson PG. The use of a single pulse of intravenous methylprednisolone in the treatment of corneal graft rejection. a preliminary report. Eye 1991; 5: 420-4.

412. Smolin G, O'Connor GR. Ocular immunology. (2nd ed.) Boston: Little, Brown and Co, 1986:273-306.

413. Kupiec-Weglinski JW, Filho MA, Strom TB, Tilney NL. Sparing of suppressor cells: a critical action of cyclosporine. Transplantation 1984; 38: 97-101.

414. Kerman RH, Fleckner JM, Van Buren CT, et al. Immunoregulatory mechanisms in cyclosporine-treated renal allograft recipients. Transplantation 1987; 43: 205-10.

415. Snyder DS, Wright CL, Ting C. Inhibition of human monocyte antigen presentation, but not HLA-DR expression by cyclosporine. Transplantation 1987; 44: 407-11.

416. Manca F, Kunkl A, Celada F. Inhibition of the accessory function of murine macrophages in vitro by cyclosporine. Transplantation 1985; 39: 644-9.

417. Palay DA, Cluff CW, Wentworth PA, Ziegler HK. Cyclosporine inhibits macrophage-mediated antigen presentation. J Immunol 1986; 136: 4348-53.

418. Whisler RL, Lindsey JA, Proctor KVM, et al. Characteristics of cyclosporine induction of increased prostaglandin levels from human peripheral blood monocytes. Transplantation 1984; 38: 377-81.