IgG Subclasses, Specific Antibodies and Immunoglobulin Allotypes in Children with Invasive *Haemophilus Influenzae* type B and *Staphylococcus Aureus* Infections

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BUFFERS AND SOLUTIONS

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ABBREVIATIONS

α	Alpha
ABTS	2-2, -azino-d (3-ethylbenthiazline-6-sulphate
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
5-AS	5-amino salicylic acid
ß	Beta
B	Black
BSA	Bovine serum albumin
°C	Degree Celcius
CF	Coloured female
CI	Confidence interval
CLB	Central Laboratory of the Netherlands Red Cross Blood Transfusion Service
CM	Coloured male
CSF	Cerebrospinal fluid
CV	Coefficient of variation
DEA	Diethylamine
EIA	Electroimmunoassay
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
FDA	Food and Drug Administration
FPLC	Fast performance liquid chromotography
g	Gram(s)
GS	Goat serum
γ	Gamma
H+L chain	Heavy and light chain
H_2SO_4	Sulphuric acid
HAI	Haemagglutination inhibition
Hbo-HA	Haemophilus influenzae type b oligosaccharide-human serum albumin
Hib	*Haemophilus influenzae* type b
HIV	Human immunodeficiency virus
hr	Hour
HRPO	Horseradish peroxidase
HSA	Human serum albumin
Ig	Immunoglobulin
IL	Interleukin
IU	International unit
IUIS	The International Union of Immunological Societies
k	Kappa
KD	Kilo dalton
λ	Lambda
L(s)	Litre(s)
Lf/ml	Flocculation units per millilitre
Log10	Logarithm to base 10

LPS	Lipopolysaccharide
M	Molar
MBP	Mannose binding protein
mM	Millimolar
ml	Millilitre
MS	Mouse serum
Moab	Monoclonal antibody
n	Number in study, group
N	Normal (concentration)
NBTS	Natal Blood Transfusion Service
ng(s)	Nanogram(s)
nm	Nanometre
No(s)	Number(s)
OD	Optical density
OM	Osteomyelitis
OMP	Outer membrane protein
o/n	Overnight
OPD	Ortho-phenylenediamine dichloride
OR	Odds ratio
p	Probability
PACIA	Particle-counting immunoassay
PAGE	Polyacrylamide-gel electrophoresis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween 20
PCFIA	Particle concentration fluorescence immunoassay
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PLL	Poly-L-lysine
PRP	Polyribosylribitol phosphate syynonymous with capsular polysaccharide of Hib
PVC	Polyvinyl chloride
QA	Quality assurance
r	Correlation coefficient
RIA	Radioimmunoassay
RID	Radial immunodiffusion
RIS	Radioimmunosorbent assay
Rh	Rhesus
SA	Septic arthritis
SAIMR	South African Institute of Medical Research
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SS	Sheep serum
TA	Teichoic acid
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween 20
TMB	3, 3', 5, 5' tetra-methylbenzidine hydrochloride
Tris	Tris (hydroxymethyl) - aminomethane

TT	Tetanus toxoid
U	Unit(s)
μg	Microgram
WHO	World Health Organization
+ve	Positive
-ve	Negative
%	Percentage

ABSTRACT

OBJECTIVE

The principal objective of this study was to measure various aspects of immunity in children with invasive infections due to *Haemophilus influenzae* type b and *Staphylococcus aureus*. These serious infections are a significant cause of childhood morbidity and mortality in all populations and affect healthy as well as compromised children. Evidence suggests that imbalances or deficiencies in certain aspects of immunity such as IgG subclasses, the capacity to make specific subclass antibodies, antibody affinities, complement isotypes, immunoglobulin allotypes or mannose binding protein may place certain children at risk for developing invasive disease.

Investigation of these factors in a group of children with infection necessitated that normal ranges be established for children of comparable ages from the same population. A secondary objective of this study has therefore been to establish normal percentiles for the IgG subclasses in age, race and sex matched healthy controls.

METHODS

Patients admitted to the Red Cross War Memorial Children's Hospital with septic meningitis due to *Haemophilus influenzae* type b and osteomyelitis/septic arthritis due to *Haemophilus influenzae* type b or *Staphylococcus aureus* formed the study population.

Section A of this thesis describes the methods for establishing, validating and standardizing ELISAs for measuring the IgG subclasses (IgG1, IgG2, IgG3 and IgG4) and subclass antibodies specific to *Haemophilus influenzae* polyribosylribitol phosphate, *Staphylococcus aureus* teichoic acid and tetanus toxoid. The relative affinity of antibodies in these ELISAs was determined by the incorporation of diethylamine (DEA).

In order to determine the immunoglobulin allotypes ELISAs were developed to measure the G1m(f), G2m(n) and Km(3) allotypes. The frequency of these allotypic markers in the different ethnic groups was established. The relationship between immunoglobulin allotypes and IgG subclass values were investigated in both patient and control groups.

RESULTS

ELISA assays to measure IgG subclasses; IgG, IgG1 and IgG4 tetanus toxoid antibodies; IgG, IgG1 and IgG2 *H.influenzae* type b polyribosylribitol phosphate capsular polysaccharide antibodies; IgG, IgG1 and IgG2 *S.aureus* teichoic acid antibodies and G1m(f), G2m(n) and Km(3) allotypes were successfully established. Where possible the assays were standardized with reference sera and specimens were exchanged with international laboratories.

Age, race and sex related percentile charts and tables of normal ranges for IgG and IgG subclasses of Black and Coloured children were established. The IgG and IgG1 values were higher than those previously reported for children in developed countries. Black children with *H.influenzae* meningitis had significantly lower IgG1, IgG2 and IgG3 levels compared to the controls and although similar trends were seen for IgG and IgG4 levels they were not statistically significant. Coloured children with *H.influenzae* meningitis and Coloured and Black children with *H.influenzae* osteomyelitis/septic arthritis also showed a similar tendency of lower IgG and IgG subclass levels than the controls but these trends were also not significantly different.

All patients responded to tetanus toxoid antigen suggesting normal immunocompetence to protein antigens.

H.influenzae type b capsular polysaccharide antibodies were low in children with *H.influenzae* type b meningitis and osteomyelitis/septic arthritis and did not increase during the illness.

IgG and IgG1 teichoic acid antibodies were raised in patients with *S.aureus* osteomyelitis/septic arthritis although no further rise in these antibodies was seen when measured several weeks after the illness.

The antibody affinity ELISAs showed that IgG1 tetanus toxoid antibody had a greater affinity than IgG4 tetanus toxoid antibody, the IgG1 and IgG2 *H.influenzae* capsular polysaccharide antibodies were of similar affinity and the IgG1 teichoic acid antibody was of higher affinity than the IgG2 antibody.

The G1m(f) and G2m(n) positive allotypes were uncommon in Black but common in the Coloured populations whereas Km(3) was common in both groups. There was a significantly decreased frequency of the G2m(n) positive allotype in Coloured patients with *H.influenzae* type b meningitis and *H.influenzae* type b osteomyelitis/septic arthritis which was not found in patients with *S.aureus* osteomyelitis/septic arthritis. In both Coloured and Black children with *H.influenzae* meningitis there was a significantly decreased frequency of the Km(3) allotype.

No differences in C4 isotypes and mannose binding protein levels were evident in the patient and control groups.

CONCLUSION

This study has developed simple, specific and reproducible ELISAs to measure IgG subclasses and subclass antibodies specific to tetanus toxoid, *H.influenzae* polyribosylribitol phosphate and *S.aureus* teichoic acid. Age, sex and race related normal ranges for IgG subclasses in the local Black and Coloured populations have been established. Black children with *H.influenzae* type b meningitis had significantly lower IgG1, IgG2 and IgG3 levels compared to the controls. There was a clear association between a decrease of the

G2m(n) allotype and the Km(3) allotype and susceptibility to invasive infections caused by *H.influenzae*.

CHAPTER 1

ENZYME-LINKED IMMUNOSORBENT ASSAY

(A review of methodology)

Enzyme-linked immunosorbent assay (ELISA), first introduced in 1971, has gained wide acceptance and application for the detectioin of antigens and antibodies. This is largely due to the sensitivity, specificity and reproducibility of these assays (Engvall and Perlmann 1971, Van Weemen and Schuurs 1971). They also require relatively limited equipment, can accommodate large numbers of samples and are reasonably economical.

The ELISA technique has been comprehensively and extensively reviewed in the literature (Kurstak 1985; Voller and Bidwell 1986; Kemeny and Chantler 1988; Kemeny 1992; Porstmann and Kiessig 1992). This chapter reviews the important technical aspects that must be taken into consideration in order to establish the ELISA assays used in this thesis and is divided into the following sections:

1.1 ELISA SYSTEMS

1.2 CHOICE OF SOLID PHASE

1.3 SOLID PHASE IMMOBILIZATION

1.4 INTERFERENCE

1.5 WASHING

1.6 SAMPLES, BUFFERS AND INCUBATION TIMES

1.7 SAMPLE DILUTION AND DISPENSING

1.8 ANTIBODY REAGENTS

1.9 ENZYME DETECTOR SYSTEMS

1.10 SUBSTRATES

1.11 PLATE FORMAT

1.12 DATA ACQUISITION

1.13 DATA INTERPRETATION AND QUALITY CONTROL

1.1 ELISA SYSTEMS

There are many different ways of configuring ELISAs and the choice depends on the nature of the sample, availability of reagents and the precision and sensitivity required. Two fundamentally different solid phase ELISAs can be distinguished, the non-competitive and competitive assays.

1.1.1 Non-Competitive ELISA

1.1.1.1 *Antigen coated plate*

These are probably the simplest type of assays, which are also known as indirect ELISAs and are commonly used for the detection of antibodies to an antigen which is immobilized on the solid phase. The antigen will capture specific antibodies from the test serum before the addition of a class or subclass specific monoclonal antibody which can then be detected by addition of an enzyme labelled antibody specific for the bound monoclonal antibody. The signal generated from the enzyme label is proportional to the amount of analyte bound to the solid phase. This assay design is used in the development of the IgG subclass specific ELISAs in this study.

1.1.1.2 *Antibody coated plate*

In these immunoassays the immunoglobulin class of interest is isolated from other immunoglobulin classes (antibody capture). This design was used in the IgG subclass ELISAs where the microtitre plate is coated with monoclonal antibody specific for the respective IgG subclass. The bound anti-IgG subclass monoclonal antibody is used to absorb its respective subclass in the sample. A labelled antibody is used to detect the quantity of captured antibody.

1.1.2 Competitive ELISA

Competitive assays use antigen coated plates where a fixed level of enzyme labelled antibody competes with variable amounts of unlabelled antibody in the test sample. Competitive assays were not used in the assays established for this thesis.

1.2 CHOICE OF SOLID PHASE

Although plastic is the most generally used solid phase in enzyme immunoassays it has a lower capacity for binding proteins (10%) than nitrocellulose (100%). Plastic microtitre plates have low background binding and are therefore more suitable for the measurement of IgG antibodies (Grant et al 1981). Different plastics have distinct uptake characteristics, polystyrene causing lower background signals than polyvinyl chloride (PVC) as it takes up less protein. It can therefore be used where a low coating level is acceptable and where non-specific uptake from the sample (e.g. serum) is to be minimized (Voller and Bidwell 1986). Attachment of antibody to the polymer surface appears to occur by a process of adsorption (Ansari et al 1985) and is dependent upon the nature of both protein and polymer. The nature of interaction of the antigens or antibodies with the plastic is not known but both hydrophobic binding and the charge of the antibody or antigen play an important role. Binding of antibody to polystyrene increases with antibody concentration, and the time of incubation before reaching a maximum value. The stable binding of antigen or antibody to the solid phase is of central importance since if this is not successful the rest of the assay will fail.

The following requirements of solid phases are common to all assays:

i. a high binding capacity for antigen or antibody which does not interfere with immunological activity after binding

ii. binding stability on storage

iii. bound material should not become detached in the course of the assay.

The most common layout for solid phase ELISAs are microtitre plates with 12 x 8 wells (round or flat bottomed). With these plates an 'edge' effect (differences in binding of the sample to the outer wells) has been described. Much of this edge effect may be due to the poor conduction of heat by polystyrene. This can be reduced by carrying out the incubation steps in an incubator or increasing the incubation times. Non-usage of the outer wells would result in a loss of about 40% of the plate. Although a theoretical possibility, the 'edge' effect has not been a problem in the ELISA's described in this thesis.

1.3 SOLID PHASE IMMOBILIZATION (COATING OF ANTIGEN OR ANTIBODY)

1.3.1 Direct

The easiest and most popular method to coat the microtitre plate is directly by passive adsorption to the surface of the plastic (Cantarero et al 1980). Observations over the past 30 years have shown that adsorption of proteins on hydrophobic synthetic surfaces result in protein alteration. There are no reports contradicting this. Butler et al (1992) have shown that polyclonal capture antibodies retain only 5-10% of their binding sites whereas the most stable monoclonal antibodies have $\leq$ 3% of functional binding sites after adsorption.

1.3.2 Indirect

Bacterial polysaccharides carry a net negative charge due to many acid groups and absorb poorly to plastic. This problem has been overcome by

covalently binding the polysaccharide to poly-L-lysine using cyanogen chloride as the coupling agent (Gray 1979). The poly-L-lysine protein binds more efficiently to the plastic thus immobilizing the coupled polysaccharide.

1.3.3 Concentration

Coating conditions must be optimized for each ELISA system. The optimal coating concentration depends on the antigen or antibody used but it is usually between 1 and 10 μg/ml protein (McLaren et al 1981, Kemeny 1992). Ideally all the binding sites on the solid phase should be occupied by the antigen molecules during coating. Overcoating with antigen is a disadvantage as a monolayer of antigen is not maintained and antigen molecules may later become detached. This will affect the assay especially if the antigen has already bound the analyte as these complexes are unlikely to re-attach to the solid phase.

1.3.4 Buffer and pH

Buffers generally have little or no effect on the immobilization of proteins (Kurstak 1985). Carbonate (0.05 M, pH 9.6), Tris-saline (0.01 M), Tris-HCL, pH 8.5, with 0.1 M NaCl) and phosphate buffered saline (PBS) (0.01 M phosphate buffer pH 7.2, with 0.1 M/NaCl) are widely used. Aqueous diluents of neutral or alkaline pH (especially carbonate or phosphate buffers) have been successfully used for most proteins.

1.3.5 Time

The coating incubation period is usually 4°C overnight but can be shortened, if incubated at room temperature or 37°C.

1.4 INTERFERENCE

1.4.1. Blocking of unreacted sites

A common problem in ELISAs is non-specific, or specific undesirable binding of different test serum or other proteins to the solid phase. To prevent non-specific adsorption it is common practice to block unreacted sites after the primary coating step with an irrelevant protein e.g. 1-5% bovine serum albumin (BSA), casein or gelatin diluted in PBS, or a non-ionic detergent (Tween 20, Triton x100). The blocking protein is usually added in excess (0.1-5.0 gm/100 ml) of the well's protein binding capacity (typically 1 μg/well). The choice of blockers is not universally suitable for all assays. As human serum albumin is invariably contaminated with a small percentage ($< 1\%$) of immunoglobulins it is unsuitable as a blocking agent for IgG and IgG subclass assays.

Blocking agents can reduce sensitivity by interfering with antigenic determinants of the coated proteins but provided this is not excessive is outweighed by the benefits of reduced background binding. If BSA or other common ingested proteins are used to block the plates, they may capture naturally occurring antibodies for these proteins that are present in the test sample. If the blocking protein is included in the assay diluent this effect can largely be prevented.

Despite the widespread usage of blocking agents there are reports of this being an unnecessary or even harmful step (Mohammad and Esen 1989; Gosling 1990).

1.4.2 Heterophile antibodies

Heterophilic antibodies are endogenous antibodies found in patients' serum that can bind to immunoglobulins from other species, including the species used to raise the antibody reagents for the ELISAs.

The use of $F(ab')_2$ fragments of the antibody reagent has been advocated as a means of decreasing heterophilic antibody interference (Schroff et al 1985; Clark and Price 1987) but may not always be effective (Boscato and Stuart 1988). Interference with heterophilic antibodies can be decreased by adding excess non-immune immunoglobulin, generally obtained from the same species as the reagent antibody.

1.4.3 Other

Other interfering substances include complement, rheumatoid factors, lysozyme and enzymes that are potential contaminants of antigen preparations (Gosling 1990).

ELISA assays always involve some non-specific adsorption which is one of the major factors limiting the accurate measurement of very small amounts of antigen or antibody.

1.4.4 Serum background

The OD measurements obtained in assays included values for both the specific antibody/antigen reaction plus non-specific background values. These non-specific values can be determined by including blank wells with serum dilutions but minus antigen, and wells with antigen but minus serum.

Many serum specimens display 'background' i.e. significant OD readings in the 'minus antigen' control well.

'Background' values may be due to reactivity of antibodies, other than the test antibody with antigenic determinants in the reagents or to non-reactive serum components that are detected by second antibodies or conjugate antibodies. The background values from wells with no serum but with antigen may be due to impurities in the antigen solution which react with second or conjugate antibody. It can also be due to adherence of unreacted conjugate to the well surface because of inadequate blocking of all the well surfaces. This is usually overcome by using at least double the volume of blocking solution to fill the wells. It is common practice to subtract the OD background readings of the minus antigen wells from each serum dilution. In all the assays described in this thesis the total of the 'minus antigen' and the 'minus serum' background values were subtracted from each serum dilution.

1.5 WASHING

Efficient washing is important if reliable results and good replicate values are required. The aim of washing is to minimize non-specific binding by removing reagents which are either unbound or weakly absorbed to the solid phase. Wash steps are necessary after each incubation step to remove weakly immobilized immunoreactants, to decrease non-specific reactivity and to prevent carry over of unbound immunoreactants.

Phosphate buffered saline (0.01 M PBS, pH 7.2) with 0.05% of polysorbate -20 e.g. Tween 20 (PBST) is most commonly used as a washing solution. Tap water may be substituted for the PBS, provided it has a neutral pH and a low chlorine content. In this thesis either PBST or Tris buffered saline (TBS) with 0.01% Tween 20 (TBST) was used as the washing fluid depending on the buffers used in the various assays developed. The

concentration of Tween 20 is not critical and a range from 0.005% to 0.5% was found to be equally effective in this study.

It has been suggested that the solid phase should be washed by rinsing wells at least three times for 3-5 minutes each (McLaren et al 1981). It is important that there is no carry over from one washing step to the next and that the carrier surface is thoroughly washed. Plates can either be washed by hand or using automated machines.

In the assays described in this study hand washing was preferred as it is gentler and less time consuming. For each wash the plate's contents are emptied by inverting them over a sink with a flicking action of the wrist. The plate is then held at a 45° angle and the washing buffer poured from a beaker across the plate in a right to left and left to right fashion working down the plate. This ensures that all wells are flooded. After the final rinse the contents of the plate are shaken out and the inverted plate firmly blotted several times onto a pad of paper towelling.

After coating and blocking, plates are washed once with PBS. After the addition of either serum, monoclonal antibody or conjugate, incubation plates are washed five times with PBST.

1.6 SAMPLES, BUFFERS AND INCUBATION TIMES

Serum and plasma samples can both be used in ELISA. In these studies blood was collected under sterile conditions. Serum was stored at -70°C in small aliquots to avoid repeated freezing and thawing of samples. It is important that samples are measured in the concentration range in which the standard curve has steep dose-response curve characteristics. To decrease non-specific binding of irrelevant proteins in the test sample a detergent

(e.g. Tween 20) can be added to the diluent in addition to blocking agents such as BSA or gelatin. It is well known that detergents prevent attachment of proteins to plastic surfaces (Gripenberg and Kuruki, 1986). Buffers used for dilution generally contain 0.05% Tween and/or inert proteins (BSA, gelatin) to prevent non-specific binding to the solid phase. The most common buffers are PBST (PBS, pH 7.4, with 0.05% Tween-20) or Tris-T (0.02 M Tris-HCL, pH 7.4, with 0,15 M NaCl, 0.005 M KCL and 0.05% Tween-20). Tris-T is particularly useful when the enzyme system is alkaline phosphatase. Optimal incubation conditions for the binding of test samples should be established for each particular system. For example incubation at 37°C may be excellent for one system but will give poor results with another owing to the nature of the antibody/antigen interaction. Generally incubation for 1-2 hours is sufficient but shorter incubations in some assays are possible.

The assumption that non-specific binding is constant over the complete range of dilutions is not valid (Kurstak 1985). Negative control sera should therefore be tested at the same dilutions as the test and positive sera.

Pooled serum makes a practical reference standard as when stored at $\leq$ -20°C it retains its antibody activity because of the stable natural environment of proteins in serum and the minimum proteolytic activity for antibodies in serum (Butler 1988). Various pooled serum samples were used for the standard curves and reference standards in all the assays described in this thesis.

Despite reports of background binding increasing with serum storage time at -20°C (Shillitoe 1982) this was not found in the assays described in this thesis using sera stored at -70°C.

1.7 SAMPLE DILUTION AND DISPENSING

The step with the greatest potential for creating assay imprecision and inaccuracy is the physical preparation of sample dilutions. Inadequate mixing of highly diluted samples is a major source of error in ELISAs. It is recommended that the same pipetting device should be used for the preparation of dilutions of the reference standard and the test specimens. In these assays dilutions were made using Gilson pipettes to dispense volumes of less than 1 ml. Graduated glass pipettes where used to dispense larger volumes manually. All dilutions were prepared in 10 x 75 mm disposable glass test tubes or 20 ml glass scintillation vials. Samples were vortexed before transfer to the appropriate wells of microtitre plates using Gilson pipettes. Providing that the weakest dilution was transferred first, the same pipette tip was used sequentially to transfer the stronger dilutions of the same sample. An initial dilution of standard curve serum was made, from which the other 8 standard curve dilutions were independently made.

Multi-channel-pipettes (Eppendorf) were used to add blocking solutions, antibody dilutions, substrate and reaction stopping solutions.

Solutions must be pipetted into each well in a timed sequence. This is particularly important for the conjugate, substrate and reaction stopping solutions where incubation times must be accurate and the end results are read immediately after stopping the reaction.

1.8 ANTIBODY REAGENTS

Both monoclonal and polyclonal antibodies can be used in ELISAs. Monoclonal antibodies are highly specific as capture reagents. However as they represent a single antibody lineage within a polyclonal immune

response it is sometimes necessary to use a mixture of antibodies with different epitope specificities. Although antibodies lose activity on adsorption to a solid phase, most antibodies retain their ability to bind antigen. The binding ability of some monoclonal antibodies may be improved by chemically coupling to BSA (Papadea et al 1985) or by treatment of the antibody with either low pH, high temperature or urea (Conradie et al 1983).

A disadvantage of coating wells with whole serum or ascitic fluid reagents rather than an immunoglobulin fraction is the increased potential of non-specific reactions due to the many other proteins present in serum or ascitic fluid. Even with affinity purified reagents unwanted cross-reactions must be controlled. In several assays developed in this study affinity purified conjugate antibodies gave poorer signals and higher non-specific binding than non-affinity purified antibodies.

The storage and handling of antibody reagents is important. Whole serum and ascitic fluid should be stored in aliquots at -20°C to minimise repeated freezing and thawing.

The antibody reagents used in this study were all purchased from commercial suppliers.

1.8.1 Antibody reagents for IgG subclass ELISAs

Polyclonal anti-IgG subclass antibodies have restricted application as reagents for IgG subclass assays. Polyclonal antisera that are monospecific for IgG subclasses are difficult to prepare and are only available from limited sources. Repeated immunization and bleeding, may over time affect

the affinity and specificity of the polyclonal antisera making it difficult to produce large quantities of standardized reproducible antibody.

Murine monoclonal antibodies specific for IgG subclasses were first reported in the mid 1980's. The International Union of Immunological Societies (IUIS) in collaboration with the World Health Organisation (WHO) evaluated monoclonal antibodies with putative specificity for the human IgG subclasses that had been produced by several laboratories (Jefferis et al 1985). Each clone evaluated was given an HP designation. The monoclonal subclass specific antibodies used in this study were selected from the panel of antibodies reviewed in the IUIS/WHO report because they displayed good specificity and were commercially available. These monoclonal antibodies all have a clone number and an IUIS/WHO study number and are referred to using the HP designation of the IUIS/WHO. Details of the anti-human IgG monoclonal antibodies used in this study are listed in Appendix A. Quality control information on the subclass specific monoclonal antibodies is not supplied by commercial firms necessitating assessment of new vials of antibody in the assay system prior to depletion of old stocks.

1.9 ENZYME DETECTOR SYSTEMS

In immunoassays, a detector is any molecule that has specificity for the sample which it is used to detect (Kemeny 1992). The final step of the ELISA is an enzyme catalysed reaction. These enzymes are conjugated to a detection antibody that binds either to the test antigen or antibody or is coupled to streptavidin which then binds to biotinylated antibody. The kinetics of the enzyme reaction can be affected by a number of factors e.g. concentration of enzyme, temperature, the solvent of the enzyme substrate and the pH of the solution. The working dilution of the conjugate is determined to give both high detection values and low background readings.

To obtain reproducible results in ELISAs, it is important to control as many factors as possible, particularly if the end point is based on time as commonly occurs in many ELISAs. This is controlled by critically stopping the enzyme substrate reaction. Many different enzymes have been used in ELISAs, of which horseradish peroxidase (HRPO), alkaline phosphatase and ß-D-galactosidase are the most commonly used.

Horseradish peroxidase is used in about 50% of described ELISA methods and alkaline phosphatase in about 25% (Gosling 1990; Porstmann and Keissig 1992). Horseradish peroxidase has a high turnover rate, is pure, relatively cheap and readily available. In the ELISAs developed in this study horseradish peroxidase was used in preference to alkaline phosphatase because of cost and availability.

The quality of the detector antibody used for enzyme conjugation and the efficacy of conjugation is directly related to assay performance. Cross-reactivity with components in the assay must be removed by pre-adsorption of the unlabelled antibody or neutralizated by the addition of the appropriate material in the conjugate diluent. Anti-species immunoglobulin cross-reactivity may be effectively decreased by addition of 1-2% of serum from the reacting species.

There are a number of approaches designed to reduce the loss of antibody and enzyme activity on conjugation, the most widely used being the avidin-biotin complex. Avidin has an extremely high affinity for biotin with an association constant of $10^{-15}M$. Biotin is linked to antibody and used as a primary label with enzyme conjugated streptavidin or avidin as the final assay reagent. As the affinity of avidin for biotin is high, the enzyme

labelled conjugate binds efficiently to the biotinylated antibody. Streptavidin is often preferred because it has a lower isoelectric point (5.5-6.5) and is not glycosylated, which helps to decrease non-specific binding (Hart and Taaffe 1987). The streptavidin-biotin interaction has been used to amplify the signal in some of the IgG subclass specific ELISAs where a biotin-labelled antibody reacts with an immobilized antigen and is then revealed by the addition of streptavidin conjugated horseradish peroxidase.

1.10 SUBSTRATE

Although horseradish peroxidase is one of the most frequently used markers in ELISAs its reaction kinetics are highly complex and have not been fully elucidated (Porstmann et al 1981). Horseradish peroxidase catalyses the reduction of H_2O_2 with the concurrent oxidation of a chromagen producing an optically measurable colour change in the ELISA. A large number of chromogens are commercially available, several of which may be used with the horseradish peroxidase conjugates. The most commonly used are ortho-phenylenediamine (OPD), 2,2-azino-di(3-ethylbenzthiazline-6-sulphate) (ABTS), 5-aminosalicylic acid (5-AS) and 3,3', 5,5' tetra-methylbenzidine hydrochloride (TMB). OPD is light sensitive. It produces a strong yellow colour and when the enzyme reaction is stopped by sulphuric acid the colour conveniently changes to orange-brown. The recommended peroxide concentrations for these chromogenic solutions varies from 0.003% for 5-AS to 0.02% for OPD. It is advisable to store hydrogen peroxide in aliquots at 4°C. Peroxidase is sensitive to micro-organisms, sodium azide and methanol. This must be borne in mind as many of the antibodies have azide added as a bacteriocidal agent.

It has been clearly shown that the sensitivity of the ELISA can be strongly affected by the choice of chromogen (Porstmann et al 1981). OPD has been reported to be the substrate that gives the most sensitive and reliable measurements (Porstmann et al 1981; Avrameas 1983). It was found in this thesis that OPD was more sensitive than ABTS giving a signal 8 times and thus it was used for all the ELISAs. With this substrate the optical density was measured with an ELISA photometer at a wavelength of 492 nm using a reference wavelength of 620 nm.

1.11 PLATE FORMAT

The plate format used for all assays in this study except the Km(3) ELISA is shown in Figure 1.1. Every assay plate included 9 dilutions of the standard for the construction of calibration curves, background reaction wells (minus serum, minus antigen and minus monoclonal antibody wells where applicable) and dilutions of controls for monitoring reproducibility.

	1	2	3	4	5	6	7	8	9	10	11	12
A			C	A L	I B	R A	T I	O N		B	C	C
B				S T	A N	D A	R D	S		B	C	C
C										B	C	C
D		M I	N U	S	A N	T I	G E	N W	E L	L S		
E												
F		U	N K	N O	W N	S	A M	P L	E S			
G												
H		M	I N	U S	A N	T I	G E	N	W E	L L	S	

B = Minus serum and minus monoclonal antibody blanks C = Controls

Figure: 1.1: Elisa Template

Rows A, B, C, E, F and G were coated with antigen whereas rows D and H were not (minus antigen wells). Nine dilutions of the standard serum were added to columns 1-9 rows A-D. The two dilutions of the positive controls were in columns 11 and 12 - rows A-D. Unknown serum samples were placed in columns 1-12 rows E-H with blanks in column 10 rows A-D. In the IgG ELISA these blanks were minus serum blanks. The blanks of column 10 in the subclass specific assays were as follows:

Row A: Minus serum, plus monoclonal antibody

Row B: Plus serum, minus monoclonal antibody

Row C & D: Minus serum, minus monoclonal antibody

This format allows samples to be run in triplicate on a plate and for the appropriate background corrections to be made. Antigen blanks and the highest value of the minus serum or minus monoclonal antibody blanks were added together and subtracted from the mean of the triplicate of each of the standard and sample dilutions. Initially these blanks were carried out in triplicate but if they were consistently low ($\leq$ 4% of signal), they were only included as a single blank in routine runs to allow for more unknown samples to be included.

1.12 DATA ACQUISITION

In this study two ELISA readers were used: i. SLT 210 photometer (SLT - Lab Instruments, Austria); ii. Anthos Reader 2001 (Labtec). Each of these is linked to a personal computer. The data can be stored and analysed at a suitable time.

There is no standardized method for the analysis of ELISA data. Various relationships between OD and log (serum dilution) have been assessed which

include: OD as a linear function of log (serum dilution), log (OD) as a linear function of log (serum dilution); OD as a quadratic or quartic polynomial function of log (serum dilution); and 4-parameter log - logistic function. A variety of estimation procedures have also been applied including visually drawn straight lines, weighted and simple least squares and weighted and unweighted non-linear least squares (Karpinski et al 1987). In evaluating analysis of ELISA data Karpinski et al (1987) found that OD as a linear function of log serum dilution is inappropriate. It involves subjective judgement of the range and adequacy of the straight line relationship. The weighted non-linear least squares analysis of 4 parameter logit was also shown to be inappropriate because it requires the specification of an arbitrary variance function.

The most appropriate procedure for determining the concentration of antigens or antibodies by the ELISA method is to represent OD as a 4-parameter logistic function with estimation carried out on the log scale. This approach was shown to provide excellent fits of data over a wide range of serum dilutions and is recommended in a variety of systems for the determination of the concentration of antigens or antibodies (Karpinski 1987).

The standard curve is assumed to be sigmoidal and is described by the following equation: $OD = C + \frac{D - C}{1+e^{-2(\alpha + \beta x)}}$

where α, β, C and D are the coefficients which determine the curve. C and D are the lower and upper asymptotes respectively while α and β are indicators of the location and steepness of the curve. The error function is the sum of the squares of the differences between each measured value and the corresponding calculated value, using the current coefficients. The initial values for the coefficients are based on an assumption of linearity.

The programme then uses formulas for first and second partial derivatives of the above function with respect to the four coefficients to repeatedly improve the initial guess. At each step a vector in four dimensions is generated in the direction of the steepest descent of the error function, and the second derivative is used to approximate the step required to obtain the extrapolated position of the minimum. Using this method a software programme was written by P W Becker (Custom Software Solutions, South Africa). This programme was used to analyse all the routine assays in this study. The user may specify a wide range of formats to be used for interpreting the data. Each format specifies:

i. The area to display on the graph and the distance between the ticks of the x and y axes

ii. The neat standard concentration

iii. The units (grams or units) and magnitude (e.g. 6 for micrograms) for the standard and the magnitude of the units to be used on the printouts

iv. The standard dilutions

v. The sample dilution used

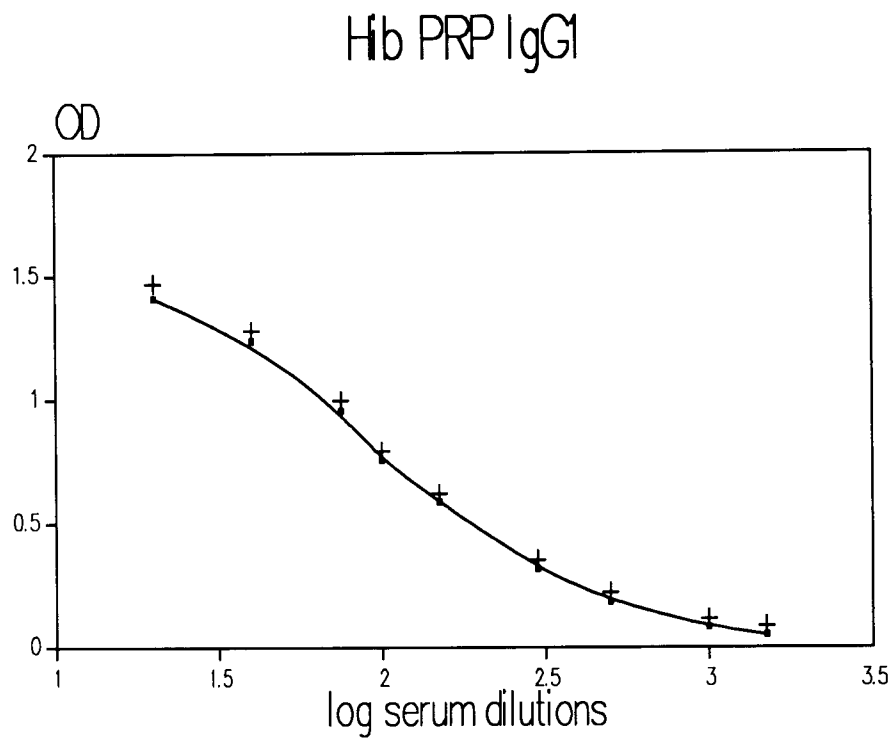
The system is programmed to calculate a mean of the triplicate dilutions and to subtract the appropriate background controls. The programme fits a sigmoidal curve to the 9 standard OD readings. Once the best fit is achieved, the resultant sum of the errors squared is reduced to < 0.02. The values of the samples are read off the curve and the final concentration calculated correcting for initial dilutions of the specimen. Figure 1.2(a) and Figure 1.2(b) below is an example of a computer printout from this programme.

A	B	C	Blank	Mean	SD	CV	X value	Calc x	gx10^-3/ml	% Err
1.489	1.459	1.460	0.039	1.410	0.014	0.9	1.301	1.329	0.944	-1.087
1.310	1.272	1.263	0.025	1.236	0.020	1.6	1.602	1.575	0.480	1.954
1.028	0.992	0.972	0.019	0.958	0.023	2.3	1.875	1.851	0.254	2.886
0.811	0.782	0.785	0.015	0.757	0.013	1.6	2.000	2.023	0.170	-3.936
0.631	0.636	0.608	0.016	0.588	0.012	2.0	2.176	2.176	0.120	0.023
0.348	0.361	0.354	0.016	0.319	0.005	1.5	2.477	2.473	0.061	1.000
0.215	0.232	0.224	0.019	0.184	0.007	3.1	2.699	2.695	0.036	0.988
0.122	0.109	0.118	0.014	0.082	0.005	4.7	3.000	2.990	0.018	2.792
0.091	0.085	0.096	0.017	0.050	0.003	3.0	3.176	3.155	0.013	6.580

The standard curve data: (from left to right)

i. **A, B, C are the triplicates of each dilution**
ii. **'Blank' denotes uncoated well of each dilution**
iii. **'Mean' indicates the mean OD of the triplicates with each individual uncoated blank subtracted**
iv. **SD and CV are the standard deviation and coefficients of variation of the triplicates**
v. **'x value' is the log of the inverse of the sample dilution**
vi. **'Calc x' is the calculated log value of the dilution**
vii. **g x 10^{-6}/ml is the calculated value of each sample**
viii. **'% Err' is the % error between the known and calculated value of each sample.**

Figure 1.2(a): Computer printout of the standard curve data for the measurement of IgG1 antibodies to Haemophilus influenzae b PRP using the plate format described in Chapter 1.11



α = 2.771 ß = -1.404 C = -0.008 D = 1.642
Sum of errors squared: 0.0025
Mean of blanks : 0.021

Figure 1.2(b): Standard curve of IgG1 antibody to *Haemophilus influenzae* type b (Hib) polyribosylribitol phosphate (PRP). Graph of OD on y axis vs log serum dilution on x axis with (+) representing data points before background blanks are subtracted. The sigmoidal curve is fitted to the 9 standard points (▪) after the background blanks have been subtracted. α, ß, C and D are the coefficients used to determine the curve (see Chapter 1.12 for details).

The format for the sample data is the same as for the standard except for the addition of a column indicating the absolute value of the sample extrapolated from the standard curve before correction for dilution.

1.13 DATA INTERPRETATION AND QUALITY CONTROL

Assay precision is of great importance and is usually expressed as a coefficient of variation (CV). This is performed by testing at least 3

dilutions of the appropriate reference to cover the upper, middle and lower parts of the standard curve in triplicate in several assays performed on different occasions. The CV is obtained by using the following equation:

$$CV = \frac{\text{standard deviation} \times 100}{\text{mean}}$$

Various CVs can be measured:

i. Intra-assay (within assay) variation, determined by testing one sample many times in one assay which tends to give an over-estimation of precision; and

ii. Inter-assay (between assay) variation based on testing one sample in a sequential number of different assays on different days (McLaren et al 1981).

iii. Inter-dilutional (between dilution) variation achieved by testing one sample of different dilutions in one assay.

The aim is to achieve an intra-assay CV $< 10\%$ and inter-assay CV $< 15\%$ (Porstmann and Kiessig 1992). Inter-dilutional CV's of $\leq 20\%$ were taken as evidence of assay parallelism. For quantitative measurements of antibody a greater degree of precision is needed, particularly if a change in antibody level is being investigated. Precision performance of an assay can be monitored by plotting the means and standard deviations of positive and negative reference sera for each test on standard quality control charts (Batty 1981). Technical performance can be checked by placing reference samples or samples of known antibody activity amongst samples for routine testing in each run. This ensures precision of the test from run to run within the laboratory and enables the mean and standard deviations (SD) of these internal standards to be plotted.

To ensure uniformity of a test it is necessary:

i. to accurately calibrate local standards with international standards; or

ii. to exchange coded samples with other laboratories if no international standard exists.

Theoretically there should be a linear relationship between optical density and analyte concentrations. As this is the exception rather than the rule, it is undesirable to express results as absolute optical density units. Where reference preparations are available, the results can be extrapolated from a standard curve run on each plate and given a value. Where reference preparations are not available a pool of serum or antigen extract with a high concentration of analyte can be used as an internal reference preparation and given arbitrary unitage. Results can only be recorded from a reference curve if the reference material and sample have parallel dilution curves.

Parallelism is shown when the same final concentration of the sample is estimated following correction for the serum dilution. When non-parallelism is seen in samples with low analyte concentration, quantitation must be interpreted with caution.

Parallelism is also useful for identifying any changes in assay performance secondary to reagent variability and stability.

Dose-response curves for antigen or antibody usually have a sigmoidal form on which a level has to be set to discriminate between positive and negative results. Establishing a lower limit or cut-off of the assay always presents problems in ELISA due to the large variation in absolute absorbance values obtained on different occasions. The cut off has been derived in different ways by different groups and expressed as a multiple of mean background,

as an absorbance value above mean background, or relative to a known reactive sample (Kemeny and Chantler 1988). In the assays developed in this thesis the cut-off values were determined as twice the mean of the background readings (wells with diluent alone). The end point is not the least amount of antibody or antigen present but the smallest quantity that can reliably be detected by the methods used. End points that are too close to the background are unreliable because small differences in concentration give equally small changes in colour. End points have been defined as 0.20 OD units above background (Kemeny 1992). The end points of assays developed in this thesis were determined from serial dilution of the standards until the results were no longer reproducible. The OD of the greatest dilution giving accurate quantitation of the standard was taken as the end point of the assay. The analyte in samples with an OD greater than this end point OD could be accurately quantitated.

CHAPTER 2

IgG SUBCLASS IMMUNOASSAYS

The accurate measurement and interpretation of IgG subclass values have become increasingly relevant to clinical medicine in the diagnosis of humoral immunodeficiencies. Sensitive and reproducible methods are required to measure IgG subclasses. This chapter discusses the need for and the various methods of IgG subclass measurement and describes the development of optimal assay conditions and the selection of the methods employed. The assay methodology is detailed and results of experiments relating to the precision and standardization of the assays are described. Finally aspects of the assays and future application of the assays are highlighted.

2.1 INTRODUCTION

2.2 DETERMINATION OF OPTIMAL ASSAY CONDITIONS

2.3 METHODS

2.4 ASSAY STANDARDIZATION

2.5 SUMMARY

2.1 INTRODUCTION

The clinical significance of IgG subclass deficiencies has been studied for the past 15 years. Sensitive assays are required to measure IgG subclass levels particularly in children as levels are relatively low. Various methods have been used to quantitate these proteins.

IgG subclasses were originally defined by serological methods but their measurement was hampered because of the difficulty in producing antibodies specific for each of the IgG subclasses (Natvig and Kunkel 1973). Even when a specific antibody is produced its use may be restricted to the assay method used for developing the antibody (Jefferis et al 1985).

Initially assays for IgG subclass proteins used polyclonal precipitating antisera. The need for antibody specificity, reproducibility and availability was resolved with the advent of monoclonal antisera.

Immunodiffusion was one of the first methods applied to the quantitative measurement of each subclass protein in serum (Yount et al 1968, Shakib et al 1975, van der Giessen et al 1975, Mancini et al 1965). Radial immunodiffusion (RID) is based upon the precipitation of immune complexes as serum is allowed to diffuse into an agar matrix containing anti-IgG antibodies. Wells are cut in thin agarose gel which contain subclass specific antiserum. Serum is pipetted into the wells. As the IgG subclass protein diffuses radially, it binds and precipitates with antibody, forming a white precipitin ring where the antigen-antibody complexes reach equivalence. The concentration of immunoglobulin is proportional to the ring of precipitation. A small error in such a measurement may result in a large error in the determination of the immunoglobulin concentration.

Leibl et al (1992) have recently shown that RID with polyclonal antibodies is suitable for reliable quantification of IgG subclasses. When comparing RID using either polyclonal reagents or monoclonal reagents, the results for IgG1 and IgG2 subclasses were comparable, but were discrepant for IgG3 and IgG4 subclasses (Leibl et al 1992). RID is simple, but its major disadvantages are the need for relatively large volumes of expensive antiserum and limited sensitivity.

To improve sensitivity, rocket immunoelectrophoresis was attempted. Here IgG subclass proteins are forced by electrophoresis, into a gel containing subclass-specific antiserum (Oxelius 1978). At equivalence a precipitin is formed in the shape of a rocket, the height of which is proportional to the concentration of IgG subclass protein in the human serum. As with RID, rocket immunoelectrophoresis requires large volumes of antiserum and requires precipitating antibody.

Nephelometric (Beck and Kaiser 1981) and turbidimetric (Maynard et al 1986) assays have been reported for the quantification of IgG subclasses but are probably not sensitive enough for the detection of IgG3 and IgG4 when using the HP series of monoclonal antibodies. In these assays IgG subclass protein in serum and subclass-specific antibody react in solution and the extent of immune-complex formation is measured by the degree of light scattering or absorption of light.

Haemagglutination and haemagglutination inhibition assays have been used in the semi-quantitative measurement of IgG proteins and subclass typing of IgG myelomas (Jefferis et al 1985). Particle-counting immunoassay (PACIA) is a quantitative agglutination method involving agglutination inhibition of latex particles coated with purified monoclonal IgG subclass

proteins by subclass-specific antisera (Magnusson et al 1984). IgG subclass proteins in human serum compete with solid phase IgG that inhibits agglutination in a concentration dependent manner. The disadvantages of PACIA are possible interference by rheumatoid factor and C1q, limited sensitivity and that it requires high affinity precipitating antisera. Particle concentration fluorescence immunoassay (PCFIA) has been adapted to measure IgG subclasses (Mayus et al 1986). This method uses fluorescence-labelled antigen in competition with unlabelled antigen for antibody that has been coated on latex particles. Following incubation, unbound antigen is removed by filtration and latex particles bearing captured antigens (both fluorescent and unlabelled) are concentrated to a small area before their fluorescence is measured. The method is sensitive and precise but requires special instrumentation.

The technique most suited for detection and quantification of IgG subclass proteins and antibodies is ELISA. No internationally standardized method exists, resulting in inter-laboratory variations which may be attributed to reagents, working conditions or genetic and environmental variables in population samples. Several ELISA formats have been used to quantify IgG subclasses in human serum (Hamilton 1987). One format employs an IgG capture antibody to extract all 4 subclasses of IgG from serum and a conjugated anti-IgG subclass monoclonal antibody to detect only one subclass. A disadvantage of this method is that an excess of capture antibody is needed to extract IgG of all 4 subclasses from serum. This is difficult to achieve in a microtitre plate that has limited protein binding capacity. In addition, in the presence of a predominance of IgG1 and IgG2 there is poor binding of IgG3 and IgG4.

The most widely used ELISA format for total IgG subclass measurement has employed IgG subclass-specific monoclonal antibody to capture a single IgG subclass and an enzyme-conjugated IgG specific antibody to detect bound IgG. Variations between the reported assays include the source of capture and detection monoclonal antibodies, the purity of the coating monoclonal antibody (ascitic fluid vs purified), incubation times, types of microtitre plates, working dilutions of the serum and the conjugated anti-human IgG detection antibody. These factors make the results of different clinical studies difficult to compare.

In this study the four IgG subclasses were initially measured by RID using commercially available monoclonal IgG subclass antisera (Kumararatne et al 1990). Briefly test sera were added at several dilutions to wells cut in agarose gel (1.4% in 0.1 M barbitone buffer pH 8.6) which contained 6% PEG 3000 and the appropriate monoclonal antiserum. A standard curve was established using a standard reference serum from the World Health Organization (WHO) (67/97). The gels were incubated for 3 days at 4°C. A standard curve was plotted on semi-log paper plotting the diameter of the precipitin ring against serum dilution. The unknown values were read off the standard and the actual concentration calculated.

Measuring the subclasses in this way proved to be very expensive as it required large volumes of commercially available monoclonal antibodies. The method worked well and was easy to perform but it was not accurate enough for the quantitative results needed in this project. Therefore an ELISA method for the measurement of the subclasses was established. The advantages of the ELISA in terms of simplicity, safety and sensitivity are well documented.

Initially immunoassays using polyclonal capture antibodies were assessed. A chicken anti-human polyclonal antibody (gift from Dr Bellstred, University of Stellenbosch) and a commercial goat anti-human IgG H and L chain (Cappel, Catalogue No 55087) were used as capture coating antibodies for the subclass specific assays. However, background blanks ranged between 30% and 50% of the signal and the maximal signal achieved for all 4 subclasses was low. There was very little signal drop off with decreasing serum concentration (1:100 - 1:5 000) suggesting high non-specific binding. These assays were not developed further and efforts were concentrated on developing IgG subclass ELISAs using monoclonal IgG subclass specific antibodies as capture antibodies.

The most important technical aspects of the assay include selection of ideal capture and detection antibody combinations; the concentration of monoclonal anti-human IgG subclass reagent insolubilized on the solid phase; the methods used for binding monoclonal antibody to the solid phase (adsorption vs covalent binding); the assay buffers; and the test and reference serum dilutions used in the assay. Although the general protocol for performing the different IgG subclass assays is similar, each assay was independently standardized and quality-controlled. These assays and their development will be described in this chapter.

2.2 DETERMINATION OF OPTIMAL ASSAY CONDITIONS

In each ELISA a number of assay conditions were analysed to determine optimal functioning and assay conditions. The time and temperature of incubation, the buffers used for the washing, the incubation steps and the type and concentration of reagents were chosen so that background (non-specific) absorbance was reduced by preventing non-specific interactions but at the same time favouring the immunological and enzymatic reactions.

2.2.1 Coating Monoclonal Antibody

2.2.1.1 *Selection*

Although the development of these assays was aided by the availability of well characterized monoclonal antibodies, various unbiotinylated and biotinylated monoclonal antibodies specific for the individual subclasses IgG1, IgG2, IgG3, IgG4 were evaluated to select the most suitable. Details of these antibodies are listed in Appendix A.

2.2.2 Backgrounds

Table 2.1 shows the effect of the various monoclonal antibodies on non-specific binding. The background readings were obtained from:

- minus monoclonal antibody i.e. minus coating monoclonal antibody but including serum and conjugated antibody
- minus serum i.e. wells coated with monoclonal antibody and conjugated antibody but minus serum
- minus monoclonal antibody and serum blanks i.e. wells without monoclonal antibody or serum but with conjugated antibody

For all the assays the minus monoclonal antibody and serum blank values were all very low (Table 2.1) and were therefore not subtracted from the signal.

Minus monoclonal antibody blanks and minus serum blanks were both subtracted from each serum dilution before the standard curves were constructed.

2.2.2.1 *IgG1 subclass ELISA*

The minus serum blanks were high (56.7%) when coating with the unbiotinylated monoclonal antibody HP6012 (Table 2.1). This was not decreased by changing the serum diluent from 0.25% BSA in PBST to 2% albumin in PBS, 0.5% casein in PBS or 0.25% gelatin in PBST (the minus serum blanks being 32.8%, 55.3% and 51% respectively). Unbiotinylated monoclonal antibodies HP6070 and HP6069 gave reasonable sigmoid curves (Figure 2.1) with acceptable background blanks.

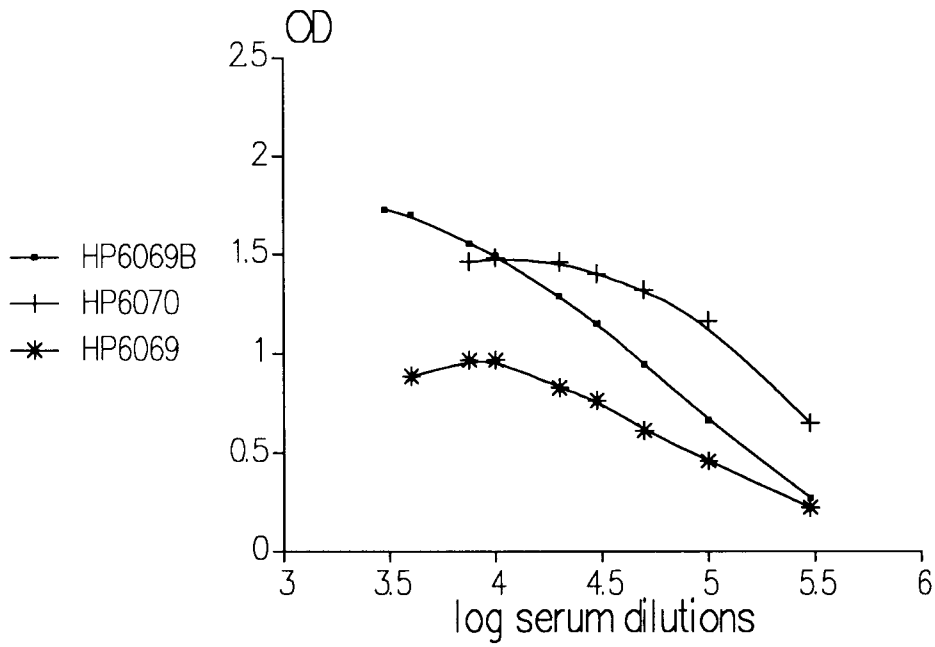


Figure 2.1: Standard curves for IgG1 using in-house AB serum standard, coating with biotinylated monoclonal antibody HP6069B (▪) and unbiotinylated monoclonal antibodies HP6070 (+) and HP6069 (*) diluted 1:1000

Coating the plate with streptavidin and then using the biotinylated anti-IgG1 antibody HP6069 (HP6069B) gave a steep standard curve and low minus monoclonal and minus serum blanks (Table 2.1). This system was selected for further assays.

TABLE 2.1: RESULTS OF OD DETERMINATIONS OF THE BLANKS IN THE IgG SUBCLASS ANTIBODY ELISAs USING BIOTINYLATED AND UNBIOTINYLATED MONOCLONAL ANTIBODIES

	Moab		Conjugate	AB Standard		OPTICAL DENSITY (OD) OF BACKGROUND BLANKS (% of highest OD of AB standard)		
Assay	Clone	Dilution	HRPO g α human ab	Dilution	OD	Minus Moab	Minus Serum	Minus Moab and Serum
IgG1	HP6012*	1:1 000	1:1 000	1:500	2.002	0.012 (0.6)	1.136 (56.7)	0.011 (0.6)
	HP6070*	1:1 000	1:1 000	1:500	2.029	0.203 (10.0)	0.316 (15.6)	0.041 (2.0)
	HP6069*	1:1 000	1:1 000	1:500	2.194	0.234 (10.7)	0.239 (10.9)	0.055 (2.5)
	HP6069**	1:1 000	1:1 500	1:4 000	1.976	0.138 (7.0)	0.141 (7.1)	0.063 (3.2)
IgG2	HP6008*	1:500	1:2 500	1:500	2.177	0.462 (21.2)	0.115 (5.3)	0.002 (0.1)
	HP6008*: HP6014*	1:500	1:1 500	1:3 000	1.860	0.058 (3.1)	0.161 (8.7)	0.009 (0.5)
	HP6002**	1:1 000	1:3 000	1:500	0.662	0.577 (87.2)	0.233 (3.5)	0.021 (3.2)

Table 2.1 continued

	Moab		Conjugate	AB Standard		OPTICAL DENSITY (OD) OF BACKGROUND BLANKS (% of highest OD of AB standard)		
Assay	Clone	Dilution	HRPO g α human ab	Dilution	OD	Minus Moab	Minus Serum	Minus Moab and Serum
IgG3	HP6010*	1:500	1:24 000	1:4 000	2.087	0.005 (2.6)	0.075 (3.6)	0.009 (0.4)
	HP6047**	1:1 000	1:3 000	1:500	1.933	0.575 (29.8)	0.029 (1.5)	0.023 (1.2)
IgG4	HP6013*	1:500	1:12 000	1:1 000	2.033	0.314 (15.5)	0.303 (14.9)	0.008 (0.4)
	HP6025**	1:1 000	1:3 000	1:500	1.892	0.592 (31.3)	1.055 (55.8)	0.078 (4.1)

* Unbiotinylated monoclonal antibodies (see text for details)

** Biotinylated monoclonal antibodies (see text for details)

HRPO g α human ab = Horseradish peroxidase goat anti-human conjugate (see text for details)

The minus monoclonal wells in these assays were coated with streptavidin but had no biotinylated monoclonal antibody. In preliminary experiments blank values of wells without streptavidin and with serum but without monoclonal antibody were also included. Readings from these wells were negligible (OD all less than 0.026).

Initial experiments were done to determine the optimum coating concentrations of the streptavidin and biotinylated antibody. Streptavidin was tested from 0.025 μg/ml - 10.0 μg/ml diluted in PBS. There was no difference in the shape of the standard curve using streptavidin 0.5 - 2.0 μg/ml but at concentrations above and below this the signal was lower and the curve was flatter. A coating concentration of 0.5 μg/ml was found to be optimal and selected for further experiments (Figure 2.2).

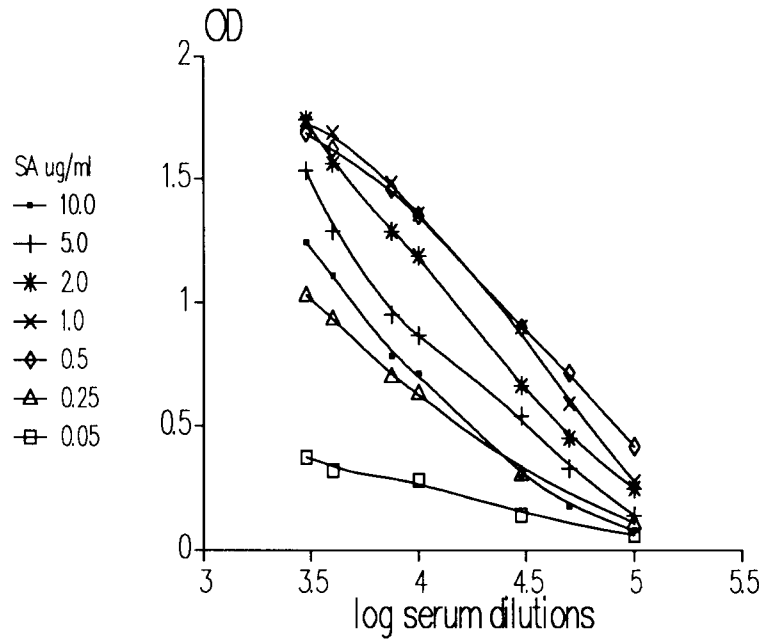


Figure 2.2: Standard curves for IgG1 using in-house AB serum standard, coating with decreasing streptavidin (SA) concentrations: 10.0 μg/ml (▪); 5.0 μg/ml (+); 2.0 μg/ml (*); 1.0 μg/ml (x); 0.5 μg/ml (◇); 0.25 μg/ml (Δ); 0.05 μg/ml (□)

The performance of the biotinylated anti-IgG1 monoclonal antibody was assessed at dilutions of 1:500 to 1:10 000. Results show that a coating dilution of 1:1 000 was suitable (Figure 2.3).

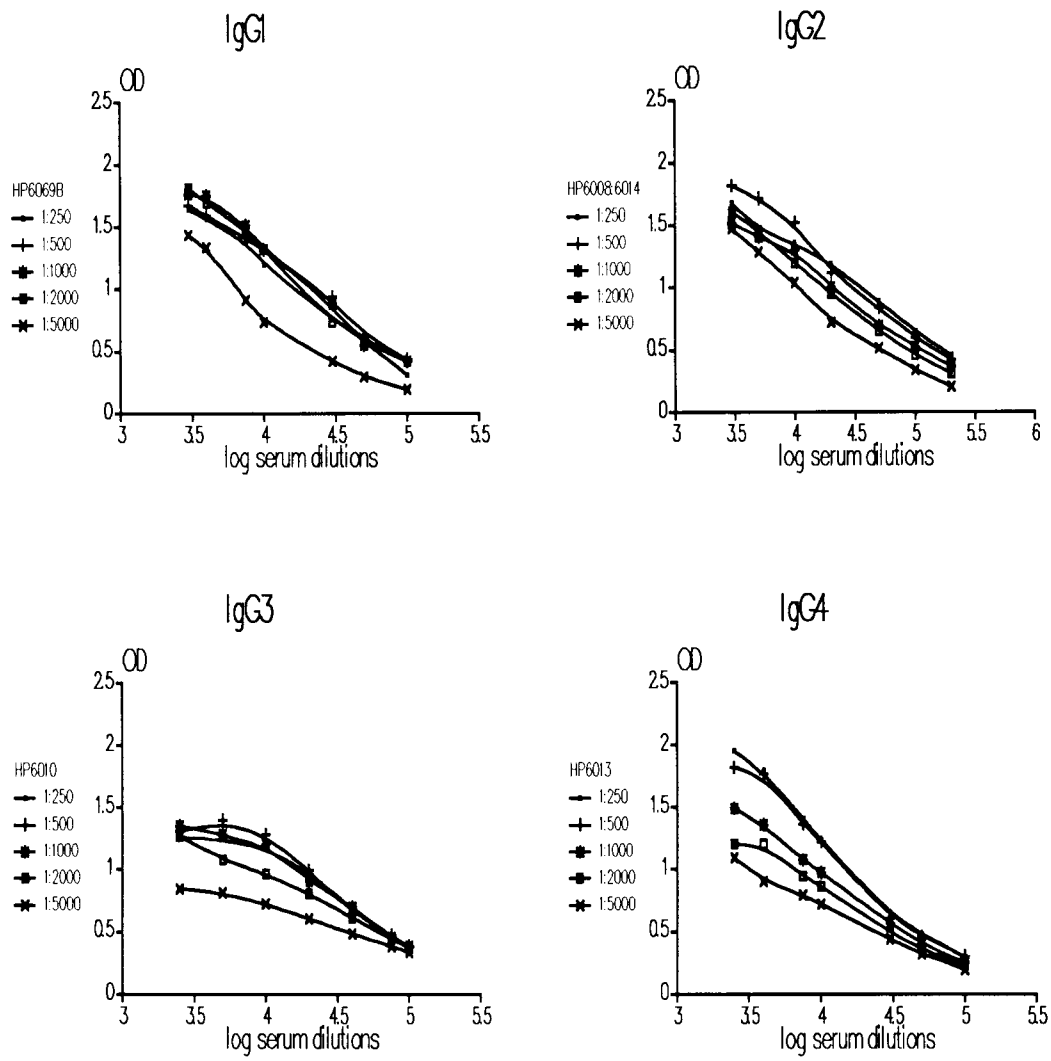


Figure 2.3: Standard curves for IgG1, IgG2, IgG3 and IgG4 using the monoclonal antibodies: biotinylated HP6069 for IgG1, HP6008:HP6014 for IgG2, HP6010 for IgG3 and HP6013 for IgG4 at dilutions of 1:250 (•); 1:500 (+); 1:1000 (*); 1:2000 (▪); 1:5000 (x)

2.2.2.2 *IgG2 Subclass ELISA*

Unlike the IgG1 ELISA the minus serum blanks in these assays were low whether a biotinylated or unbiotinylated monoclonal antibody was used (Table 2.1). However, the minus monoclonal blanks were high in the IgG2 assays when the biotinylated monoclonal antibody was used. Thus the assay format of coating the plates with unbiotinylated anti-IgG2 antibody was selected for IgG2 subclass measurement. As the minus monoclonal antibody background blanks were unacceptably high (fluctuating between 15% - 25% of maximum signal) these assays were developed further. A combination of two unbiotinylated monoclonal antibodies, HP6008 and HP6014 was used to coat the plates. The optimum combination of the mixture was found to be a 2:1 ratio of HP6008:HP6014 (Figure 2.4) and a 1:500 dilution of both antibodies (Figure 2.3). This caused a shift to the right of the curve which enabled the most concentrated serum dilution to be decreased from 1:1 000 to 1:3 000 which accounted for the reduction in readings of the minus monoclonal antibody blanks. The minus serum blanks increased marginally using the monoclonal antibody combination (Table 2.1) but the sum of the minus monoclonal antibody and minus serum blanks as a percentage of the most concentrated standard was significantly lower with the HP6008:HP6014 mixture when compared to HP6008 alone.

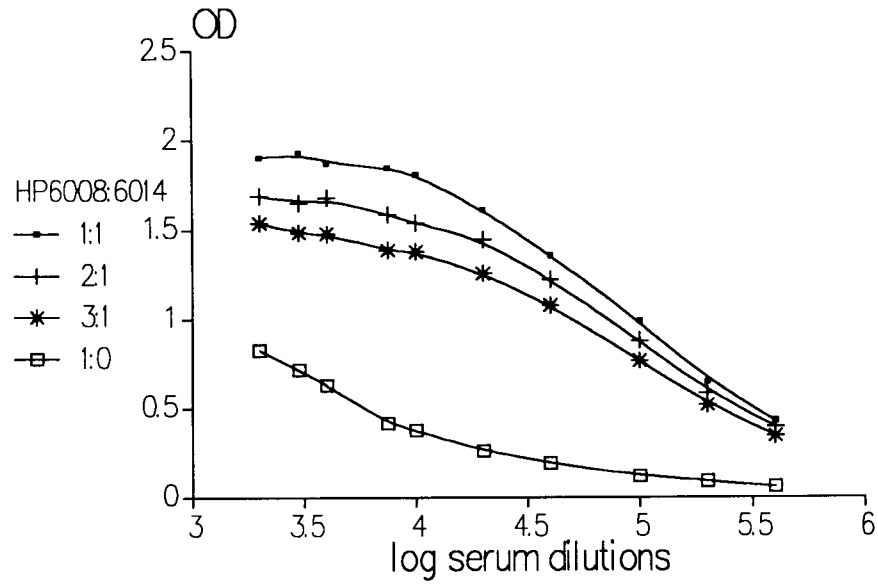


Figure 2.4: Standard curves for IgG2 using in-house AB serum standard, coating with monoclonal antibodies HP6008 and HP6014, diluted 1:500, in combinations 1:1 (▪); 2:1 (+); 3:1 (*); 1:0 (□)

2.2.2.3 *IgG3 Subclass ELISA*

Both biotinylated or unbiotinylated anti-IgG3 monoclonal antibodies gave low minus serum blank readings. The readings of the minus monoclonal antibody blanks were significantly lower (Table 2.1) and the standard curve steeper when using the unbiotinylated antibody to coat the plates. Hence the unbiotinylated anti-human IgG3 antibody (HP6010) was used to coat the plates in the IgG3 subclass assays. Results show that a coating dilution of 1:500 was optimal (Figure 2.3).

2.2.2.4 *IgG4 Subclass ELISA*

Unbiotinylated antibody HP6013 was selected in preference to the biotinylated antibody for the capture antibody as it gave lower minus monoclonal antibody and minus serum blanks (Table 2.1). Results show that a coating dilution of 1:500 was optimal (Figure 2.3).

The unbiotinylated monoclonal antibody HP6011 was evaluated but gave a much lower positive signal when compared to HP6013 even when used at a greater concentration (Figure 2.5). The signal could be improved using it in combination with HP6013 but was not better than HP6013 alone (Figure 2.5). The unbiotinylated monoclonal antibody HP6013 was selected for use in the IgG4 subclass assays in this study.

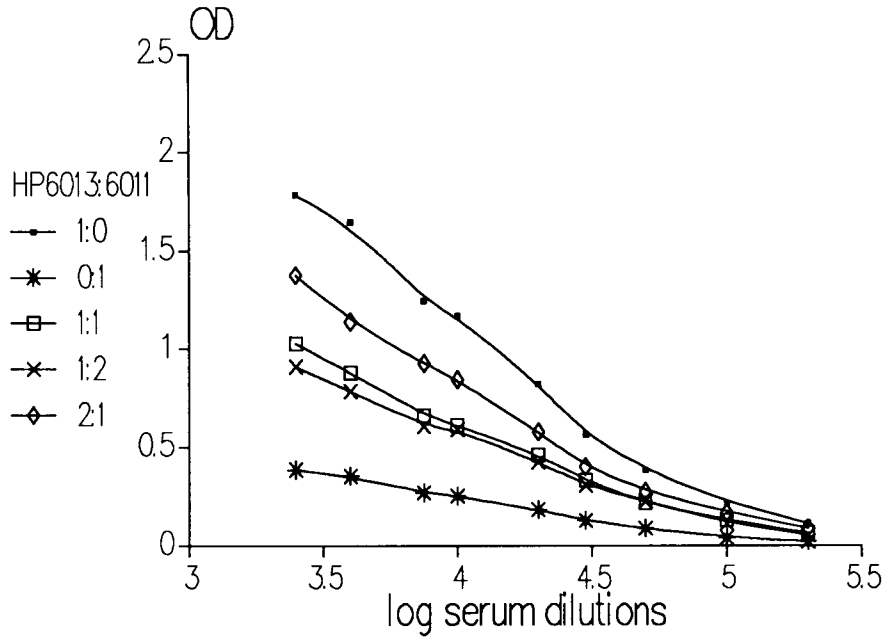


Figure 2.5: Standard curves for IgG4 using in-house AB serum standard coating with monoclonal antibodies HP6013 and HP6011, concentration 1:500, in combinations 1:0 (▪); 0:1 (*), 1:1 (□); 1:2 (x); 2:1 (◇)

2.2.2.5 *Conclusion and discussion*

Standard curves for the four IgG subclasses could be produced in a system which used monoclonal antibodies to coat the plates to trap specific serum IgG isotypes, and polyclonal anti-IgG antibodies as detection conjugates. The selection of the most appropriate monoclonal antibodies is dependent in part on the system they are to be used in. In an extensive study of 59

monoclonal antibodies of supposed specificity for human IgG subclasses, tested by different methods the results were discouraging with IgG1 and particularly IgG2, demonstrating assay restriction (Jefferis et al 1985). It has also been shown that the murine monoclonal antibodies recognize only a limited number of unique epitopes of the IgG subclasses (Jefferis 1986). A critical starting factor in the IgG subclass ELISAs is the stable binding of functional monoclonal antibodies to a solid-phase support. Most antibodies retain their ability to bind antigen when coated to plastic, despite losing a portion of their activity (Kemeny 1992). However, some monoclonal antibodies lose their reactivity when absorbed directly onto plastic material. Hussain et al (1986) were unable to obtain calibration curves with HP6012, HP6022, HP6021 and HP6006 bound to a polystyrene surface and meaningful calibration curves could not be obtained with HP6012 attached directly to the plates. Papadea et al (1985) were able to bind these same monoclonal antibodies to polystyrene wells that had been precoated with BSA and then cross-linked with glutaraldehyde before addition of the four monoclonal antibodies. Kemeny (1992) found that the binding capacity of HP6012 was enhanced when linked to the plate with anti-mouse antibodies than when bound to the plate directly.

In this study it was also found that anti-IgG1 monoclonal antibody HP6012 functioned poorly when adsorbed directly to the plates. Covalently coupling the biotinylated monoclonal antibody (HP6069) to streptavidin coated plates enhanced the assay sensitivity. Butler et al (1992) have shown that immobilization of capture antibodies via a streptavidin bridge resulted in preservation of antibody sites when compared to direct absorption.

HP6014 is known to preferentially bind lambda (λ) over kappa (k)-bearing IgG2 protein when used in limiting concentrations (Hamilton 1987,

Madassery et al 1988, Aucouturier et al 1992a). HP6008 is also said to have a slightly higher detection efficacy for IgG2 λ than for IgG2 k and a low sensitivity (Madassery et al 1988). Although HP6008 has a lower affinity for IgG2 than does HP6014 it is a better reagent for IgG measurement. HP6008 is widely used in IgG2 ELISAs. It was found in this study that a combination of HP6008 and HP6014 in a ratio of 2:1 increased the sensitivity of the IgG2 assay.

HP6010 and HP6047 have both been used in previous reports of IgG3 subclass ELISAs (Table 2.2). In this study HP6010 was selected as the anti-IgG3 antibody in preference to the biotinylated HP6047 as it has lower minus monoclonal antibody blanks.

Although HP6011 has been used in previous reports of IgG4 subclass assays (Aucouturier et al 1985) it gave poor results in the experiments reported here. HP6013 was selected as the best anti-IgG4 antibody.

The selected monoclonal antibodies used in this study were unpurified and still in ascitic fluid. Chemical purity of the monoclonal antibody has been found by Hamilton et al (1988) to be the most important variable influencing the binding of anti-human IgG monoclonal antibody to the plate. They found the use of unpurified monoclonal antibodies in ascitic fluid as primary capture antibodies, produced large variation and low binding capacities when compared to the direct coating of chromatographically purified antibody. There are not many references directly comparing the use of purified and unpurified ascitic fluid as coating antibodies. However, ascitic fluid has been used successfully to establish IgG subclass ELISAs (Table 2.2).

TABLE 2.2: SUMMARY OF MONOCLONAL ANTIBODIES USED IN REPORTED IgG SUBCLASS ELISAs

REFERENCE	MONOCLONAL ANTIBODY					BLOCKER
	CLONE		PURITY*	COATING CONCENTRATION	DILUENT	
Aucouturier et al 1985	IgG1	HP6012	A	1:30 000	Carbonate Buffer pH 9.6	0.2% BSA
	IgG2	HP6014: HP6008	A	1:30 000 1:15 000		
	IgG3	HP6010	A	1:10 000		
	IgG4	HP6011	A	1:3 000		
Papadea et al 1985	IgG1	HP6012	A	1:300	Pre-coated BSA-glutaraldehyde Plates	0.5% BSA
	IgG2	HP6014	A	1:3 000		
	IgG3	HP6048	A	1:3 300		
	IgG4	HP6022	A	1:30 000		
Hussain et al 1986	IgG1	HP6001	P	1:800	PBS pH 7.5	5% BSA
	IgG2	HP6002	P	1:100		
	IgG3	HP6047	A	1:2 000		
	IgG4	HP6023	A	1:1 000		

Table 2.2 continued

REFERENCE	MONOCLONAL ANTIBODY					BLOCKER
	CLONE		PURITY*	COATING CONCENTRATION	DILUENT	
Hamilton et al 1988	IgG1	HP6069	P	1:100 - 1:300	PBS pH 7.4	0.5% BSA
	IgG1	HP6070	P	1:100 - 1:300		
	IgG2	HP6002	p	1:100 - 1:300		
	IgG2	HP6002: HP6014	P	1:100 - 1:300		
	IgG3	HP6047	P	1:100 - 1:300		
	IgG4	HP6025	P	1:100 - 1:300		
	IgG4	HP6023	P	1:100 - 1:300		
Black et al 1988 and Meissner et al 1990	IgG1	HP6069	A	1:1 000	Deionized H_2O	10% FCS
	IgG2	HP6014	A	1:1 000		
	IgG3	HP6047: HP6050	A	1:500		
	IgG4	HP6024	A	1:1 000		
Leibl et al 1992	IgG1	HP6091	A	1:1 000	Carbonate Buffer pH 9.6	2% BSA
	IgG2	HP6014	A	1:4 000		
	IgG3	HP6010	A	1:2 000		
	IgG4	HP6025	A	1:4 000		

A = Unpurified ascitic fluid
P = Purified monoclonal antibody

2.2.3 Coating Buffer

2.2.3.1 *IgG1 subclass ELISA*

The streptavidin used to coat the plates was diluted in PBS buffer pH 7.2. It made no difference to the sum of the background blanks (minus monoclonal antibody and minus serum blanks) expressed as a percentage of the maximum standard signal whether the monoclonal antibody was diluted in PBS, glycine/Tris pH 7.0-7.5 or 0.25% BSA in PBST. The biotinylated monoclonal antibody was diluted in 0.25% BSA in PBST as this diluent resulted in the highest positive signal with acceptable background blanks (Table 2.3).

2.2.3.2 *IgG2 subclass ELISA*

Coating of monoclonal antibody in a glycine/Tris buffer pH 7.0-7.5 gave a standard curve with a higher signal and steeper slope than that obtained using a carbonate coating buffer pH 9.6 (Figure 2.6). As it had no detrimental effect on the background blanks when they were expressed as a percentage of the maximum signal the glycine/Tris buffer was selected for use in this assay (Table 2.3).

2.2.3.3 *IgG3 subclass ELISA*

The glycine/Tris coating buffer pH 7.0-7.5 gave a greater signal and produced a standard curve with a steeper slope than that obtained with carbonate coating buffer pH 9.6 (Figure 2.6). The coating buffer did not affect the minus monoclonal antibody or minus serum blanks and the glycine/Tris was used as the coating buffer in the IgG3 ELISA (Table 2.3).

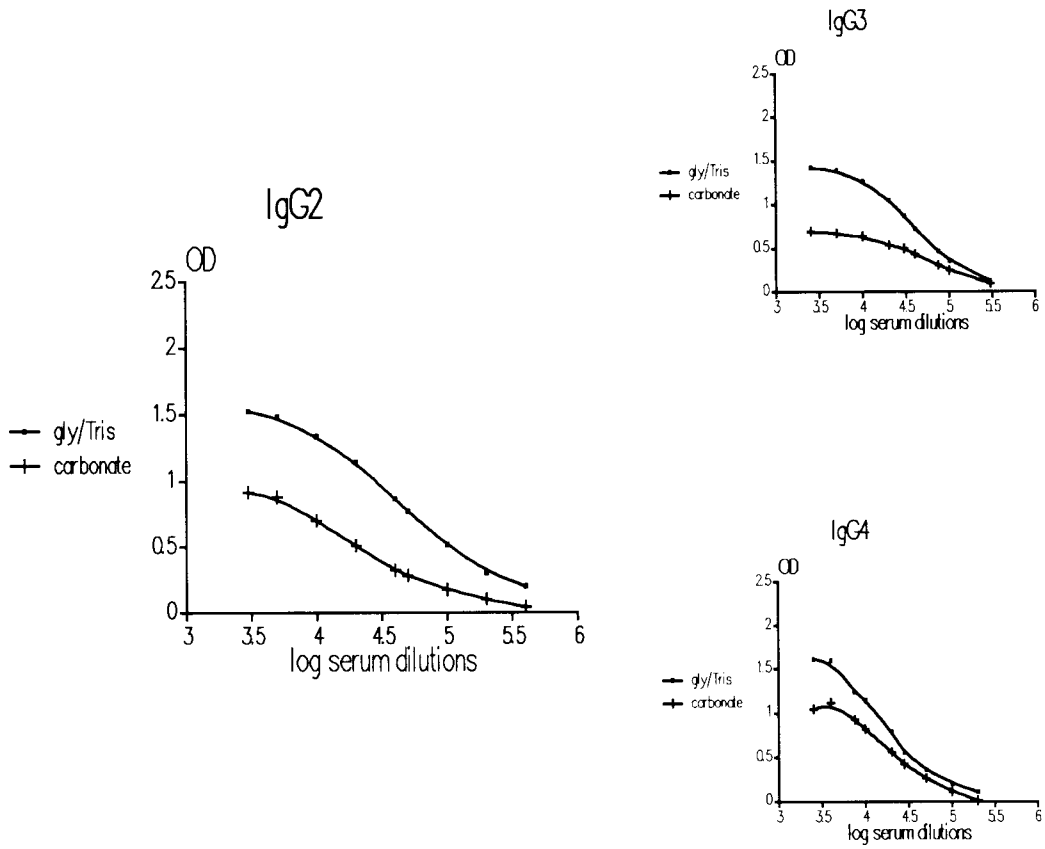


Figure 2.6: Standard curves for IgG2, IgG3 and IgG4 using in-house AB serum standard, coating with monoclonal antibodies in glycine/Tris buffer pH 7.0-7.5 (▪), and carbonate buffer pH 9.6 (+)

2.2.3.4 *IgG4 subclass ELISA*

The glycine/Tris coating buffer pH 7.0-7.5 gave a higher signal than the carbonate buffer in the IgG4 ELISA as it had in the IgG2 and IgG3 ELISAs, although the differences were not as marked and the shapes of the curves were similar (Figure 2.6). Coating in the glycine/Tris buffer pH 7.0-7.5 resulted in lower minus monoclonal antibody blanks without increasing the minus serum blanks (Table 2.3) and was therefore also found most suitable for the IgG4 assay.

TABLE 2.3: EFFECT OF COATING BUFFERS ON THE IgG SUBCLASS ASSAYS

SUBCLASS ASSAY	COATING BUFFER	OD MAX* SIGNAL	OPTICAL DENSITY (OD) OF BACKGROUND BLANKS (% of Maximum Signal)	
			Minus Moab	Minus Serum
IgG1	PBS	1.526	0.301	0.179
HP6069			(19.7)	(11.7)
biotinylated	0.25% BSA in PBST	2.000	0.331	0.204
			(16.5)	(10.2)
	gly/Tris pH 7.0-7.5	1.116	0.298	0.082
			(25.6)	(7.0)
IgG2	gly/Tris pH 7.0-7.5	1.887	0.090	0.241
HP6008:			(4.8)	(12.8)
HP6014	Carbonate pH 9.6	1.080	0.040	0.117
			(3.8)	(10.8)
IgG3	gly/Tris pH 7.0-7.5	1.580	0.46	0.125
HP6010			(2.9)	(7.9)
	Carbonate pH 9.6	0.790	0.026	0.066
			(3.3)	(8.4)
IgG4	gly/Tris pH 7.0-7.5	1.891	0.074	0.135
HP6013			(3.9)	(3.9)
	Carbonate pH 9.6	1.450	0.299	0.101
			(20.6)	(7.0)

* **OD max signal = highest optical density reading of AB standard**

2.2.3.5 *Conclusion and discussion*

The binding of individual monoclonal antibodies depends largely on the nature of the solid phase support and on the coating buffer.

Antibody-antigen reactions normally take place between pH 6.0 and 9.0. The pH of the coating buffer can influence the amount of mouse immunoglobulin that binds to the plate (Kemeny 1992).

In these assays, some monoclonal antibodies (HP6008:HP6014 and HP6010) gave a much higher signal when they were coated on polystyrene plates in a glycine/Tris buffer pH 7.0-7.5 than when they were diluted in the usual carbonate buffer pH 9.6. Hussain et al (1986) found that monoclonal antibodies HP6003 and HP6024 gave higher signals when coated in a pH 7.5 buffer. Hamilton et al (1988) found no difference in antibody binding capacity or sensitivity when the coating monoclonal antibody was diluted in carbonate buffer or PBS and selected PBS because it was more physiological than the carbonate buffer. In the IgG2, IgG3 and IgG4 subclass assays developed in this study the glycine/Tris buffer pH 7.0-7.5 was selected as it gave better antibody binding than the more commonly used carbonate buffer pH 9.6.

Black et al (1988) found that coating monoclonal antibody with relatively high dilutions of ascitic fluid in distilled water maintained antigen binding capability when dried on plastic surfaces.

2.2.4 Plates

Ninety six well flat bottomed polystyrene Nunc certificated (Denmark, Catalogue No 39454) and flat bottomed polyvinyl chloride Falcon (Catalogue No 3912) plates were compared for these immunoassays.

2.2.4.1 *IgG1 subclass ELISA*

In the IgG1 assays (Figure 2.7) no significant differences in the shapes of the standard curve or background values were found when using Nunc or Falcon plates.

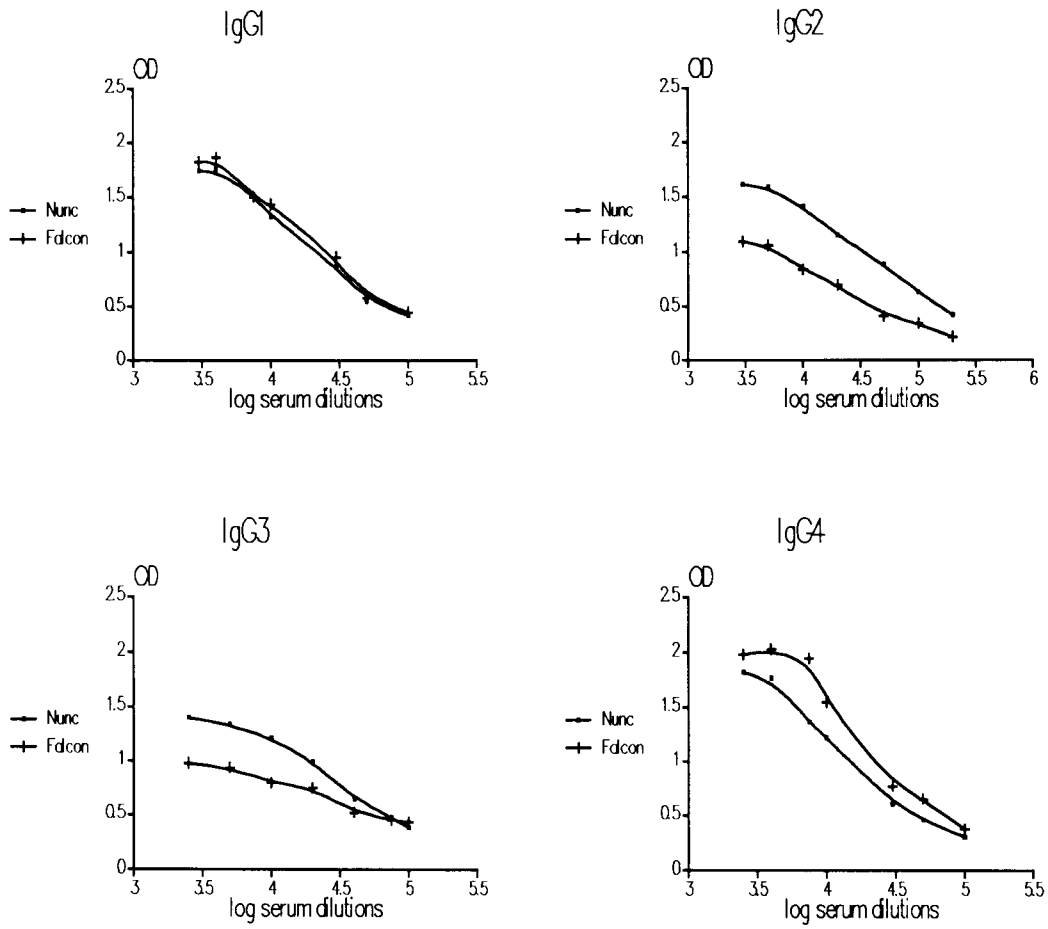


Figure 2.7: Standard curves for IgG1, IgG2, IgG3 and IgG4 using in-house AB serum standard on Nunc (flat bottomed polystyrene certificated) plates (▪) or Falcon (flat bottomed polyvinyl chloride) plates (+).

2.2.4.2 *IgG2 subclass ELISA*

The signal of the IgG2 standard curve on the Falcon plates was lower (65%) than on the Nunc plates (Figure 2.7). There was no significant difference in minus monoclonal antibody or minus serum blank backgrounds between the two plates.

2.2.4.3 *IgG3 subclass ELISA*

The shape of the IgG3 standard curve was flatter and the signal lower (± 31%) on the Falcon plates (Figure 2.7). There was no significant difference in the background values on the two plates.

2.2.4.4 *IgG4 subclass ELISA*

In the IgG4 assays the signal and shape of the curve were similar between the two plates (Figure 2.7). However, the minus serum blanks were significantly higher on the Falcon plates compared to the Nunc plates.

2.2.4.5 *Conclusion and discussion*

In previously described IgG subclass ELISAs various plates have been used: Nunc Immunolon I (Hamilton et al 1988, Leibl et al 1992), Dynatech (Aucouturier et al 1985, Hussain et al 1986, Hamilton et al 1988, Meissner et al 1990), Limbro (Hamilton et al 1988) and Falcon (Hamilton et al 1988). Hamilton et al (1988), compared Dynatech, Falcon and Limbro plates and found that these plates all bound purified antibody maximally at the same concentration and produced equivalent final assay performance when compared directly. Other authors have not commented as to why particular plates were selected or whether other plates were assessed. The expensive high binding plates advocated for ELISA have been found to give high backgrounds due to non-specific binding (Aucouturier et al 1984).

The results from this study indicate that the type of plate used may affect the ELISA sensitivity, this effect varying from one subclass assay to another.

The Falcon plates were more difficult to work with and gave greater variation in triplicate samples than did the Nunc plates. Nunc plates were selected for use in all four subclass assays as they were easier to load without splashing, cheaper (1/5 of the cost) and overall gave steeper standard curves and lower background blanks than the Falcon plates.

No significant differences were observed when Nunc certificated and non-certificated flat bottomed plates were compared.

2.2.5 Blocking Agents

Various blocking agents used to prevent non-specific binding to the microtitre plates were assessed in these assays:

- Bovine serum albumin (BSA), albumin fraction V - Boehringer Mannheim, Catalogue No 735086
- Fetal calf serum (FCS) - Flow, Catalogue No 29-101-49
- Gelatin - Biorad, Catalogue No 170-6537
- Casein - Unilab Saarchem, Catalogue No 152813
- Casein - Hammarsten, Catalogue No 44020

2.2.5.1 *IgG1 subclass ELISA*

The background readings of the minus monoclonal antibody and minus serum blanks ranged between 5-10% of the maximum signal and were unaffected by altering the blocker. There was also no difference to either of the blanks when the plates were blocked pre- or post- the monoclonal antibody incubation step or when they were not blocked at all (Figure 2.8).

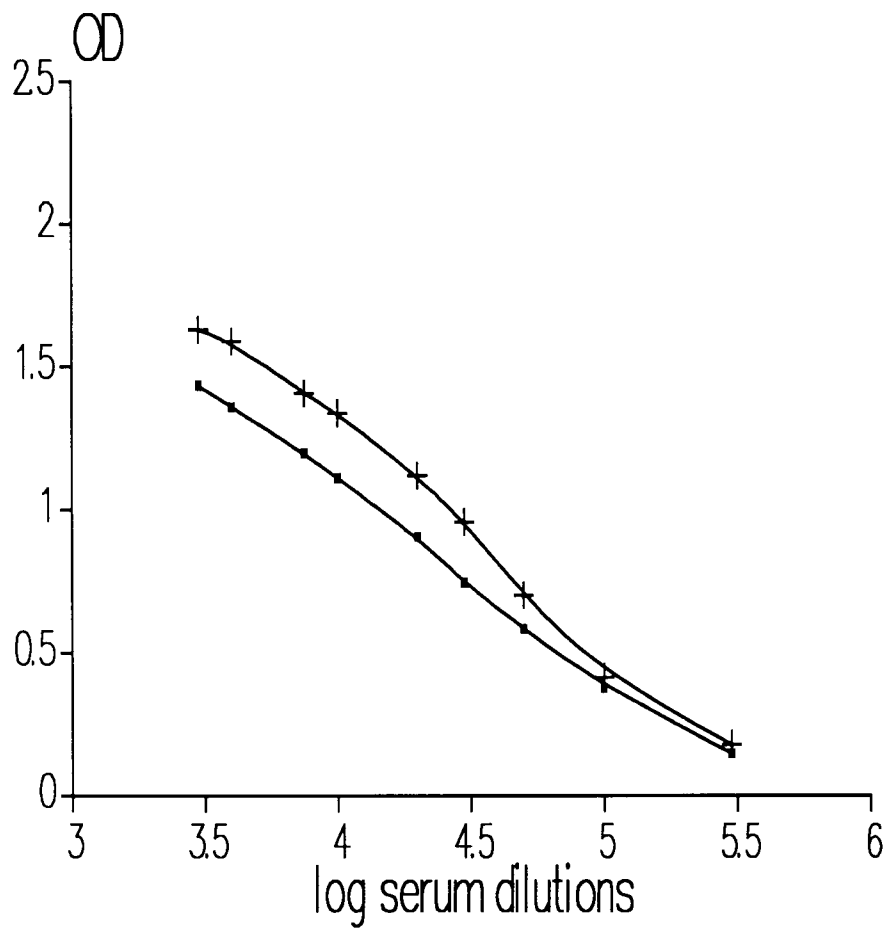


Figure 2.8: Standard curves for IgG1 using in-house AB serum standard with (▪) and without (+) blocking with 1% casein

2.2.5.2 *IgG2 subclass ELISA*

There was no difference in minus monoclonal antibody blanks when 0.5%, 1%, 2% or 5% BSA in PBS was used as a blocking agent but the minus monoclonal antibody backgrounds increased when 0.2% BSA was used. The effect of various blockers on the background readings in the IgG2 subclass assays when the plates were coated with 1:500 dilution of HP6008 or HP6014 or a 2:1 mixture of HP6008:HP6014 monoclonal antibodies is

shown in Table 2.4. Differences were most noticeable with the minus monoclonal antibody blanks. Although the addition of detergents to blocking agents has been used to decrease non-specific binding to the solid phase the minus monoclonal antibody blanks increased significantly when 0.5% Tween 20 was added to 2% BSA as the blocking agent (Table 2.4). 1% casein consistently gave the lowest background readings. The Unilab casein was just as effective as the higher grade Hammarsten casein and was less expensive.

2.2.5.3 *IgG3 subclass ELISA*

The various blockers assessed made little difference to the minus monoclonal antibody or minus serum blanks which were consistently less than 3% and 7% respectively of the signal of the maximum standard dilution.

2.2.5.4 *IgG4 subclass ELISA*

There was no significant difference in readings or blanks with the various blockers.

TABLE 2.4: EFFECT OF BLOCKING AGENT ON THE BACKGROUND BLANKS IN THE IgG2 ELISA

IgG2 HP6008			
BLOCKING AGENT	OD Maximum* Signal	OPTICAL DENSITY (OD) OF BACKGROUND BLANKS (% of Maximum Signal)	
		Minus Moab	Minus Serum
2% BSA	1.308	0.356 (27.2)	0.086 (6.6)
5% BSA	1.213	0.284 (23.4)	0.032 (2.6)
2% BSA + 10% FCS	1.294	0.421 (32.5)	0.036 (2.8)
2% BSA + 0.5% Tween-20	1.449	1.303 (89.9)	0.087 (6.0)
10% FCS	1.121	0.246 (21.9)	0.039 (3.5)
1% Gelatin	1.215	0.369 (30.4)	0.089 (7.3)
1% Casein Unilab	1.025	0.151 (14.7)	0.033 (3.2)
1% Casein Hammersten	0.705	0.078 (11.1)	0.022 (3.1)

* OD maximum signal = highest OD reading of AB standard

Table 2.4 continued

IgG2 HP6014			
BLOCKING AGENT	**OD Maximum* Signal**	**OPTICAL DENSITY (OD) OF BACKGROUND BLANKS (% of Maximum Signal)**	
		Minus Moab	**Minus Serum**
2% BSA	2.245	0.367 (16.4)	0.268 (11.94)
5% BSA	2.180	0.363 (16.7)	0.251 (11.5)
2% BSA + 10% FCS	2.009	0.242 (12.1)	0.113 (5.6)
2% BSA + 0.5% Tween-20	ND	ND	ND
10% FCS	1.959	0.155 (7.9)	0.113 (5.8)
1% Gelatin	2.070	0.133 (6.4)	0.232 (11.2)
1% Casein Unilab	2.139	0.062 (2.9)	0.092 (4.3)
1% Casein Hammersten	1.829	0.051 (2.8)	0.057 (3.1)

*** OD maximum signal = highest OD reading of AB standard**
ND = not done

Table 2.4 continued

IgG2 HP6008:HP6014
2:1

BLOCKING AGENT	OD Maximum* Signal	OPTICAL DENSITY (OD) OF BACKGROUND BLANKS (% of Maximum Signal)	
		Minus Moab	Minus Serum
2% BSA	2.094	0.210 (10.0)	0.224 (10.7)
5% BSA	1.928	0.145 (7.5)	0.230 (11.9)
2% BSA + 10% FCS	1.770	0.210 (11.9)	0.75 (4.2)
2% BSA + 0.5% Tween-20	1.914	0.970 (50.7)	0.108 (5.7)
10% FCS	1.833	0.153 (12.0)	0.078 (4.3)
1% Gelatin	1.962	0.176 (9.0)	0.120 (6.1)
1% Casein Unilab	1.911	0.050 (2.6)	0.059 (3.1)
1% Casein Hammersten	1.700	0.072 (4.2)	0.041 (2.4)

*** OD maximum signal = highest OD reading of AB standard**

2.2.5.5 Conclusion and discussion

It is common practise to block unreacted sites with an irrelevant protein after the primary coating step. In the IgG1 assays the plates were blocked after the streptavidin coating and before the addition of the monoclonal antibody. Human serum albumin is not suitable as a blocking protein in IgG subclass assays because it contains a small percentage ($\leq$ 1%) of immunoglobulins that produce high background values. BSA is the most common blocker used in IgG subclass ELISAs (Table 2.1) and although both BSA and 1% casein were found to be equally suitable in the IgG1, IgG3 and IgG4 subclass assays developed in this study, 1% casein gave lower minus monoclonal antibody blanks in the IgG2 assays. 1% casein was therefore selected as the blocker for all the subclass assays. The minus monoclonal antibody blanks were significantly reduced in the IgG2 subclass assay when the incubation time of the blocking step was increased from 1 hour at 37°C to overnight at 37°C but in the IgG1, IgG3 and IgG4 subclass ELISAs the one hour incubation at 37°C was adequate. In order to coordinate the addition of the sera to all 4 subclass assays, the coating times were adjusted.

For the IgG1 assay, after the streptavidin coating overnight at 4°C, the plates were blocked for 1 hour at 37°C and then the monoclonal antibody was left on for 2 hours at room temperature.

For the IgG2 assay, the monoclonal antibody was left on for 2 hours at room temperature and then the plates were blocked overnight at 37°C. For the IgG3 and IgG4 assays, the monoclonal antibodies were left on overnight at 4°C and the plates were then blocked for 1 hour at 37°C.

2.2.6 Serum Diluents

2.2.6.1 *IgG1, IgG3 and IgG4 subclass ELISAs*

1% casein was compared to 0.25% BSA in PBST as a serum diluent. No difference in serum blanks was observed in the IgG1, IgG3 and IgG4 assays.

2.2.6.2 *IgG2 subclass ELISA*

When 1% casein was used as a serum diluent in the IgG2 assay, lower blanks (2-7% of the signal) were observed than when 0.25% BSA in PBST was the diluent (10-20%).

2.2.6.3 *Conclusion and discussion*

A 1% casein solution was selected for use in all four of the subclass assays as the blocking solution and serum diluent, as there was a significant decrease in the minus monoclonal antibody blanks of the IgG2 subclass assays and it did not adversely affect the other IgG subclass assays.

2.2.7 Serum Standards

A large pool of serum, blood group AB (Rh +ve) was obtained from the Western Province Blood Transfusion Service and used as the in-house laboratory standard. This pool was calibrated against the WHO immunoglobulin reference preparation 67/97 obtained from the Department of Health and Human Services, Atlanta, USA. WHO 67/97 is produced from pooled serum from normal adult donors with no history of malaria or hepatitis (Rowe et al 1970). WHO 67/97 was calibrated (Rowe et al 1972) as a secondary standard against the WHO primary standard for human IgG, IgA and IgM (WHO 67/86). WHO 67/97 reference preparation is widely recognized as a reference for the determination of IgG subclasses. A collaborative study (Klein et al 1985) re-examined the calibration of the IgG subclass concentrations in the WHO 67/97 reference serum using purified

human myeloma IgG proteins in immunodiffusion with polyclonal sheep anti-human IgG subclass antisera. They proposed WHO 67/97 be used as a calibrator for IgG subclasses with the designated values of 5.0 g/l for IgG1, 2.6 g/l for IgG2, 0.4 g/l for IgG3 and 0.5 g/l for IgG4. The concentrations of the 4 subclasses in the WHO 67/97 reference standard received from Geneva or Atlanta vary slightly (Table 2.5). The in-house standard was calibrated with the WHO subclass values received from Geneva. The subclass concentrations of the AB in-house standard were calculated by using 5 dilutions with absorbance readings on the linear section of the curve and taking the mean concentration of them. This was repeated over 12 consecutive runs and the results are shown in Table 2.5. At intervals the in-house standard has been recalibrated but there has been no drift in the concentration of the subclasses.

TABLE 2.5: IgG SUBCLASS VALUES OF THE REFERENCE SERUM POOLS (g/l)

SAMPLES	IgG1	IgG2	IgG3	IgG4
WHO 67/97, Geneva	5.10	2.50	0.55	0.35
WHO 67/97, Atlanta	5.00	2.60	0.40	0.50
In-House AB Standard[a]	9.85	3.31	0.73	0.40

a = AB standard was calibrated from the WHO 67/97 Geneva reference

2.2.7.1 *IgG1 subclass ELISA*

Pooled human AB serum (the in-house standard) and the WHO reference standard serum 67/97 (international standard) were compared. The standard curves were parallel (Figure 2.9). The pooled AB serum was used as the calibrator for the standard curve. The WHO standard was included in two dilutions as a positive control in each assay. Various dilutions of the control and samples were compared for their ability to give reproducible results. Calculated antibody concentrations were similar for dilutions read off any part of the linear section of the curve. Dilutions of 1:15 000 and 1:20 000 were selected for the control samples. The unknown samples were diluted to read off the linear portion of the standard curve. This dilution was 1:50 000 in the majority of samples.

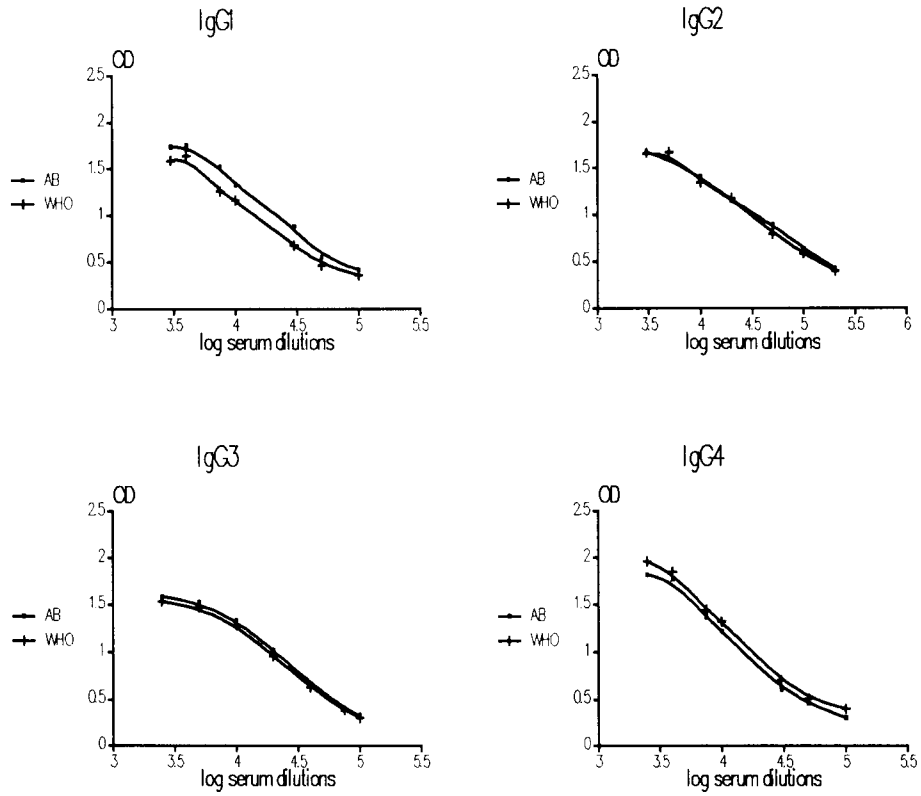


Figure 2.9: Standard curves for IgG1, IgG2, IgG3 and IgG4 using in-house AB serum standard (▪) and WHO serum standard (+).

2.2.7.2 *IgG2 subclass ELISA*

Titration curves of the in-house standard and the international reference standard were parallel (Figure 2.9). Control samples were diluted to 1:30 000 and 1:50 000. The unknown samples were diluted to 1:30 000 (except serum from very young children which was diluted to 1:20 000).

2.2.7.3 *IgG3 subclass ELISA*

Titration curves of the in-house standard and international reference standard were parallel (Figure 2.9) and the control samples were diluted to 1:20 000 and 1:30 000. The unknown samples were diluted to 1:30 000.

2.2.7.4 *IgG4 subclass ELISA*

Titration curves of the standards were parallel (Figure 2.9) and control samples were diluted 1:10 000 and 1:20 000. The unknown samples were diluted to 1:5 000 except samples from very young children which were diluted to 1:1 000.

2.2.7.5 *Conclusion and discussion*

The World Health Organization International Reference Standard for Human Serum Immunoglobulins: IgA, IgG and IgM (WHO 67/97) was used to establish the quantitative basis for the IgG subclass standardization of these assays. Non-standardized materials used to calibrate assays have ranged from purified myeloma proteins to pooled human sera. A large pool of serum, blood group AB (Rh +ve) was used as the in-house standard in these assays and was calibrated from WHO 67/97. WHO 67/97 is an official standard for IgA, IgG and IgM but not for the subclasses. Morell et al (1972a), van der Giessen et al (1975) and Klein et al (1985) independently established the mass concentration of the IgG subclasses in this preparation and these 'standard' values have been used by a number of groups studying

IgG subclasses (Chapter 9, Table 9.6). Use of this reference allows interlaboratory comparison of subclass assays.

2.2.8 Serum Incubation times

2.2.8.1 *IgG1, IgG2, IgG3 and IgG4 subclass ELISAs*

The shapes of the standard curves when the serum was incubated for 2.5 hours at room temperature or overnight at 4°C were parallel but the signal was higher and the curve was shifted to the right with the overnight incubation (Figure 2.10). There was no difference in the minus monoclonal antibody or minus serum blanks between the two incubation periods.

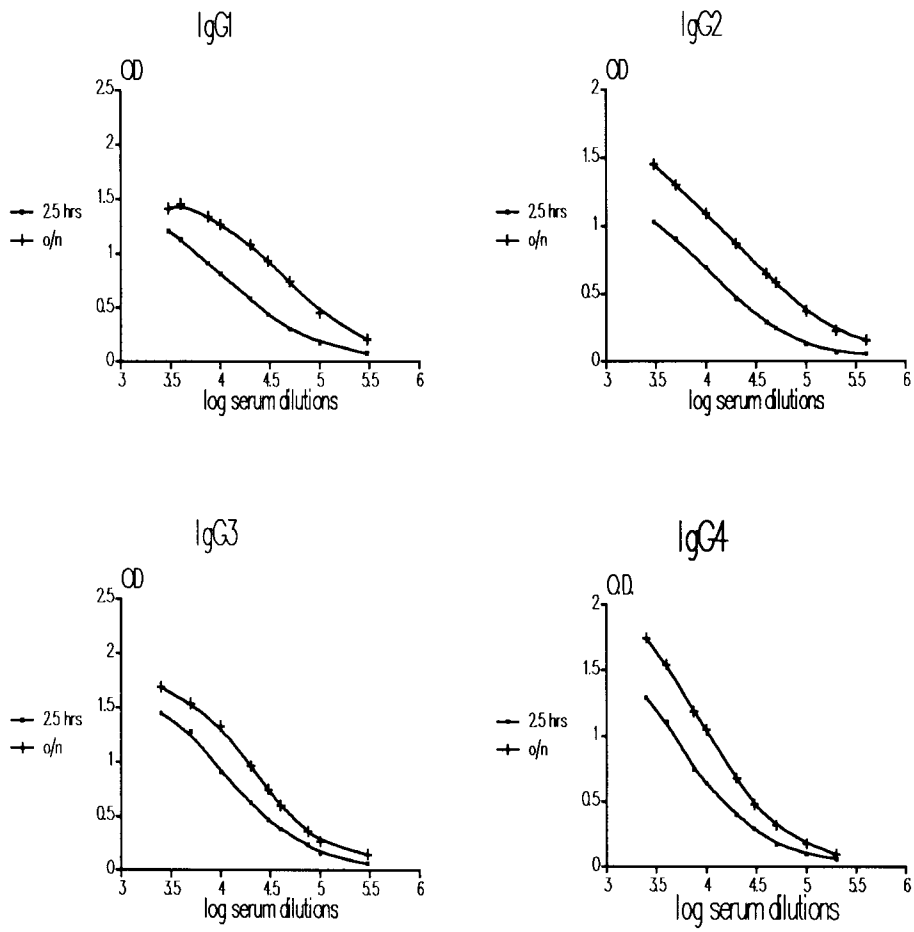


Figure 2.10: Standard curves for IgG1, IgG2, IgG3 and IgG4 using in-house AB serum standard, incubating the serum for 2.5 hours at room temperature (▪) and overnight (o/n) at 4 °C (+).

2.2.8.2 *Conclusion and discussion*

The overnight incubation period at 4°C was chosen as it gave a higher signal and was more convenient.

2.2.9 Evaluation of the Enzyme Detection System

The following conjugated antibodies were evaluated:

- Non-affinity purified goat anti-human gamma chain specific HRPO (Cappel, Catalogue No 3301-0121)
- Affinity purified goat anti-human gamma chain specific HRPO absorbed with mouse serum proteins (Zymed, Catalogue No 62-8420)
- Sheep anti-human IgG (H and L) specific HRPO (Binding Site, Catalogue No PP003).

2.2.9.1 *IgG1 subclass ELISA*

The Zymed goat anti-human conjugate diluted at 1:1 500 in 0.25% BSA/PBST was selected for use in these assays as it gave the lowest minus monoclonal antibody and minus serum blank readings of the conjugates assessed (Table 2.6). The Cappel and Binding Site anti-human conjugates gave high background readings (Table 2.6).

TABLE 2.6: EFFECT OF VARIOUS CONJUGATES ON THE BACKGROUND READINGS IN THE IgG1, IgG2, IgG3 and IgG4 SUBCLASS ASSAYS

SUBCLASS ASSAY	ANTI-HUMAN CONJUGATES[a]	BACKGROUND BLANKS %*	
		Minus Moab	Minus Serum
IgG1	Zymed	6.8	2.0
	Zymed + 1% MS[b]	8.6	2.0
	Binding Site	34.2	33.0
	Binding Site + 1% SS[c]	30.3	31.8
	Cappel	24.2	51.8
IgG2	Zymed	14.7	46.9
	Zymed + 1% MS[b]	14.3	14.3
	Zymed + 1% GS[d]	14.3	46.7
	Binding Site	15.5	12.9
	Binding Site + 1% SS[c]	16.7	13.1
	Cappel	16.1	45.4
	Cappel + 1% MS[b]	3.3	23.8
IgG3	Zymed	7.3	5.3
	Cappel	2.5	5.3
IgG4	Zymed	12.8	16.5
	Zymed + 1% GS[b]	10.6	15.6
	Zymed + 1% MS[b]	12.2	17.4
	Cappel	2.1	9.1
	Cappel + 1% MS[b]	2.8	4.8

a See text for details of conjugates
b 1% MS = addition of 1% mouse serum to the conjugate
c 1% SS = addition of 1% sheep serum to the conjugate
d 1% GS = addition of 1% goat serum to the conjugate
* = OD of Background blanks expressed as a % of the highest OD reading of AB standard

2.2.9.2 *IgG2 subclass ELISA*

The addition of 1% mouse serum to the Zymed goat anti-human HRPO conjugate significantly decreased the minus serum blanks (46.9% to 14.3%) (Table 2.6). The addition of 1% goat serum to the Zymed goat anti-human conjugate did not decrease the high minus serum blank. The addition of sheep serum to the Binding Site sheep anti-mouse conjugate made no difference to minus monoclonal antibody or minus serum blanks. The addition of 1% mouse serum to the Cappel goat anti-human conjugate significantly decreased the high minus serum blanks and decreased the minus monoclonal antibody blanks (Table 2.6). The Zymed goat anti-human HRPO conjugate diluted 1:1 500 in 0.25% BSA/PBST with 1% mouse serum was selected for use in these assays.

2.2.9.3 *IgG3 subclass ELISA*

There was no difference in the minus serum blanks when using either the Zymed or Cappel goat anti-human conjugates (Table 2.6). The minus monoclonal antibody blanks were slightly lower using the Cappel conjugate than with the Zymed conjugate (Table 2.6). The Cappel conjugate could be used at half the concentration of the Zymed conjugate so was more economical. The Cappel goat anti-human HRPO conjugate diluted 1:24 000 in 0.25% BSA/PBST was selected for use in this assay.

2.2.9.4 *IgG4 subclass ELISA*

The Cappel conjugate gave a steeper standard curve and lower minus monoclonal antibody and minus serum blanks than the Zymed conjugate (Table 2.6). The addition of 1% goat serum or 1% mouse serum to the Zymed conjugate made no difference to these background blanks. The addition of 1% mouse serum to the Cappel conjugate lowered the minus serum blanks.

2.2.9.5 *Conclusion and discussion*

Selection of the optimal enzyme conjugated anti-human IgG detection antibody is complicated by the fact that some of the HP series murine monoclonal anti-human IgG subclass antibodies react with sheep or goat immunoglobulin (Jefferis et al 1985). In this study various polyclonal HRPO conjugated anti-human IgG antibodies were assessed with and without the addition of animal sera to decrease non-specific binding. Results obtained in the various assays showed that the Zymed conjugate was suitable for IgG1 and IgG2 assays and that the Cappel conjugate was best for the IgG3 and IgG4 assays. The addition of 1% mouse serum to the conjugates in the IgG2 and IgG4 assays significantly reduced background values. As a result of these experiments the following conjugates and diluents were used in the assays:

IgG1 and IgG2	Zymed goat anti-human HRPO diluted 1:1 500 in 0.25% BSA/PBST (with 1% mouse serum for IgG2)
IgG3	Cappel goat anti-human HRPO conjugate diluted 1:24 000 in 0.25% BSA/PBST
IgG4	Cappel goat anti-human HRPO conjugate diluted 1:12 000 in 0.25% BSA/PBST with 1% mouse serum.

0.25% BSA in PBST was selected as the diluent for the conjugates in all 4 subclasses as it had been used successfully when determining optimal assay conditions. When the conjugates were diluted in 1% casein there was a significant increase in the minus serum blanks.

2.3 METHODS

Optimum reaction conditions and reagents had been determined in preliminary experiments. All reagents and samples were added to the wells in 100 μl volumes except the 2N H_2SO_4 where 50 μl volumes were used. After serum incubation and conjugate incubation respectively plates were washed five times with PBST. A summary of the reagents used in all of the IgG1, IgG2, IgG3 and IgG4 subclass assays is given in Table 2.7. All incubations were in a moist chamber.

2.3.1 IgG1 Subclass ELISA

Microtitre wells were coated with streptavidin (0.5 μg/ml) (Zymed, Catalogue No 43-4301) diluted in PBS. Plates were incubated overnight at 4°C. The contents were discarded and non-specific binding sites were blocked with 250 μl/well of 1% casein for 1 hour at 37°C. Plates were then washed once with PBS and the biotinylated mouse monoclonal antibody (HP6069) diluted 1:1 000 in 0.25% BSA in PBST was added to the wells and incubated for 2 hours at room temperature. Plates were washed once with PBST and sera diluted in 1% casein were added to triplicate wells. Standard curves were generated using the in-house standard of human AB serum in 9 serial dilutions ranging from 1:3 000-1:300 000 (1:3 000, 1:4 000, 1:7 500, 1:10 000, 1:20 000, 1:30 000. 1:50 000, 1:100 000, 1:300 000). Positive controls (International WHO reference standard) were added in two dilutions of 1:15 000 and 1:20 000. Test samples were diluted so as to be read on the linear part of the curve, usually 1:50 000. The samples were incubated overnight at 4°C. Minus serum blanks i.e. wells containing only diluent were included on every plate.

TABLE 2.7: REAGENTS USED IN IgG SUBCLASS ASSAYS

	IgG1	IgG2	IgG3	IgG4
Antibody coating Streptavidin	0.5 μg/ml	Nil	Nil	Nil
Moab	HP6069B*	HP6008:HP6014	HP6010	HP6013
Moab dilution	1:1 000	2:1 @ 1:500	1:500	1:500
Blocking 1% casein	1 hr 37°	o/n at 37°	1 hr 37°	1 hr 37°
Serum dilutions (μg/ml) AB Standard	1:3 000-1:300 000 (0.0328-3.283)	1:3 000-1:400 000 (0.0082-1.10)	1:2 500-1:300 000 (0.0024-0.292)	1:2 500-1:200 000 (0.0020-0.160)
WHO control	1:15 000 (0.255) 1:20 000 (0.340)	1:30 000 (0.050) 1:50 000 (0.083)	1:20 000 (0.018) 1:30 000 (0.028)	1:10 000 (0.018) 1:20 000 (0.035)
Samples	1:50 000	1:30 000	1:30 000	1:5 000
Conjugate	goat α human Zymed 1:1 500	goat α human Zymed 1:1 500 + 1% mouse serum	goat α human Cappel 1:24 000	goat α human Cappel 1:12 000 + 1% mouse serum
Substrate	OPD	OPD	OPD	OPD

*** HP6069B = biotinylated HP6069**

The plates were then washed five times with PBST. Affinity purified goat anti-human IgG gamma chain specific horseradish peroxidase conjugate (Zymed) diluted 1:1 500 in 0.25% BSA in PBST was added to the wells and incubated for 90 minutes at room temperature. After washing 5 times with PBST, OPD diluted in 0.1 M citrate buffer with 0.1% hydrogen peroxide was added and incubated in the dark for 15 minutes at room temperature. The colour reaction was stopped by the addition of 50 μl 2N H_2SO_4 and the OD was read on an ELISA photometer at a wavelength of 492 nm.

2.3.2 IgG2 Subclass ELISA

Microtitre wells were coated with a 2:1 mixture of monoclonal antibodies HP6008 and HP6014 diluted 1:500 in a glycine/Tris buffer pH 7.0-7.5. This was left for 2 hours at room temperature. The contents of the plates were discarded and the plates were blocked with 1% casein overnight at 37°C. The plates were washed once with PBS and dried. Standard AB serum in 9 dilutions ranging from 1:3 000-1:400 000 (1:3 000, 1:5 000, 1:10 000, 1:20 000, 1:40 000, 1:50 000, 1:100 000, 1:200 000, 1:400 000) and positive controls (WHO reference standard) were added in two dilutions (1:20 000 and 1:30 000). Test samples were diluted to read on the linear part of the curve, usually 1:30 000 for adults and 1:10 000 and 1:20 000 for children. The samples were incubated overnight at 4°C. Further steps were as for the IgG1 ELISA.

2.3.3 IgG3 Subclass ELISA

This was similar to the method described above for the IgG2 subclass ELISA except for the following dilution and antibody differences:

- blocking step was at 37°C for 1 hour

- the monoclonal antibody used to coat the plates was an anti-human IgG3 antibody HP6010 diluted 1:500 in glycine/Tris buffer pH 7.0-7.5 and it was left on overnight at 4°C
- the standard curve dilutions of the pooled standard AB serum ranged from 1:2 500-1:300 000 (1:2 500, 1:5 000, 1:10 000, 1:20 000, 1:30 000, 1:40 000, 1:75 000, 1:100 000, 1:300 000).
- Positive WHO controls were diluted to 1:20 000 and 1:30 000 and test samples were diluted to 1:30 000.
- The Cappel affinity purified goat anti-human IgG gamma chain specific horseradish peroxidase was diluted 1:24 000 in 0.25% BSA in PBST.

2.3.4 IgG4 Subclass ELISA

This was similar to the IgG3 subclass ELISAs with the following dilution and antibody differences:

- The mouse anti-human IgG4 monoclonal antibody HP6013 diluted 1:500 in glycine/Tris buffer pH 7.0-7.5 was used to coat the microtitre plates and it was left on overnight at 4°C
- The standard curve dilutions of the pooled human AB serum ranged from 1:2 500-1:200 000 (1:2 500, 1:4 000, 1:7 500, 1:10 000, 1:20 000, 1:30 000, 1:50 000, 1:100 000, 1:200 000)
- Positive WHO controls were diluted 1:10 000 and 1:20 000 and test samples were diluted to 1:5 000 for adults and 1:1 000 for children
- Cappel goat anti-human peroxidase conjugated antibody was diluted 1:12 000 in 0.25% BSA in PBST with 1% mouse serum.

2.3.5 Design of Plate

Every assay plate included 9 dilutions of the standard pooled human AB serum for the calibration curves, dilutions of the WHO International

Standard for monitoring reproducibility and blanks (wells minus monoclonal antibody, wells minus serum and wells minus both monoclonal antibody and serum). All reactions were performed in triplicate. For the routine analysis of unknown samples the design of the plate is the same for each subclass ELISA and is shown in Chapter 1, Figure 1.1. In these assays the blanks in column 10, rows A-D and are as follows:

Column 10: Row A-C: Minus serum, plus monoclonal antibody

Column 10: Row D: Minus serum, minus monoclonal antibody

2.3.6 Calculation of Results

The minus monoclonal antibody blank and the minus serum blank were subtracted from the mean of the triplicate of the standard/sample dilutions. Standard curves relating absorbance to the concentration of the 4 subclasses in the AB serum standard were constructed using the curve fitting four parameter logistic programme (Chapter 1.12).

2.4 ASSAY STANDARDIZATION

2.4.1 Standard Curves

An in-house standard of pooled AB serum quantitated against an International WHO standard 67/97 was used to construct standard curves for IgG1, IgG2, IgG3 and IgG4 subclasses relating absorbance to subclass concentration (Figure 2.9). Each standard curve was constructed from nine different triplicate dilutions of standard serum on each plate and included in every ELISA plate. The titration curves of the in-house standard and WHO International Standard were parallel for all four subclasses (Figure 2.9).

The concentrations of immunoglobulin used to construct a standard curve for IgG1 = 0.033-3.283 μg/ml; IgG2 = 0.008-1.100 μg/ml; IgG3 = 0.0024-0.292 μg/ml and IgG4 = 0.0020-0.160 μg/ml.

2.4.2 Precision and Sensitivity

2.4.2.1 *Coefficients of variation*

To determine assay precision, the coefficient of variation (CV) of assay results of a single sample was determined using one assay plate (intra-plate) or multiple plates in the same run (intra-assay) or 3 samples on different runs (inter-assay).

The inter-assay variation was assessed with 3 samples assayed in triplicate in 16 different runs. The sera selected were the standard WHO control and 2 random samples one of which had low total IgG. The CV ranged from 3.6 to 18.0% with a mean of 8.75% (Table 2.8).

The intra-assay variation was obained for the WHO standard serum. These samples were assayed 14 times in one run and the coefficient of variation ranged from 6.0 to 8.1% with an average of 6.93% (Table 2.8).

2.4.2.2 *Running means*

If the values of the controls inserted in each run fell outside the 2 SD range, the run was repeated. Running means of these controls were calculated to detect trends. Mean values were calculated and added onto cumulative data. The running means calculated in this way were plotted and the variation from the value of the control observed.

TABLE 2.8: EVALUATION OF IgG SUBCLASS ASSAY PRECISION

	IgG1[a]			IgG2[a]			IgG3[a]			IgG4[a]		
	Mean	**SD**	**CV**	**Mean**	**SD**	**CV**	**Mean**	**SD**	**CV**	**Mean**	**SD**	**CV**
INTER-ASSAY PRECISION[b,c]												
WHO	5.336	0.193	3.6	2.46	0.153	6.2	0.533	0.024	4.5	0.362	0.020	5.5
Sample A	8.87	1.300	14.7	3.46	0.457	13.0	0.730	0.070	9.5	0.400	0.030	7.5
Sample B (low titre)	0.77	0.108	14.0	0.15	0.023	15.3	0.790	0.007	8.9	0.010	0.002	18.0
INTRA-ASSAY PRECISION[d]												
WHO	4.78	0.36	7.6	2.48	0.20	8.1	0.540	0.03	6.0	0.329	0.019	6.0
INTRA-PLATE PRECISION[e]												
WHO	5.1	0.20	3.9	2.5	0.16	6.4	0.49	0.26	5.0	0.340	0.17	5.0

a Mean and standard deviation (SD) in mg/ml and coefficient of variation (CV) in percent
b This was taken from plates assayed over 16 consecutive weeks
c Average CV's = 8.75%
d Average CV's = 6.93%
e Average CV's = 5.08%

2.4.2.3 *Correlation with total IgG*

The prerequisite of a reliable IgG subclass determination is its correlation to the total IgG value. Using the data of the controls, a linear regression analysis was used to estimate the relationship between the sum of the subclasses and total measured IgG. The four subclasses and total IgG were quantitated in 937 samples. In each case the values for the IgG subclasses were summed to obtain the calculated total concentration which was then compared with results from an accepted standardized method of total IgG measurement by nephelometry. A scatter plot of calculated versus measured IgG values showed a highly significant correlation (r = 0.90) between measured total IgG and the sum of the IgG subclasses when applying Pearson's correlation coefficient estimation (Figure 2.11).

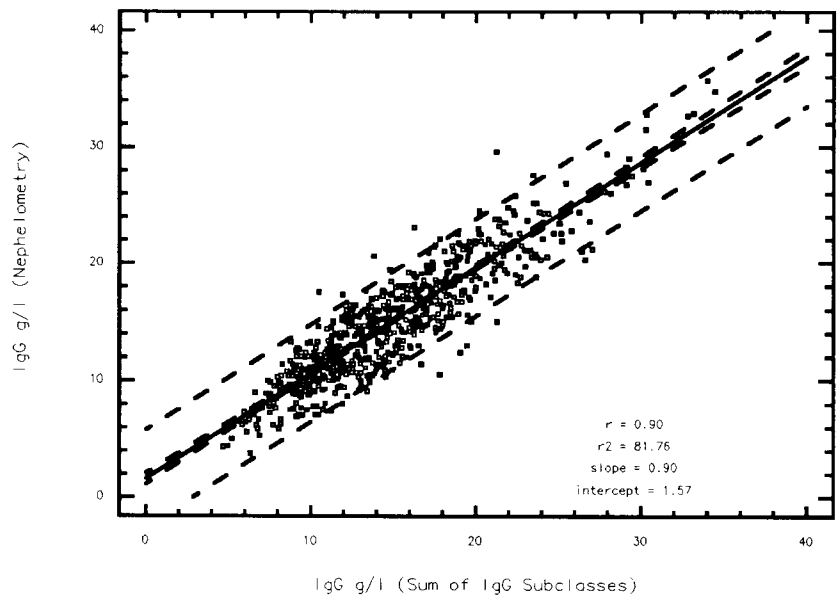


Figure 2.11: Regression analysis of IgG measured by nephelometry on the sum of the four IgG subclasses measured by ELISA

Standard deviations about the regression line between the 'gold standard' method (as y) and the new technique (as x) provides information about the range of true values which are consistent with a particular reading obtained by the new technique. When this is done on these data (Figure 2.11) it can be seen that most readings fall within the $\pm$ 2 SD range about the regression line. It is also noted that for a unit increase in 'x' there is not a unit increase in 'y'. At low IgG levels, the IgG measured by nephelometry is greater than the IgG obtained from the sum of the four subclasses whereas at high levels the opposite is true. The intercept does not go through the origin and is calculated as 1.57.

When IgG was calculated as the sum of the IgG subclasses, 45.7% of the samples had higher value than when IgG was measured by nephelometry. Although the slope of the regression line between measured and calculated total IgG concentrations of the different sera was 0.90 (Figure 2.11) there was a slight positive bias. This may indicate that the calculated value includes the errors of four assays or simply that the total IgG and IgG subclasses are measured with different assay methods.

In these experiments this positive bias may have been eliminated if an ELISA was used to measure total IgG and if the monoclonal antibodies used for the IgG subclasses and total IgG assays were of the same murine isotype to ensure comparable reaction with the secondary antibody.

2.4.2.4 *Differences between the sum of the IgG subclasses and the total IgG*

It has been argued that correlation coefficients should not be used to compare two methods of measurements as this measures the strength of the relationship between them and not the agreement between them. It would be expected that the data would be highly correlated (Bland and Altman 1986).

To assess agreement between methods, differences between the pairs of data can be plotted against the means of the pairs (Figure 2.12) (Bland and Altman 1986). If the mean of the differences is zero then there is perfect agreement between the methods. In Figure 2.12 the central horizontal line corresponds to the mean difference and the two outer horizontal lines correspond to plus or minus two standard deviations. If the differences have a Normal distribution, 95% of the differences will be between these limits. The measurements themselves do not have to follow a Normal distribution and in the case of the IgG subclasses, do not. The histogram of the differences of these data has a Normal distribution. The mean difference between the IgG measured by nephelometry and the sum of the subclasses is 0.375 (IgG nephelometry > subclasses summed). The differences are distributed fairly symmetrically about the mean difference.

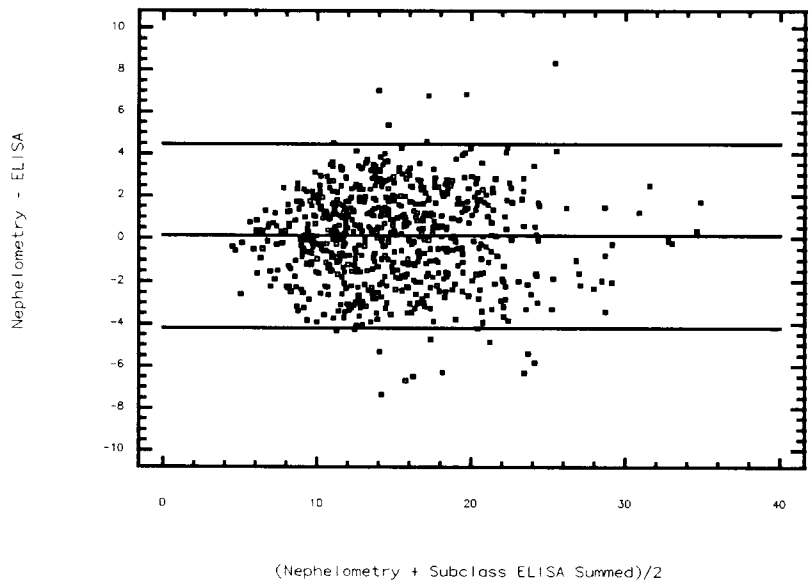


Figure 2.12: The difference between the IgG measured by nephelometry and the total IgG calculated by the sum of the four subclasses (ELISA) is plotted against the mean of the measured and calculated IgG. The central horizontal line corresponds to the mean difference and the two outer horizontal lines correspond to the mean difference + and - 2 SD (standard deviations)

The correlation between pair differences and pair means provides a comparison of the variances of the IgG measured by both methods. This correlation ($r = 0.03$; $p = 0.60$) indicated no significant difference between the variances of the two methods used to determine IgG levels.

2.4.3 Quality Control

2.4.3.1 *Inter-laboratory Correlations*

The results of 15 specimens measured in this laboratory using IgG subclass ELISAs described here correlated (r: IgG1 = 0.90; IgG2 = 0.93; IgG3 = 0.63 and IgG4 = 0.75) with the results obtained on the same 15 specimens measured by Dr Rabson in the Department of Pathology, Boston, using an ELISA method (Black et al 1988).

2.4.3.2 *Quality assurance scheme*

This laboratory participates in an international quality control programme run by the Binding Site, Birmingham, United Kingdom. A sample is sent from Binding Site 5-6 times a year. Results of the sample measured in this laboratory are forwarded to Binding Site within a given time and once the results of all the participants have been analysed they are returned to the participating laboratories. Results of 7 runs participated in since May 1992 are given in Table 2.9 and it can be shown that the IgG subclass results from these assays are accurate.

TABLE 2.9: COMPARISON OF RESULTS OF IgG SUBCLASSES MEASURED IN THIS LABORATORY AND THE QA* SCHEME

Binding Site Sample No	Assay	IgG1 g/l	IgG2 g/l	IgG3 g/l	IgG4 g/l
6/Q106	Laboratory	4.63	2.47	0.41	0.29
	QA Scheme	4.68	2.74	0.45	0.30
7/Q107	Laboratory	8.74	1.20	1.20	0.35
	QA Scheme	8.26	1.66	1.60	0.40
8/Q108	Laboratory	Not done	2.19	0.39	0.33
	QA Scheme	Not sent	2.18	0.37	0.33
9/Q109	Laboratory	7.17	2.50	0.49	0.44
	QA Scheme	5.63	3.0	0.56	0.43
10/Q110	Laboratory	5.99	1.77	0.41	0.29
	QA Scheme	5.24	2.78	0.49	0.31
11/Q111	Laboratory	27.52	0.84	0.13	0.05
	QA Scheme	20.45	1.05	0.15	0.05
12/Q112	Laboratory	5.98	3.35	0.45	0.22
	QA Scheme	5.12	3.63	0.49	0.22

QA* = quality assurance

2.5 SUMMARY

The importance of IgG subclass determinations in clinical practice is generally accepted, but the choice of methods for their determination remains controversial. Although numerous kits are commercially available, they are very expensive and studies have shown them not to be fully reliable (Meissner et al 1990; Aucouturier et al 1992b). RID kits using monoclonal

antibodies lack accuracy and show poor interlaboratory and interbatch reproducibility (Aucouturier et al 1992b). In addition these IgG2 ELISA kits all use the monoclonal antibody HP6014 which has a preference for binding λ rather than k IgG2. The RID kits using polyclonal antibodies are not specific particularly for IgG2. The methods developed in this study using a capture non-competitive ELISA system have proved to be quantitative, sensitive and allow reproducible measurement of IgG subclasses. In this study a panel of antibodies that have been well characterized were selected from the IUIS/WHO Report (Jefferis et al 1985). These commercially available monoclonal antibodies provide consistency and specificity not possible with polyclonal reagents and offer easy availability and standardization for comparison with other laboratories. The ELISA routinely includes in-house and international controls and has been standardized against the WHO International Serum for IgG, IgA and IgM (WHO 67/97). Such standardization with a universally available reference provides the basis for uniform calibration and permits inter-laboratory comparsions.

The ELISAs for IgG1, IgG2, IgG3 and IgG4 are similar assays in terms of methodology but different in terms of specific details (reagents, performance, serum dilutions).

In the IgG1 assay a great improvement in the attachment of the IgG1 antibody to the plastic plates was obtained when the plates were coated with streptavidin followed by biotinylated monoclonal antibody.

In the IgG2 subclass assays, prolonged serum incubation overnight resulted in an improved signal. Improved binding of the monoclonal antibodies was

observed when a glycine/Tris buffer pH 7.0-7.5 was used instead of the more conventional carbonate buffer pH 9.6.

The IgG2 monoclonal antibody HP6014 is a high affinity antibody commonly used in IgG2 measurement despite the fact that it binds IgG2 λ in preference to IgG2 k (Aucouturier et al 1992a). HP6014 may give false results and will over- or under-estimate the IgG2 depending on the λ:k ratio in the serum. The IgG2 antibody HP6008 did not perform well in IgG2 ELISA. However, a combination of HP6008:HP6014 in a 2:1 mixture performed well and overcame the problems of inaccurate measurement using HP6014 alone.

Ten microlitres of serum is sufficient to measure all four subclass levels. This microsample volume is especially desirable in testing children's sera.

Furthermore, these ELISAs are suitable for the accurate quantitation of IgG subclasses in children's sera where low levels are to be expected. The sensitivity of these assays varies from 0.010 g/l to 30 g/l depending on the assay system used. Working ranges (ng/ml) for IgG subclass detection are: IgG1 = 32.8 - 3283 using HP6069; IgG2 = 8.2 - 1110 using a 2:1 mixture of HP6008 and HP6014; IgG3 = 2.4 - 292 using HP6010 and IgG4 = 2.0 - 160 using HP6013. Inter- and intra-assay precision was within acceptable limits for clinical immunoassays and quality control studies with other laboratories (Binding Site and Boston) showed good correlations.

There was good agreement between the sum of the four IgG subclasses measured by ELISAs and total IgG measured by nephelometry.

CHAPTER 3

TETANUS TOXOID IMMUNOASSAYS

The measurement of tetanus toxoid (TT) has long been used to evaluate specific humoral immune responses. This chapter describes in detail the methodology involved in establishing the optimal conditions and reagents for IgG, IgG1 and IgG4 tetanus toxoid ELISAs. With the use of a chaotropic agent it was possible to measure the antibody affinity of IgG1 and IgG4. These assays were applied to assess specific antibody responses to tetanus toxoid in controls and patients. This chapter is subdivided into the following sections:

3.1 INTRODUCTION

3.2 DETERMINATION OF OPTIMAL ASSAY CONDITIONS

3.3 METHODS

3.4 ASSAY STANDARDIZATION

3.5 ANTIBODY AFFINITY

3.6 SUMMARY

3.1 INTRODUCTION

Immunisation with tetanus toxoid has been used for many years to evaluate the ability to mount a specific humoral immune response (Knutsen et al 1981; Litwin 1981; WHO Scientific Group 1983).

In 1890 Von Behring and Kitasto originally demonstrated tetanus toxoid antibodies using toxin neutralisation tests in mice and guinea pigs (Farzad et al 1986). This test measures the ability of anti-tetanus antibodies to protect mice from a standard innoculum of tetanus toxin. Subsequently numerous methods have been developed for the quantitation of anti-tetanus antibodies including passive agglutination (Barr et al 1975; Wang et al 1982), reversed rocket immunoelectrophoresis (Höyeraal et al 1975), latex agglutination (De Saint Martin et al 1975), radioimmunoassay (Repetti and Gill 1980) and ELISA (Hagenaars et al 1984; Virella et al 1985).

The sensitivity, accuracy and reproducibility of these assays for tetanus antibodies vary widely. For example, the latex agglutination assay was proposed as a screening test but only detects sera with high antibody titres (Booth and Nuttall 1978). Quantitative immuno-electrophoretic assays are relatively simple and reproducible, but not very sensitive (Höyeraal et al 1975). Haemagglutination methods are simple to perform, sensitive but imprecise (Virella et al 1985).

Radioimmunoassays and ELISAs are sensitive, reproducible and easy to perform. In addition ELISAs are safe, rapid and relatively inexpensive. ELISAs are also capable of detecting tetanus antibodies of all the specific immunoglobulin classes and subclasses (French and Harrison 1985; Dengrove et al 1986; Rubin et al 1986). The ELISA detects IgG antibodies, which contain all the toxin-neutralizing activity and titres are often higher

than neutralization assays particularly in sera taken from people shortly after immunization (Hagenaars et al 1984).

The affinity of an antibody for its corresponding antigen has been shown to be an important determinant of the biological efficacy of that antibody and may play a major role in immune processes in man. Excessive production of low affinity antibody has been considered to be an expression of relative immunodeficiency (Soothill and Steward 1971). High affinity antibodies have been shown to be biologically more effective than low affinity antibodies (Steward 1981). If this is true a predominantly low affinity antibody response may be an important factor in increased susceptibility to infection. Analysis of the affinity of the subclass type of IgG antibody produced after immunization with tetanus toxoid has shown that high levels of IgG4 antibodies are associated with an overall low affinity response to tetanus toxoid whereas high levels of IgG1 antibodies are associated with high affinity responses (Devey et al 1985; 1987; 1988).

The use of chaotropic agents in ELISA methods has been shown to have an effect on the dose response curve, producing a marked shift to the left by antibodies produced early in the immune response (generally accepted as being of low affinity) but having less effect on high affinity antibodies (Inouye et al 1984; Pullen et al 1986).

The purpose of establishing an assay to quantitate IgG and IgG subclass antibodies to tetanus toxoid was to evaluate the capacity to generate antibody responses to protein antigens in children of different ages and to differentiate between high and low affinity antibody production.

3.2 DETERMINATION OF OPTIMAL REAGENTS AND CONDITIONS

The assay procedures described later (Chapter 3.3) resulted from a process of trial and error. Various technical factors and test conditions affecting the performance of the assays were assessed initially.

3.2.1 Plates

Various 96 well polystyrene and polyvinyl chloride plates were compared for these immunoassays. These included: Nunc round bottomed plates (Weil, Denmark, Catalogue No 1-63320), Nunc flat bottomed plates (Weil, Denmark, Catalogue No 4-42404), Falcon microtest flexible plastic (PVC) round bottomed (Catalogue No 3911) and flat bottomed (Catalogue No 3912) plates.

3.2.1.1 *IgG tetanus toxoid ELISA*

In the IgG assay (Figure 3.1) no significant differences were found between Nunc and Falcon flat bottomed plates or Falcon round bottomed plates. At the higher coating concentrations of tetanus toxoid (1.0 Lf/ml and 4.0 Lf/ml) Nunc round bottomed plates gave similar shaped standard curves to the other 3 plates. However, at the more dilute concentrations the shapes of the standard curves were flatter (Figure 3.1 and 3.4). Nunc flat bottomed plates were selected with a coating concentration of 0.4 Lf/ml of tetanus toxoid.

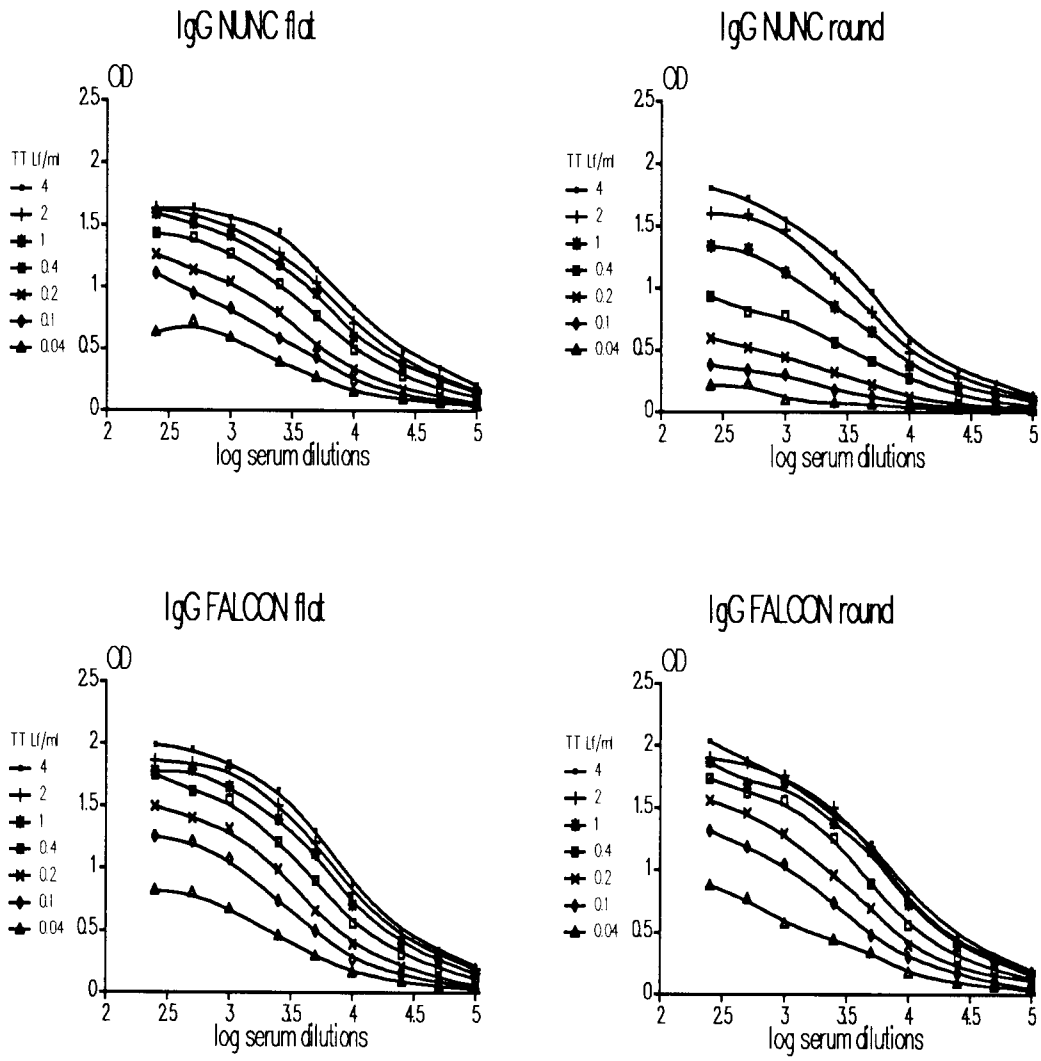


Figure 3.1: Standard curves of IgG tetanus toxoid ELISA comparing Nunc flat and round bottomed and Falcon flat and round bottomed plates, using coating concentrations of tetanus toxoid ranging from 4 to 0.04 Lf/ml: 4 Lf/ml (•), 2 Lf/ml (+), 1 Lf/ml (*), 0.4 Lf/ml (▪), 0.2 Lf/ml (x), 0.1 Lf/ml (♦) and 0.04 Lf/ml (▲)

3.2.1.2 *IgG1 tetanus toxoid ELISA*

The trends were identical to those seen in the IgG tetanus toxoid ELISA (Figure 3.1). Again flat bottomed Nunc plates were selected with a coating concentration of 0.4 Lf/ml of tetanus toxoid (Figure 3.2 and 3.4).

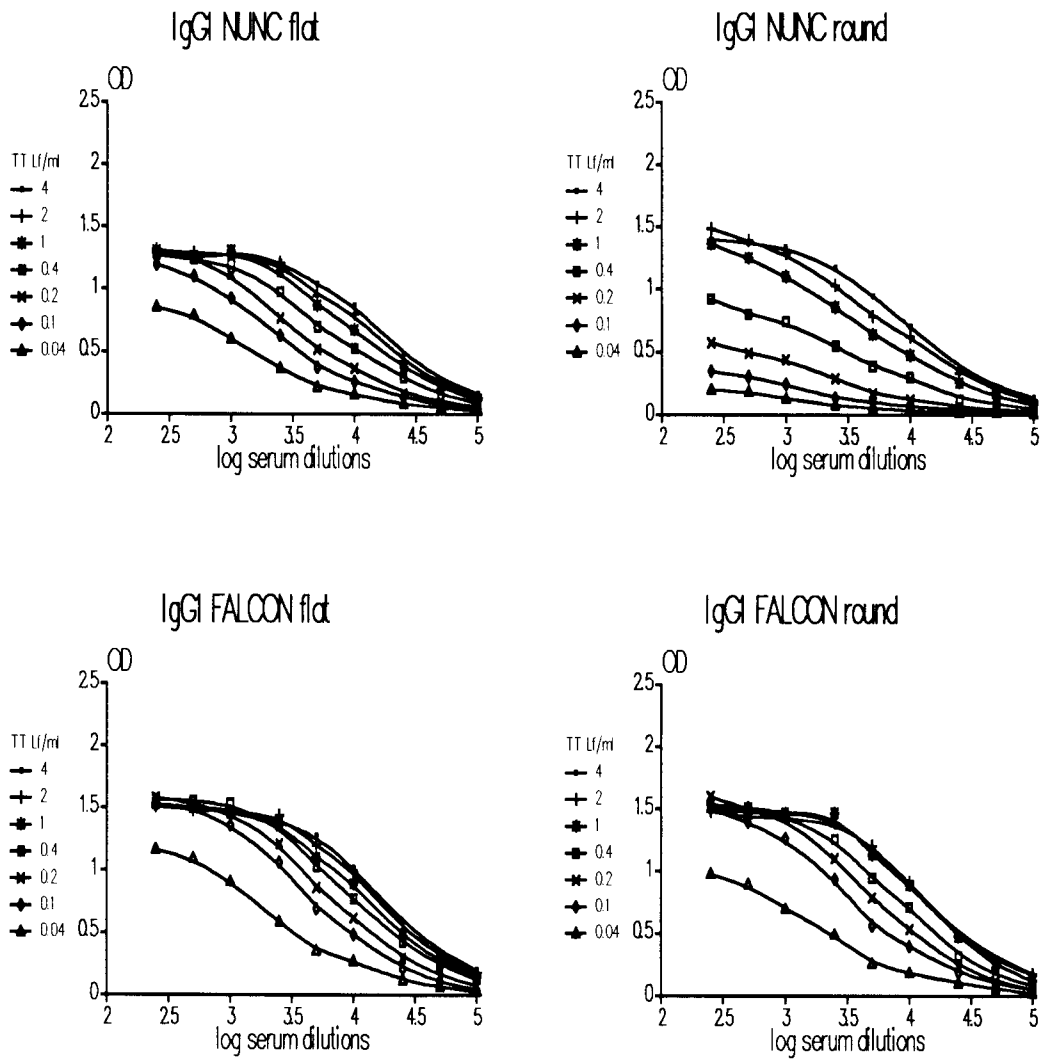


Figure 3.2: Standard curves of IgG1 tetanus toxoid ELISA comparing Nunc flat and round bottomed and Falcon flat and round bottomed plates using coating concentrations of tetanus toxoid ranging from 4.0 to 0.04 Lf/ml: 4 Lf/ml(•), 2Lf/ml (+), 1 Lf/ml (*), 0.4 Lf/ml (▪), 0.2 Lf/ml (x), 0.1 Lf/ml (♦) and 0.04 Lf/ml (▲).

3.2.1.3 *IgG4* tetanus toxoid *ELISA*

The only difference in these assays was that the shapes of the standard curves coated with dilute (0.4 Lf/ml - 0.04 Lf/ml)) tetanus toxoid were much flatter on all plates (Figure 3.3). As with the IgG tetanus toxoid and IgG1 tetanus toxoid ELISA the Nunc flat bottomed plates were selected with a

coating concentration of 0.4 Lf/ml of tetanus toxoid. Nunc round bottomed plates were unsuitable.

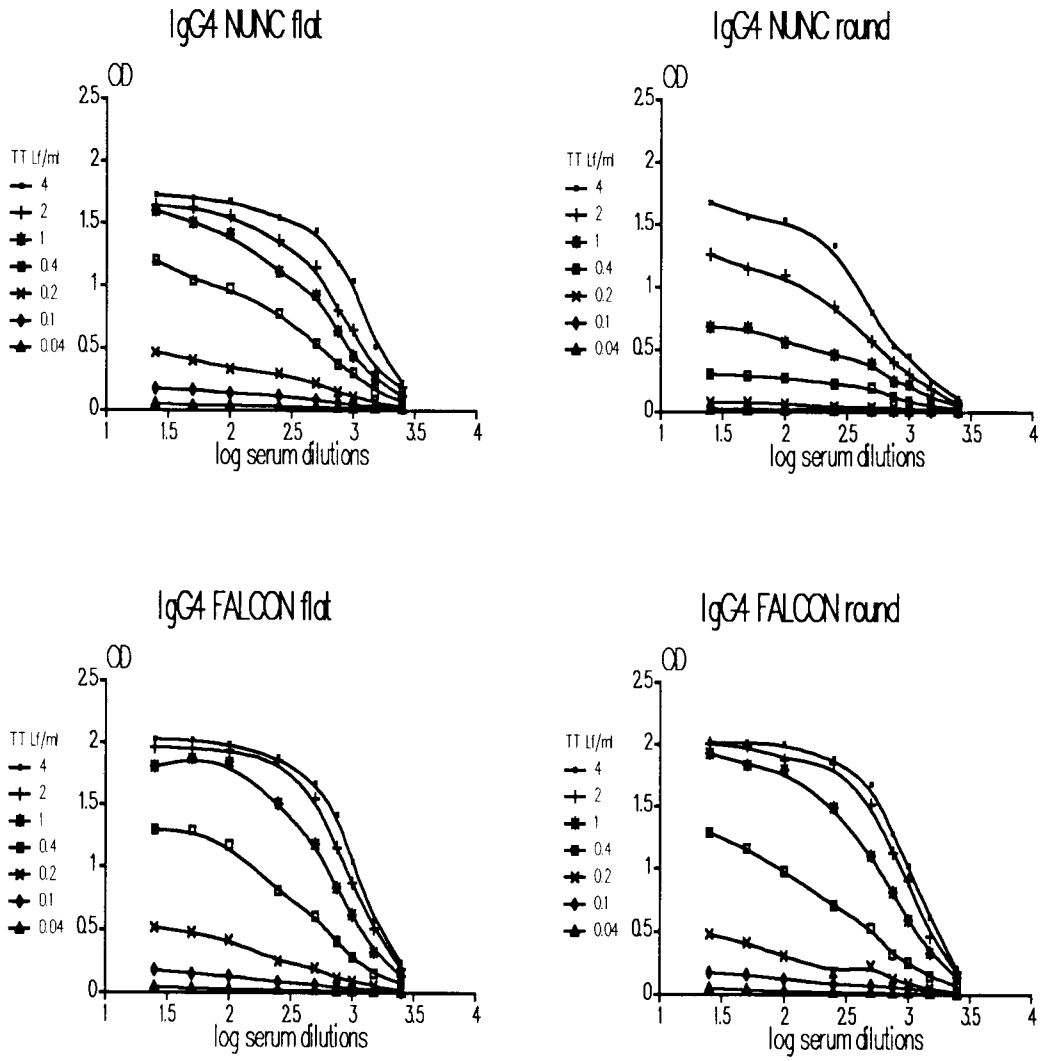


Figure 3.3: Standard curves of IgG4 tetanus toxoid ELISA comparing Nunc flat and round bottomed and Falcon flat and round bottomed plates using coating concentrations of tetanus toxoid, ranging from 4.0 to 0.04 Lf/ml: 4 Lf/ml, (•) 2 Lf/ml (+), 1 Lf/ml (*), 0.4 Lf/ml (▪), 0.2 Lf/ml (x), 0.1 Lf/ml (♦) and 0.04 Lf/ml (▲).

3.2.1.4 *Conclusion and discussion*

In previously reported ELISAs measuring IgG and IgG subclass specific antibodies various plates have been used viz, Dynatech (Dengrove et al 1986; Farzad et al 1986; Rubin et al 1986), Limbro (Sedgwick et al 1983),

Flow (Hagenaars et al 1984; Devey et al 1987; 1988), Nunc (French and Harrison 1985), and Nunc flat bottomed (Lau 1987; Simonsen et al 1987). The authors do not specify if other plates were assessed in these assays and why those particular plates were selected. Data from this study indicates that the type of plate used may affect the sensitivity and results of the assay. The Nunc flat bottomed plates were chosen for use in further assays as these plates produced steeper standard curves at optimum antigen coating concentrations (0.4 Lf/ml tetanus toxoid) than the Nunc round bottomed plates (Figure 3.4). The flat bottomed Nunc plates were selected for use over the Falcon plates as they were easier to load without splashing, readily available and much cheaper (1/5 of the cost). It was also more convenient to use the same plates in all the tetanus toxoid ELISAs.

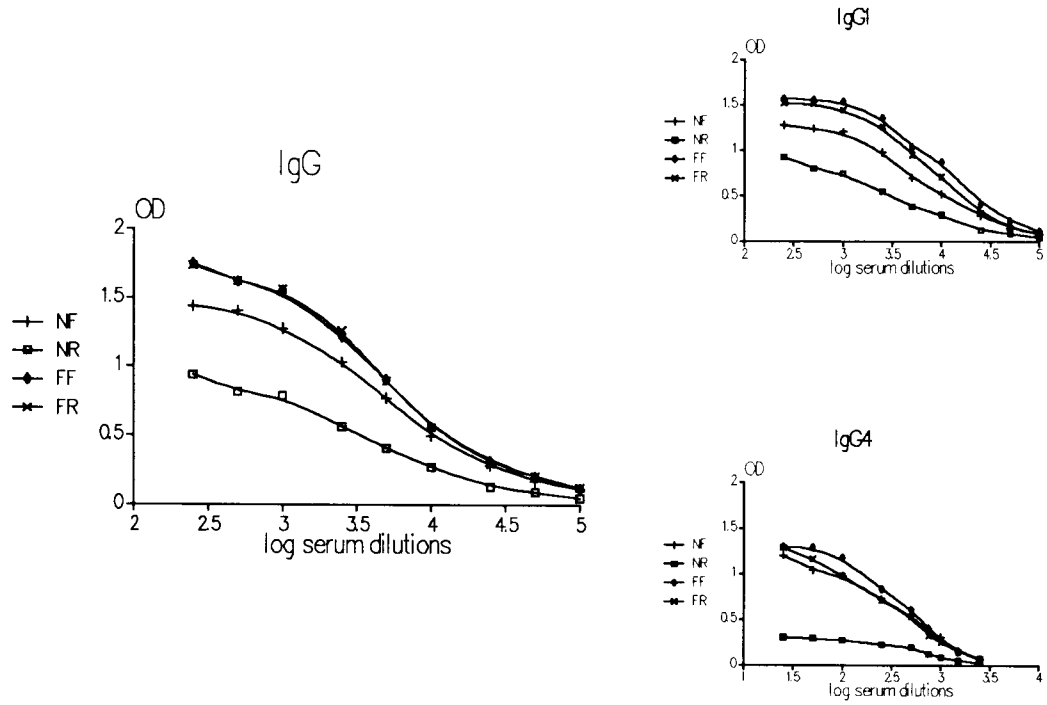


Figure 3.4: Comparison of standard curves of IgG, IgG1 and IgG4 tetanus toxoid ELISAs using various microtitre plates. The plates used were Nunc flat (NF) (+) or Nunc round (NR) (□) bottomed polystyrene plates and Falcon flat (FF) (♦) or Falcon round (FR) (x) bottomed PVC plates. The plates were coated with 0.40 Lf/ml tetanus toxoid antigen

3.2.2 Antigen Coating

The coating antigen used in these assays was purified tetanus formol-toxoid (20 Lf/ml) preserved with 0.01% thiomersal produced by the South African Institute of Medical Research (SAIMR). All dilutions of coating antigen were made in carbonate buffer pH 9.6

3.2.2.1 *IgG tetanus toxoid ELISA*

Maximal coating was obtained between 4.0 and 0.4 Lf/ml of tetanus formol-toxoid (Figure 3.1). The concentration of 0.4 Lf/ml was selected for the assays. No difference was found between coating the plates overnight at 4°C or for 1½ hours at 37°C. The standard curve of a plate stored at 4°C for a week in an air tight container shifted to the left when compared to a freshly coated plate (Figure 3.5). Thus all plates were coated just prior to use.

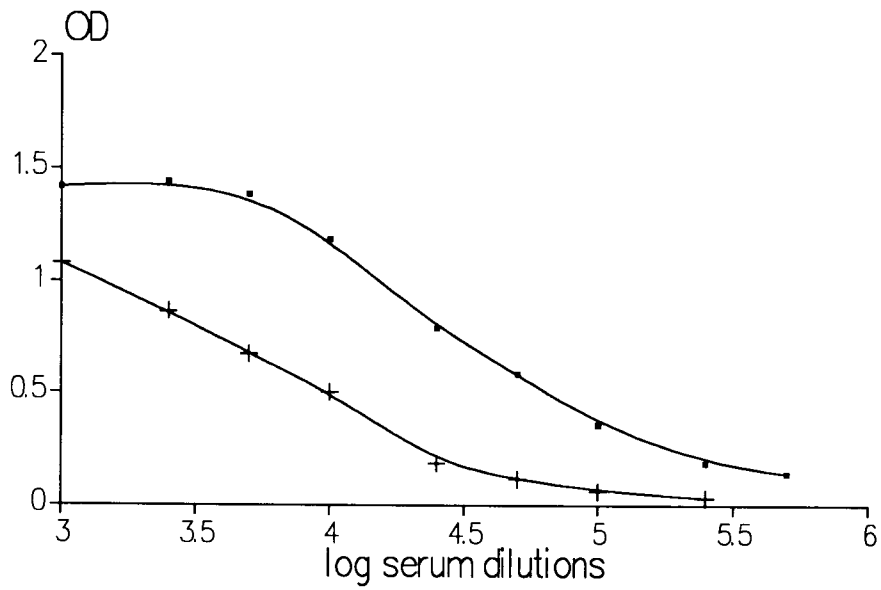


Figure 3.5: Comparison of standard curves of IgG tetanus toxoid ELISA using plates coated with antigen just prior to use (▪) and plates coated and stored for 1 week prior to use (+)

3.2.2.2 *IgG1 tetanus toxoid ELISA*

As in the IgG tetanus toxoid ELISA a coating concentration of 0.4 Lf/ml tetanus toxoid was selected for these assays (Figure 3.2).

3.2.2.3 *IgG4 tetanus toxoid ELISA*

A coating concentration of 0.4 Lf/ml was selected for these assays (Figure 3.3).

3.2.2.4 *Conclusion and discussion*

The antigen most commonly used in reported ELISAs has been either tetanus toxoid vaccine at coating concentrations between 5.0 - 0.2 Lf/ml (Sedgwick et al 1983; Hagenaars et al 1984; French and Harrison 1985; Rubin et al 1986; Lau 1987) or affinity purified tetanus toxoid at a concentration of 5-10 μg/ml (Farzad et al 1986; Devey et al 1988).

Hagenaars et al (1984) found that by decreasing the tetanus toxoid coating concentration in the ELISA from 5.0 to 0.2 Lf/ml a better correlation was obtained between ELISA results and bioassay titres.

In this study the standard curves of IgG, IgG1 and IgG4 showed little change in the shape of the curves when the plates were coated with concentrations of tetanus toxoid ranging from 4.0-0.4 Lf/ml (Figures 3.1, 3.2 and 3.3).

Decreases in toxoid concentration below this resulted in flattening of the standard curve and unacceptably low assay absorbances. Tetanus formol-toxoid vaccine at a coating concentration of 0.4 Lf/ml was selected for the IgG, IgG1 and IgG4 assays described here.

Coating times for tetanus toxoid range from 1 hour at 37°C to overnight at 4°C, the latter being the most commonly employed (Sedgwick et al 1983; Hagenaars et al 1984; French and Harrison 1985; Dengrove et al 1986; Farzad et al 1986; Lau 1987; Devey et al 1988). Voller and Bidwell's (1986) general guide to antigen coating of overnight incubation at 4°C was followed. The results showed that this period was satisfactory as well as being convenient. Although there are reports that sensitized plates can be stored for time intervals ranging from 2 weeks (Lau 1987; Devey et al 1988) to 1 year (Farzad et al 1986) without loss of sensitivity, experiments performed here were unable to reproduce this (Figure 3.5).

3.2.3 Blocking Agents

Various blocking agents were compared in these assays to prevent non-specific binding to the microtitre plates:

- Gelatin - (Biorad (EIA grade), Catalogue No 170-6537) in PBS
- Casein - (Centrolab, SAARCHEM Catalogue No 152813) in TBS
- BSA - Albumin Fraction V, (Boerhinger Mannheim, Catalogue No 735086) in PBS

3.2.3.1 *IgG tetanus toxoid ELISA*

Casein 1% was selected as the blocking agent and serum diluent as it gave the lowest uncoated well blanks without altering the capability for detecting antibody (Table 3.1). 1% gelatin and 2% BSA both gave high minus antigen blank readings (Table 3.1).

TABLE 3.1: EFFECT OF BLOCKING AGENTS ON THE 'MINUS ANTIGEN' WELL BLANKS OF THE IgG, IgG1 and IgG4 TETANUS TOXOID ASSAYS

	IgG			IgG1			IgG4		
Blocker	**Maximum OD[a]**	**Minus Antigen Blank OD**	**(%)[b]**	**Maximum OD[a]**	**Minus Antigen Blank OD**	**(%)[b]**	**Maximum OD[a]**	**Minus Antigen Blank OD**	**(%)[b]**
1% casein	1.572	0.046	(2.9%)	1.510	0.015	(1.0%)	1.731	0.012	(0.7%)
2% BSA	1.469	0.551	(37.5%)	1.371	0.403	(29.4%)	1.919	1.750	(91.2%)
1% gelatin	1.616	0.247	(15.3%)	1.980	0.311	(15.7%)	1.20	0.120	(10%)

a Optical density of the most concentrated standard

b Minus antigen well blanks as a percentage of the most concentrated standard

3.2.3.2 *IgG1 tetanus toxoid ELISA*

1% Casein was selected as the blocking agent and serum diluent for the same reasons (Table 3.1).

3.2.3.3 *IgG4 tetanus toxoid ELISA*

Very high minus antigen well blanks (91.2%) were found when BSA was used and 1% casein gave the best results (Table 3.1).

3.2.3.4 *Conclusion and discussion*

In previous reports gelatin (1%) (Rubin et al 1986; Devey et al 1988) and BSA (0.1% to 2%) (French and Harrison 1985; Farzad et al 1986; Simonsen et al 1987) were used as blocking agents for IgG and IgG subclass specific tetanus toxoid assays. In other reports the blocking step has been omitted and the sera have then been diluted in 5% BSA (Hagenaars et al 1984), 5% FCS (Lau 1987), 1% casein (Devey et al 1987) or simply in PBS buffer with 0.05% Tween 20. Comparison of the effectiveness of blocking agents in the ELISAs in these reports is not possible as the values of blank readings with the various blockers were not documented.

In the present assays 1% casein in TBS was selected as the blocking agent. It was more effective as a blocker when compared to 1% gelatin although the latter was considered acceptable. 2% BSA was unacceptable as a blocker as in all the assays the minus antigen well blanks were high ($\geq$ 29% of the maximum signal). Casein is also the cheapest agent.

3.2.4 Choice of Serum Standards and Controls

Normal pooled human immunoglobulin and hyperimmune human anti-tetanus immunoglobulin (125 IU/ml) both supplied by Natal Blood Transfusion Service (NBTS) were evaluated as standards.

3.2.4.1 *IgG tetanus toxoid ELISA*

In the IgG tetanus toxoid ELISA hyperimmune anti-tetanus immunoglobulin shifted the curve to the right compared to the normal immunoglobulin (Figure 3.6). Hyperimmune anti-tetanus immunoglobulin was selected as the calibrator for the standard curve as it could be used in greater dilutions which resulted in lower uncoated well blank readings. The normal pooled human immunoglobulin was used as the positive control in each assay.

Pooled batches of the standard and positive control were aliquotted and stored at -70°C until use. When new batches of normal human immunoglobulin were required they were recalibrated against the standard. Calculated antibody concentrations were similar for dilutions read off the linear part of the curve and control serum dilutions of 1:1 000 and 1:1 500 were selected. Unknown samples pre- and post-tetanus toxoid immunization were initially tested at dilutions of 1:200 and 1:1 000 respectively. Titration curves of the positive control serum and test samples were parallel to the standard curve (Figure 3.6).

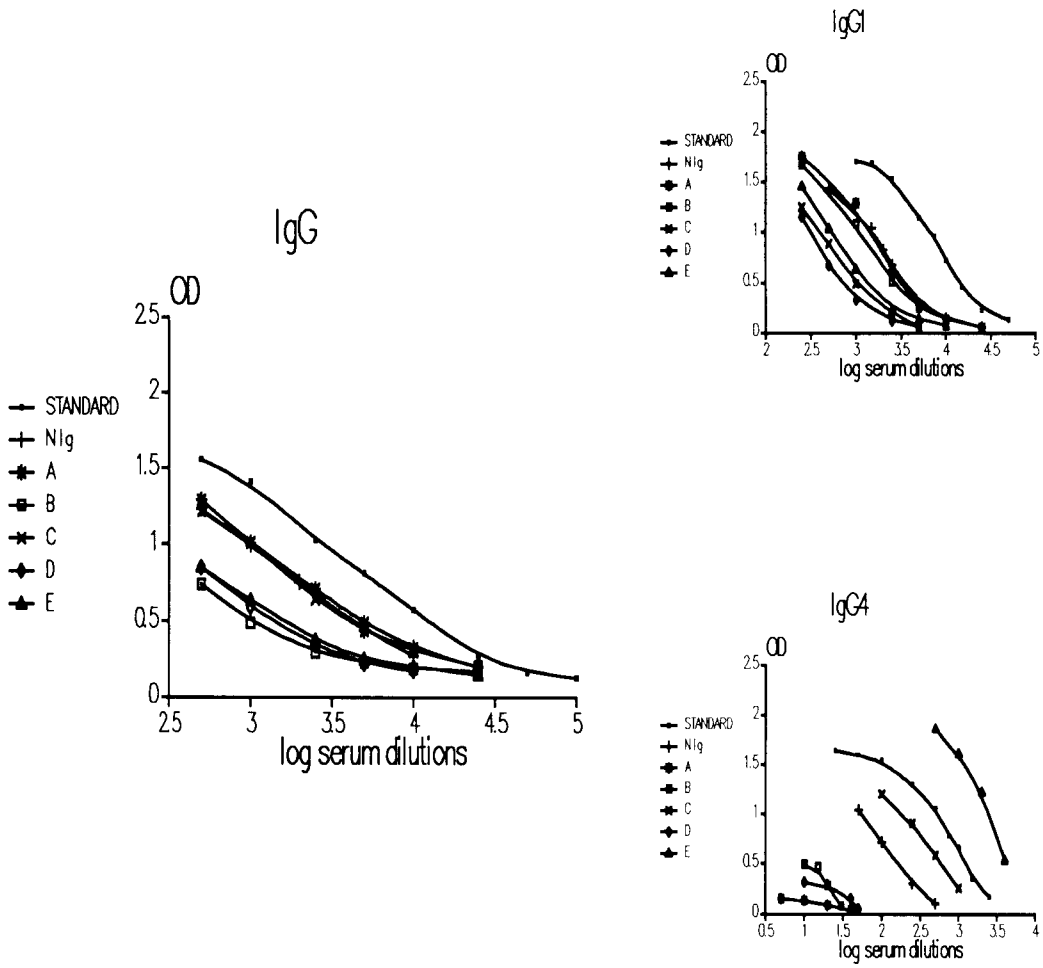


Figure 3.6: IgG, IgG1 and IgG4 tetanus toxoid ELISAs demonstrating parallelism of the standard (•) (hyperimmune human anti-tetanus immunoglobulin), the control (+) (NIg-pooled normal human immunoglobulin), and individual serum samples post tetanus toxoid immunization: A (*), B (□), C (x), D (♦), E (▲)

3.2.4.2 *IgG1 tetanus toxoid ELISA*

Results were similar to IgG tetanus toxoid ELISA and the positive control samples were diluted to 1:1 000 and 1:1 500. Unknown samples pre- and post-tetanus toxoid immunization were diluted 1:100 and 1:1 000 respectively. The titration curves of the sera samples were parallel to the standard curve (Figure 3.6).

3.2.4.3 *IgG4 tetanus toxoid ELISA*

Results were identical to the IgG tetanus toxoid ELISA except that the control samples were diluted to 1:300 and 1:400 and the unknown samples pre- and post-tetanus toxoid immunization to 1/15 and 1:1 000 respectively. Only titration curves of sera samples with high concentrations of IgG4 tetanus toxoid were parallel to the standard curve (Figure 3.6).

3.2.4.4 *Conclusion and discussion*

For accurate quantitation it is important that the titration of test samples parallel that of the standard. In the IgG, IgG1 and IgG4 tetanus toxoid ELISAs titration of sera with high titre antibodies paralleled the standard curves allowing accurate analysis of results (Figure 3.6). Due to the non parallelism observed with low levels of IgG4, the results must be interpreted with caution. The immunochemical basis for non-parallelism may be secondary to:

- reagent instability and variability or
- high non-specific binding of the specimen
- combination of isotype competition and epitope heterogeneity when multivalent antigens are used
- competitive inhibition between isotypes for the same antigen (Butler 1988)

3.2.5 Serum Incubation Periods

The role of incubation time in the assay was studied by incubating the serum for 1 hour at 37°C or overnight at 4°C. There was no difference in the shape of the curve or intensity of the signal. For convenience a serum incubation period of 1 hour at 37°C was selected for all 3 assays.

3.2.6 Monoclonal Antibody - Selection

Details of the various unbiotinylated and biotinylated monoclonal antibodies specific for the individual subclasses IgG1, IgG2, IgG3 and IgG4 evaluated for these assays can be found in Appendix A. The monoclonal antibodies were all diluted in 1% casein in TBS.

3.2.7 Monoclonal Antibody - Backgrounds

Table 3.2 shows the effect of the various monoclonal antibodies on non-specific background binding. Background readings were obtained from the following blanks:

- Minus antigen blanks i.e. minus tetanus toxoid antigen but including serum and monoclonal antibody
- Minus monoclonal antibody blanks i.e. antigen coated wells with serum but minus monoclonal antibody
- Minus serum blanks i.e. antigen coated wells with monoclonal antibody but minus serum
- Background blanks i.e. antigen coated wells with no serum or monoclonal antibody.

For all the assays the background values obtained from wells with antigen and monoclonal antibody but minus serum were very similar to those with antigen but minus serum and monoclonal antibody. These background blanks gave OD readings in the range of 0.002 - 0.040 and accounted for less than 2.5% of the total signal (Table 3.2).

Minus antigen blanks and the highest value of the serum blanks or monoclonal antibody blanks were added together and subtracted from each serum dilution before standard curves were constructed.

TABLE 3.2: OD RESULTS OF THE BLANK READINGS IN THE IgG SUBCLASS SPECIFIC TETANUS TOXOID ELISAs USING BIOTINYLATED AND UNBIOTINYLATED MONOCLONAL ANTIBODIES

	Moab		Conjugate		Maximum Standard		OPTICAL DENSITY (OD) OF BACKGROUND BLANKS (% of maximum standard)			
Assay	Clone	Dilution	HRPO[a]	Dilution	Dilution	OD	Minus Antigen	Minus Moab	Minus Serum	Minus Serum Minus Moab
IgG1	HP6012*	1:2 000	gαm[b]	1:2 500	1:1 000	2.075	0.13 (6.3)	0.45 (21.5)	0.12 (0.7)	0.12 (0.7)
	HP6069**	1:2 000	SA[c]	1:20 000	1:100	2.156	0.01 (0.5)	0.004 (0.1)	0.002 (1.8)	0.002 (1.2)
IgG2	HP6008*	1:2 000	gαm[b]	1:1 000	1:250	1.509	0.14 (8.3)	1.382 (91.6)	0.037 (2.4)	0.027 (1.8)
	HP6002**	1:250	SA[c]	1:5 000	1/10	1.549	0.769 (49.6)	0.003 (0.2)	0.007 (0.5)	0.015 (1.0)
IgG3	HP6010*	1:2 000	gαm[b]	1:1 000	1:250	1.931	0.289 (14.6)	1.493 (77.32)	0.040 (2.0)	0.032 (1.6)
	HP6047**	1:250	SA[c]	1:5 000	1/10	1.819	0.148 (8.1)	0.010 (0.6)	0.008 (0.4)	0.020 (1.0)
IgG4	HP6013*	1:2 000	gαm[b]	1:2 000	1:250	2.05	0.085 (4.1)	0.852 (41.0)	0.040 (1.9)	0.019 (0.9)
	HP6025**	1:1 000	SA[c]	1:20 000	1:100	1.461	0.002 (0.1)	0.003 (0.2)	0.004 (0.2)	0.003 (0.2)

* Unbiotinylated monoclonal antibodies (refer to text for details)

** Biotinylated monoclonal antibodies (refer to text for details)

a HRPO - horseradish peroxidase conjugates

b Cappel non-affinity purified $F(ab')_2$ goat anti-mouse IgG H + L chain

c Streptavidin peroxidase

A major reason for raised background measurements was the monoclonal antibody blank. This was due to non-specific binding of the conjugate antibody to the antigen coated wells containing test serum. Each serum dilution had its own monoclonal antibody blank in the development of the assays. For the unbiotinylated antibodies the monoclonal antibody blanks accounted for 21.45% to 91.60% of the signal (Table 3.2). The non-specific binding reflected by these monoclonal antibody blanks could not be decreased significantly by using different horseradish peroxidase conjugated mouse anti-human antibodies or by the addition of animal sera such as mouse, goat or fetal calf serum. By using biotinylated IgG1, IgG2, IgG3 and IgG4 monoclonal antibodies and streptavidin conjugated horseradish peroxidase as the detecting agent, the monoclonal antibody blanks could be significantly reduced and accounted for less than 0.2% of the maximum signal (Table 3.2).

When only the minus antigen well blanks and minus serum blanks were subtracted from the signal, sigmoidal curves were obtained for IgG1, IgG2, IgG3 and IgG4 tetanus toxoid subclass specific assays using both unbiotinylated and biotinylated monoclonal antibodies (Figures 3.7 and 3.8). However, when monoclonal antibody blanks were also subtracted from the signal, sigmoidal curves were lost in the IgG2 and IgG3 assays when unbiotinylated antibodies were employed (Figures 3.7 and 3.8). This illustrates how a failure to include subtraction of monoclonal antibody blanks can lead to erroneous standard curves.

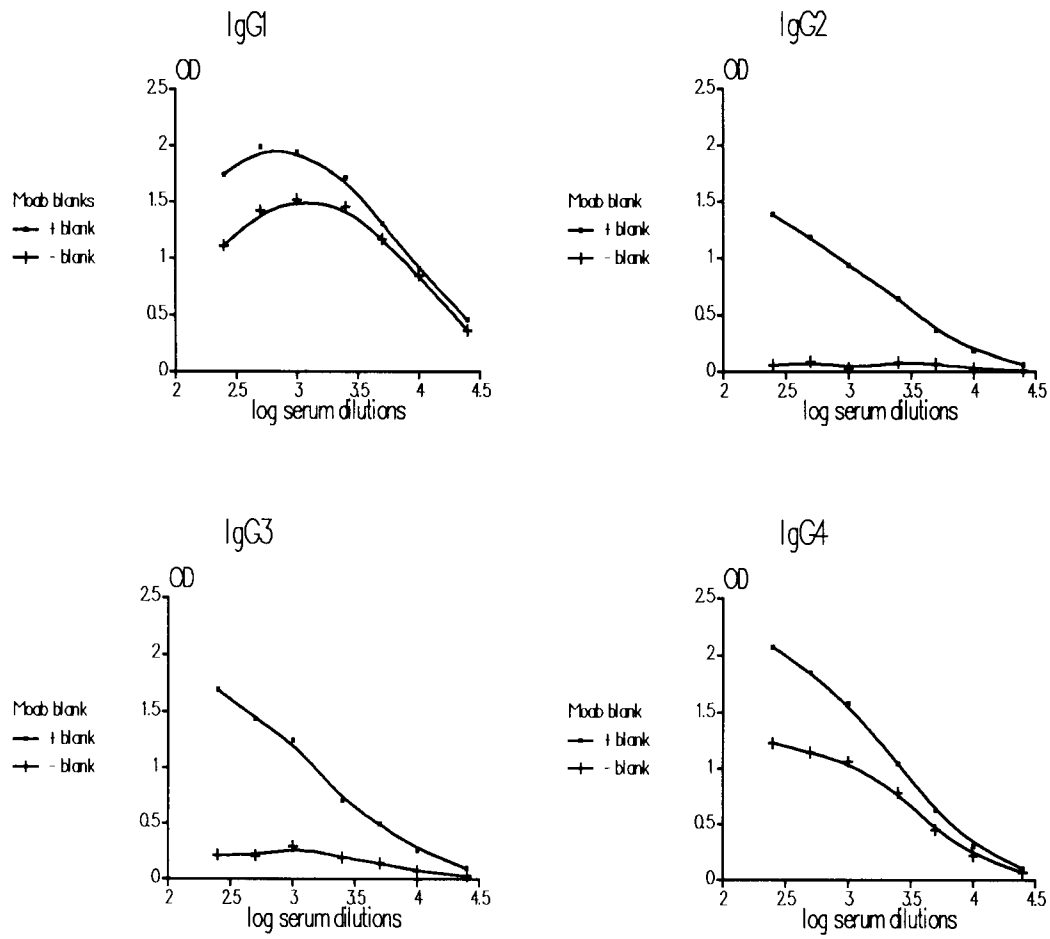


Figure 3.7: Standard curves for IgG1, IgG2, IgG3 and IgG4 tetanus toxoid ELISAs using unbiotinylated monoclonal antibodies (see text for details) without (▪) and with (+) the minus monoclonal antibody (Moab) background OD subtracted

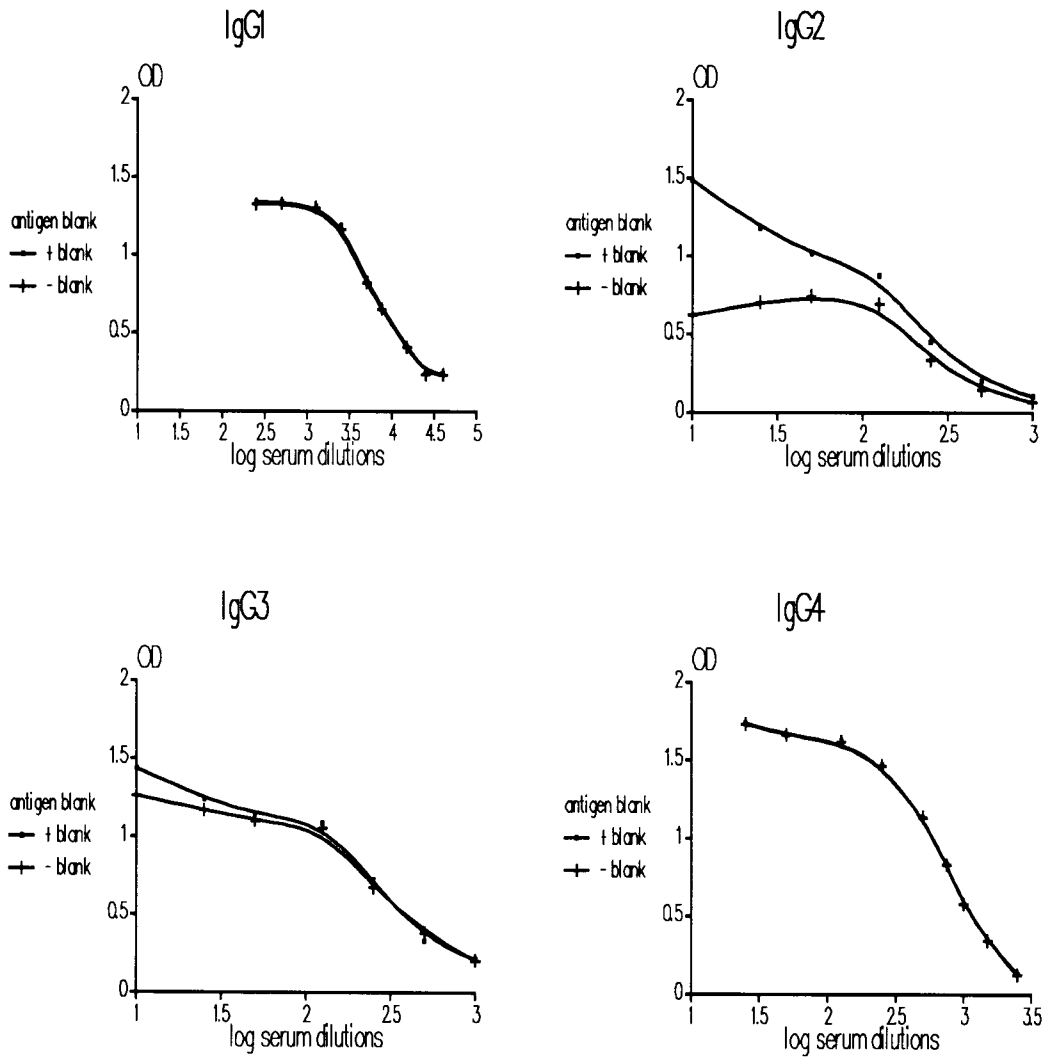


Figure 3.8: Standard curves for IgG1, IgG2, IgG3 and IgG4 tetanus toxoid ELISAs using biotinylated monoclonal antibodies (see text for details) without (▪) and with (+) the minus antigen background OD subtracted

3.2.7.1 *IgG1 tetanus toxoid ELISA*

In the IgG1 tetanus toxoid ELISAs both unbiotinylated and biotinylated monoclonal antibody gave low antigen blanks, accounting for 6.3% and 0.43% respectively of the signal of the most concentrated standard dilution. The monoclonal blanks of the biotinylated antibody were much lower than those of the unbiotinylated antibody being 0.1% and 21.45% respectively,

of the most concentrated standard dilution (Table 3.2). Therefore, the biotinylated anti-IgG1 monoclonal antibody HP6069 was selected for the IgG1 subclass specific tetanus toxoid assay.

Checkerboard titrations of monoclonal antibody, serum and conjugate concentrations were done to optimize the IgG1 tetanus toxoid antibody assays. The performance of the biotinylated anti-IgG1 monoclonal antibody was tested at 1:1 000, 1:2 000 and 1:4 000 dilutions and the results show that a 1:2 000 dilution was the optimum (Figure 3.9).

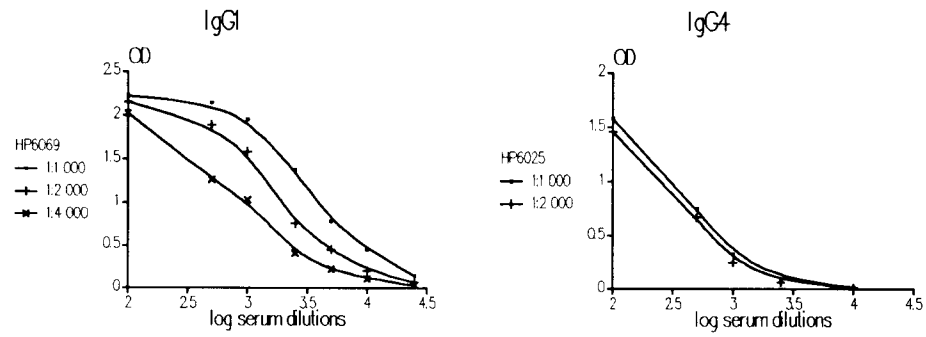


Figure 3.9: Effect of monoclonal antibody (Moab) dilutions on the standard curves of the IgG1 and IgG4 tetanus toxoid ELISAs. The biotinylated anti-IgG1 Moab HP6069 was tested at 1:1 000 (•), 1:2 000 (+) and 1:4 000 (x) dilutions and the biotinylated anti-IgG4 Moab HP6025 at 1:1 000 (•) and 1:2 000 (+).

3.2.7.2 *IgG4 tetanus toxoid ELISA*

Antigen blanks were low using either the unbiotinylated or biotinylated antibodies but monoclonal antibody blanks were high (41% of the maximum

signal) using the unbiotinylated antibody and low (0.2% of the maximum signal) using the biotinylated antibody (Table 3.2). The biotinylated anti-IgG4 antibody HP6025 was selected for these assays at a concentration of 1:1 000 (Figure 3.9).

3.2.7.3 *IgG2 tetanus toxoid ELISA*

In the IgG2 assays, the unbiotinylated monoclonal antibody HP6008, gave monoclonal blanks of 91.6% of the signal whereas using the biotinylated IgG2 antibody the antigen blanks were almost 50% of the signal. These blanks could not be lowered even with the addition of various animal sera to diluents, or by using other blockers. Because of this problem and because there appeared to be only a relatively small amount of IgG2 tetanus toxoid antibody present in the hyperimmune anti-tetanus immunoglobulin preparation (Figure 3.10), IgG2 subclass specific tetanus toxoid assays were not developed.

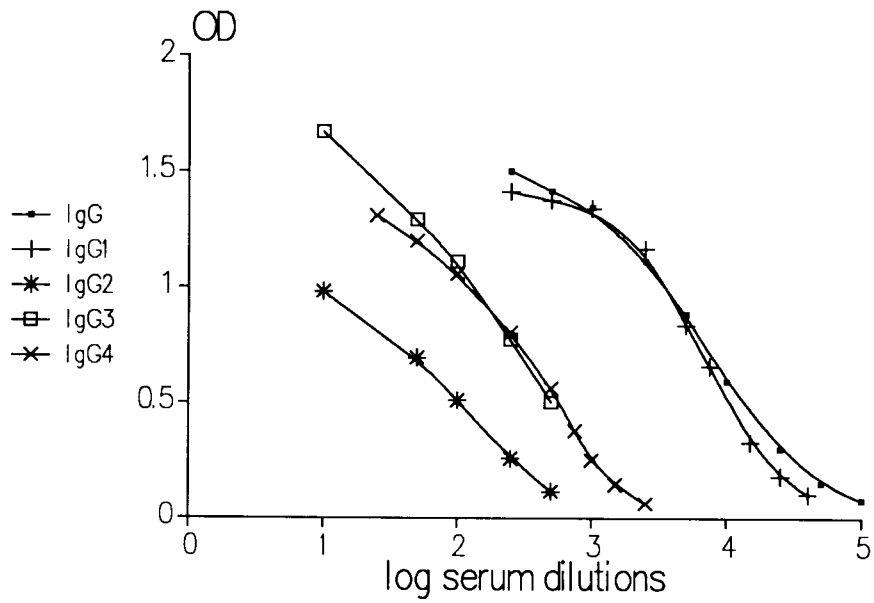


Figure 3.10: Standard curves of IgG (▪); IgG1 (+); IgG2 (*); IgG3 (□) and IgG4 (x) subclass specific tetanus toxoid ELISAs using hyperimmune human anti-tetanus immunoglobulin as the standard

3.2.7.4 *IgG3 tetanus toxoid ELISA*

In the IgG3 assays the unbiotinylated HP6010 antibody blanks were 77.32% of the signal. Using the biotinylated IgG3 monoclonal antibody all the blanks were acceptable (< 10%) (Table 3.2) but this antibody had to be used at a high concentration (1:250). For these reasons and the fact that the IgG3 response to tetanus toxoid was thought not to be as important, this assay was not standardised.

3.2.7.5 *Conclusion and discussion*

Standard curves of total IgG and IgG subclass specific tetanus toxoid antibodies were established using hyperimmune human anti-tetanus immunoglobulin as the standard (Figure 3.10). For reasons mentioned above IgG2 and IgG3 tetanus toxoid ELISAs were not standardized for routine use.

Devey et al (1987; 1988) and Rubin et al (1986) have used the monoclonal antibody HP6012 (NL16) and French and Harrison (1985) used the monoclonal antibody HP6007 (JL512) to establish IgG1 subclass specific tetanus toxoid assays (Table 3.3). There are no reports of the use of the unbiotinylated or biotinylated anti-IgG1 monoclonal antibody HP6069 used in the experiments described here. The biotinylated antibody HP6069 was selected in preference to HP6012 because it gave lower non-specific binding.

The three groups that have reported IgG4 tetanus toxoid assays have all used a different anti-IgG4 monoclonal antibody (Table 3.3) (French and Harrison 1985; Rubin et al 1986; Devey et al 1988). Again there are no reports of

the use of the biotinylated IgG4 monoclonal antibody HP6025 which was selected for the assay described above.

Although previous reports of IgG2 tetanus toxoid assays use the monoclonal antibody HP6008 (French and Harrison 1985; Rubin et al 1986; Devey et al 1987) (Table 3.3) it was found to be unsuitable in the assay system described above because of the high background monoclonal antibody blank readings obtained.

There are reports of the anti-IgG3 monoclonal antibody HP6010 (French and Harrison 1985; Devey et al 1987) and SK33 (Rubin et al 1986) being used to establish IgG3 subclass specific tetanus toxoid assays (Table 3.3). There are no reports of the use of the biotinylated antibody HP6047 which was found to be better suited to the assay system described above than the antibody HP6010. This assay was not developed further.

It is not possible to make a direct comparison of the assays developed in our laboratory with those previously reported because of the different combinations of reagents, in particular the monoclonal and conjugated antibodies used.

TABLE 3.3: SUMMARY OF REAGENTS USED IN REPORTED IgG SUBCLASS SPECIFIC TETANUS TOXOID ANTIBODY ELISAs

	MONOCLONAL ANTIBODY CLONE / DILUTION				CONJUGATE (Source)	ENZYME SUBSTRATE
Reference	**IgG1**	**IgG2**	**IgG3**	**IgG4**		
French and Harrison 1985	HP6007 1:250	HP6008 1:250	HP6010 1:250	HP6011 1:250	Alkaline phosphatase rabbit anti-mouse IgG (Sigma)	Alkaline phosphatase/ ABTS
Rubin et al 1986	HP6012 1:10 000	HP6008 1:1 000	SJ 33 1:4 000	SK 44 1:10 000	Goat anti-mouse IgG + IgM (Tago)	Horseradish peroxidase/OPD
Devey et al 1987	HP6012 1:2 500	HP6008 1:5 000	HP6010 1:5 000	HP6013 1:5 000	Affinity purified anti-mouse IgG (Jackson)	Horseradish peroxidase/OPD
Devey et al 1988	HP6012 1:2 500	-	-	HP6013 1:5 000	Affinity purified human IgG adsorbed anti-mouse IgG (Jackson)	Horseradish peroxidase/OPD

3.2.8 Evaluation of Enzyme Detector Systems

3.2.8.1 *IgG tetanus toxoid ELISA*

The detection antibody was a goat anti-human IgG gamma chain specific antibody coupled to horseradish peroxidase (HRPO). The following conjugated antibodies were evaluated:

- Non affinity purified goat anti-human gamma chain specific HRPO (Cappel, Catalogue No 3301-0121)
- Affinity purified goat anti-human gamma chain specific HRPO (Cappel, Catalogue No 3601-0121).
- Affinity purified goat anti-human gamma chain specific HRPO absorbed with mouse serum proteins (Zymed, Catalogue No 62-8420)
- $F(ab')_2$ fragment affinity purified goat anti-human gamma chain specific HRPO (Sigma, Catalogue No 62-8420)

The Cappel affinity purified goat anti-human gamma chain specific horseradish peroxidase conjugate gave a very low signal whereas the Cappel non-affinity purified goat anti-human gamma chain specific horseradish peroxidase conjugate could be used at a dilution of 1:30 000. The Zymed and Sigma goat anti-human conjugates at dilutions of 1:5 000 and 1:1 000 respectively gave lower signals than the non-affinity purified Cappel conjugate (Figure 3.11) which was selected for reasons of precision and economy. It was diluted from a 1:100 stock to 1:30 000 in 1% casein in TBS.

3.2.8.2 *IgG1 tetanus toxoid ELISA*

In the subclass specific assays where biotinylated monoclonal antibodies were selected streptavidin conjugated horseradish peroxidase was used. The effect of various concentrations (1:10 000,1:15 000, 1:20 000, 1:30 000,

1:35 000) of Streptavidin horseradish peroxidase on the detection of IgG1 tetanus toxoid antibodies using a biotinylated monoclonal antibody (at a concentration of 1:2000) can be seen in Figure 3.11. The dilution of streptavidin horseradish peroxidase of 1:30 000 was selected. The conjugate was diluted in 1% casein TBS from a stock solution of 1:1 000.

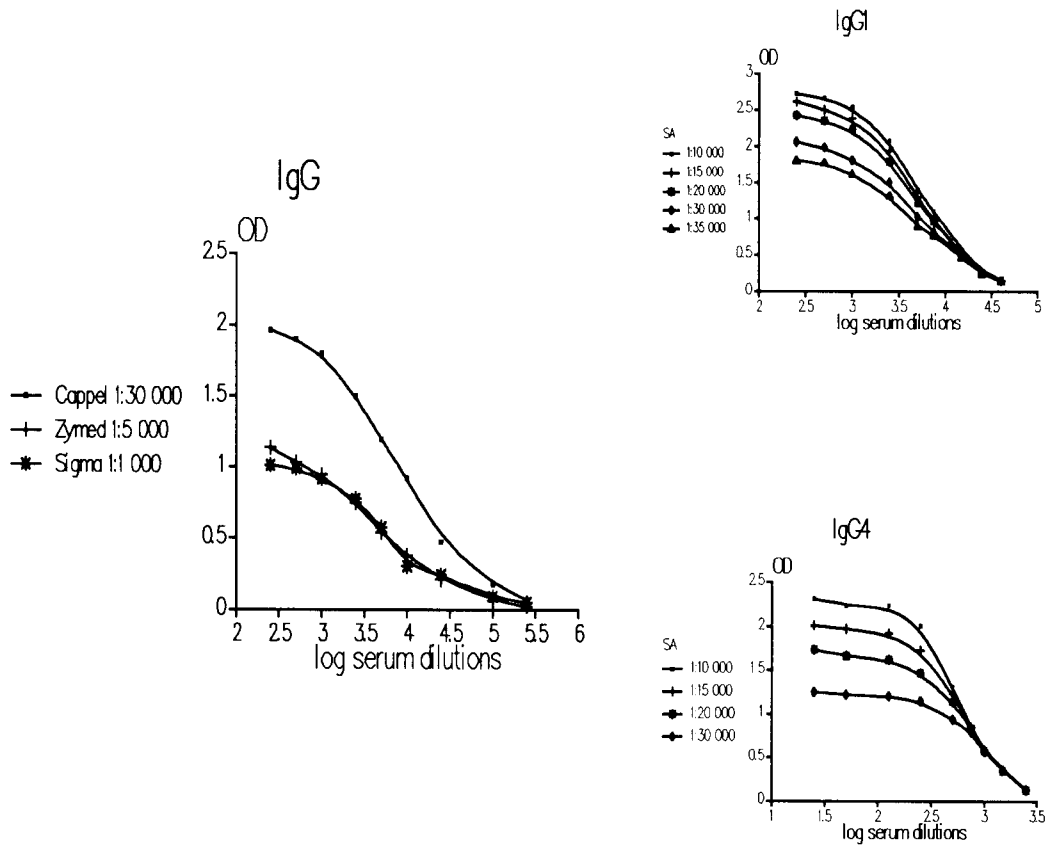


Figure 3.11: Effect of various goat anti-human IgG conjugates on the standard curve of the IgG tetanus toxoid ELISA: Cappel (•), Zymed (+) and Sigma (*) and of various dilutions of streptavidin peroxidase (SA): 1:10 000 (•), 1:15 000 (+), 1:20 000 (*), 1:30 000 (♦), 1:35 000 (▲) on the IgG1 and IgG4 tetanus toxoid ELISAs.

3.2.8.3 *IgG4 tetanus toxoid ELISA*

The results obtained in the IgG4 tetanus toxoid ELISA were very similar to the IgG1 assay above and a 1:20 000 dilution of streptavidin horseradish peroxidase was selected for routine use (Figure 3.11).

3.2.8.4 *Conclusion and discussion*

Most reports have used alkaline phosphatase or peroxidase conjugated antibodies as detection antibodies. There is little uniformity in purity or source of these antibodies. However Lau (1987) incorporated the use of the biotin streptavidin system to measure IgG tetanus toxoid antibodies. This system has proven very useful in the IgG1 tetanus toxoid and IgG4 tetanus toxoid assays described in this study.

3.3 METHODS

Optimum reaction conditions had been determined in preliminary experiments. All reagents and samples were added to the wells in 100 μl volumes except for the 2N H_2SO_4 of which 50 μl volumes were used.

3.3.1 IgG tetanus toxoid ELISA

Flat bottomed Nunc microtitre plates (Catalogue No 4-4204) were coated with tetanus formol-toxoid SAIMR (20 Lf/ml) diluted to 0.4 Lf/ml in carbonate buffer pH 9.6. This coating concentration of 0.04 Lf per well was shown to be optimal in preliminary assays. Plates were incubated overnight in a moist chamber at 4°C. Non-specific protein binding sites were then blocked with 1% casein diluted in TBS for 30 minutes at 37°C in a moist chamber. Plates were washed once with PBS and dilutions of sera diluted in 1% casein in TBS were added to quadruplicate wells. Hyperimmune human anti-tetanus immunoglobulin from NBTS (125 IU/ml) in 9 dilutions ranging from 1:250-1:100 000 (1:250, 1:500, 1:1 000,

1:2 500, 1:5 000, 1:10 000, 1:25 000, 1:50 000, 1:100 000) was used as the standard. Positive control sera (normal human immunoglobulin) (NBTS) dilutions of 1:1 000 and 1:1 500 were selected. Dilutions of pre- and post-tetanus toxoid immunization sera of 1:200 and 1:1 000 respectively were suitable to quantitate antibody both pre- and post-tetanus toxoid immunization samples in the majority of specimens. If the OD of the sample did not read off the linear section of the standard curve the samples were reassayed at appropriate dilutions. The samples were incubated for 1 hour at 37°C.

Buffer and diluent were included in each assay as negative controls. Agammaglobulinaemic sera had no detectable reaction in the assay.

The plates were washed with PBST five times. $F(ab')_2$ fragment goat anti-human IgG Fc gamma chain specific horseradish peroxidase conjugated antibody (Cappel Catalogue No 3301-0121) diluted 1:30 000 in 1% casein was added to the wells and incubated for 30 minutes at 37°C. The plates were again washed five times with PBST. OPD substrate buffer was added and incubated in the dark at room temperature for 15 minutes. The reaction was stopped with 50 μl of 2N H_2SO_4. The OD was read at 492nm in an SLT ELISA reader.

3.3.2 IgG1 tetanus toxoid ELISA

The coating, washing, blocking and substrate steps were identical to those described for the IgG tetanus toxoid ELISA. The following changes were made:

- The standard curve dilutions of human hyperimmune anti-tetanus immunoglobulin ranged from 1:1 000-1:75 000 (1:1 000, 1:2 500,

1:5 000, 1:7 500, 1:10 000, 1:15 000, 1:25 000, 1:40 000, 1:75 000)

- Positive control dilutions of 1:1 000 and 1:1 500 were selected
- Initially, patient sera pre- and post-tetanus toxoid immunization were added at two dilutions of 1:100 and 1:1 000 respectively
- The sera samples were diluted in 1% casein and incubated for 1 hour at 37°C
- Monoclonal biotinylated mouse anti-human IgG subclass specific antibody to IgG1 (HP6069) diluted 1:2 000 in 1% casein was then added to the wells and incubated overnight at 4°C
- After washing streptavidin conjugated horseradish peroxidase (Zymed Catalogue No 43-4323) diluted 1:30 000 in 1% casein was added and incubated at 37°C in a moist chamber for 1 hour.

3.3.3 IgG4 tetanus toxoid ELISA

This was similar to the method mentioned above for the IgG1 tetanus toxoid ELISA except for the following dilution and antibody differences.

- The standard curve dilutions of the human anti-tetanus immunoglobulin ranged from 1:25 - 1:2 500 (1:25, 1:50, 1:100, 1:250, 1:500, 1:750, 1:1 000, 1:1 500, 1:2 500)
- Positive control dilutions of 1:300 and 1:400 were selected.
- Initially patient sera pre- and post-tetanus toxoid immunization were added at dilutions of 1/15 and 1:1 000 respectively
- The biotinylated mouse anti-human IgG subclass specific antibody to IgG4 (HP6025) was diluted in 1% casein to 1:1 000
- Streptavidin conjugated horseradish peroxidase was diluted in 1% casein to 1:20 000

3.3.4 Design of Plate

All reactions were performed in triplicate. Every assay plate included 9 dilutions of the standard anti-tetanus toxoid immunoglobulin for the calibration curves, background reaction wells (wells minus serum, minus antigen, and minus monoclonal antibody (where applicable)) and dilutions of the sample of pooled normal immunoglobulin for monitoring reproducibility. For the routine analysis of unknown samples the design of the plate is similar for the IgG, IgG1 and IgG4 tetanus toxoid ELISAs and is shown in Chapter 1, Figure 1.1.

The blanks are in column 10 rows A-D. In the IgG tetanus toxoid ELISA these blanks are minus-serum blanks. The blanks in column 10 in the subclass specific assays are minus monoclonal antibody blanks i.e. each well contains the most concentrated standard dilution minus the relevant monoclonal antibody.

Rows A-C and E-G were coated with tetanus toxoid antigen whilst row D and H were the minus antigen blanks and were only coated with the carbonate coating buffer.

Initially serum blanks were monitored in the subclass assays but since they were consistently low (Table 3.2) only the minus monoclonal antibody blank was retained. This allowed more unknown samples to be included on each plate.

The minus serum, minus antigen and minus monoclonal antibody blanks were subtracted from the mean of the triplicates of the standard and sample dilutions.

3.3.5 Calculation of Results

The commercial human anti-tetanus immunoglobulin contains 250 IU/vial and the volume of each vial ranged between 1.5 - 2.5 ml. Six vials of this standard were pooled giving 16.25 mls containing 1 500 IU which is equivalent to 93.2 IU/ml. A value of 93.2 units/ml for IgG was assigned to the standard to allow IgG tetanus toxoid antibodies to be quantitated.

A value of 100 units/ml for IgG1 and 10 units/ml for IgG4 was arbitrarily assigned to this standard to allow the subclass specific tetanus toxoid antibodies to be quantitated. The proportion of the value of the IgG1 and IgG4 tetanus toxoid antibody units of 10:1 roughly equates the proportion of IgG1 subclass to IgG4 subclass in serum. This was done to enable the value of the arbitrary units to be realistic. Levels of antibody for each subclass were calculated relative to these references.

Standard curves relating absorbance to the concentration of IgG, IgG1 and IgG4 specific tetanus toxoid antibodies in the standard serum dilutions were constructed.

Standard dose curves were constructed from average absorbance values and concentrations that had been entered into a curve fitting four parameter logistic programme based on the mathematical model described elsewhere (Chapter 1.12). The concentration of each analyte in each sample was obtained by interpolating the average absorbance values from the standard curve and multiplying the result by the dilution factor.

These standard curves were used to calculate the antibody concentrations in samples tested by using at least 2 absorbance readings on the straight line

portion of the curve and taking the mean concentration of them. Samples which fell out of this range were repeated at appropriate dilutions.

3.3.6 Diethylamine-ELISA for IgG1 and IgG4 tetanus toxoid

These assays are based on those of Devey et al 1988, using diethylamine (DEA) as the chaotropic agent. The details of these ELISAs are identical to those described earlier except for the addition of DEA to the serum diluent. Serial dilutions of sera were made in 1% casein in the presence and absence of various concentrations of DEA (5-50 mM).

OD was plotted against serum dilution and the leftward shift in dose-response curve in the presence of DEA was measured at 50% of the maximum OD. Results were expressed as the $\log_{10}$ of this shift.

3.4 ASSAY STANDARDISATION

3.4.1 Standard Curves

Commercial human hyperimmune anti-tetanus toxoid immunoglobulin was used to construct standard curves for IgG, IgG1 and IgG4 tetanus toxoid ELISAs relating absorbance to IgG, IgG1 or IgG4 subclass concentrations (Figure 3.10). Each plate contained a standard curve constructed from triplicate samples of nine different dilutions of standard serum.

3.4.2 Precision and Sensitivity

The inter-assay variation was assessed with 3 samples assayed in triplicate in 12 different runs. The sera selected had high, intermediate and low IgG, IgG1 and IgG4 specific tetanus toxoid antibodies. For each assay the mean of each sample's results over 12 runs was calculated and the SDs and CVs were then derived. The CVs ranged from 4.5% to 12.7% with an average of 9.27% (Table 3.4).

TABLE 3.4: EVALUATION OF IgG, IgG1 AND IgG4 TETANUS TOXOID ASSAY PRECISION

INTER-ASSAY PRECISION[b]

	IgG			IgG1			IgG4		
	Mean	SD	CV[a]	Mean	SD	CV[a]	Mean	SD	CV[a]
Normal Pooled Ig	23.4	1.98	8.5	21.9	2.28	10.4	4.1	0.44	10.6
	21.1	2.60	12.7	23.2	1.23	5.3	3.8	0.43	111.2
	22.06	2.66	11.9	22.5	1.92	8.5	3.9	0.45	11.5
Sample A (low titre)	1.29	0.12	9.3	0.46	0.04	9.7	0.30	0.25	8.2
Sample B (high titre)	40.65	5.90	4.5	49.2	5.00	10.2	9.47	0.616	6.5

INTRA-ASSAY PRECISION

Normal Pooled Ig	212.4	1.62	7.9	22.9	1.83	8.0	3.6	0.14	4.7
Sample A (low titre)	1.55	0.91	12.4	0.13	0.007	5.55	0.361	.024	6.8
Sample B (high titre)	36.84	1.57	4.3	59.9	2.99	5.0	9.38	0.39	4.25

INTRA-PLATE PRECISION

Normal Pooled Ig	24.5	0.87	3.6	23.8	1.46	6.1	3.91	0.250	5.4

a **Mean and standard deviation (SD) were expressed in units/ml and coefficient of variation (CV) in percent.**
b **This was taken from plates assayed over 12 consecutive weeks**

The intra-assay variation was also obtained for high, intermediate and low titred serum. These samples were assayed 14 times in one run and the CV ranged from 4.3% to 12.4% with an average of 6.56% (Table 3.4).

The lowest detection limit for each assay was 0.003 U/ml for IgG and 0.005 U/ml for IgG1 and IgG4. These levels were determined from serial dilutions of the normal immunoglobulin control until the results were no longer reproducible. These detection limits correlated with the most dilute standard of the standard curve in all 3 assays. Samples with ODs below the OD of the most dilute standard were considered negative.

Assays in which the results of the control were more than two standard deviations from the known mean value of the normal immunoglobulin control were repeated.

3.4.3 Specificity

The specificity of the IgG, IgG1 and IgG4 tetanus toxoid ELISA assays were assessed by a competitive inhibition ELISA using either tetanus toxoid or a non-specific antigen diphtheria toxoid (SAIMR, Catalogue No P6190). These ELISAs were carried out as described in Chapter 3.3 except that the competitive antigens (tetanus and diphtheria toxoids) were mixed with the serum in dilutions ranging from 1/2-1:50 000 (10 Lf/ml - 0.0004 Lf/ml for tetanus toxoid and 25 Lf/ml - 0.001 Lf/ml for diphtheria toxoid). Two dilutions of the serum, both reading on the linear section of the sigmoid curves for the assays were selected. In the IgG tetanus toxoid ELISA these dilutions were 1:5 000 and 1:10 000, in the IgG1 tetanus toxoid ELISA 1:5 000 and 1:7 500 and in the IgG4 ELISA 1:500 and 1:750. The mixtures of the serum and competitive antigens were either added immediately to the tetanus toxoid coated wells or after pre-incubation of the serum and soluble

antigen for 30 minutes at room temperature. Wells containing serum without soluble tetanus toxoid were incubated as positive controls. Binding of the tetanus toxoid antibody to wells coated with tetanus toxoid could be inhibited by incubation with tetanus toxoid antigen but not with the non-specific antigen diphtheria toxoid. The percentage inhibition for each concentration of inhibitor was calculated (Table 3.5 and 3.6). In the ELISAs where the inhibiting antigens were mixed with the serum and added immediately to the plates, the dilution of tetanus toxoid required to cause a 50% inhibition of the signal assay ranged between 1:25 and 1:50 (0.8-0.4 Lf/ml) in the IgG and IgG1 assay, and between 1/10 and 1:25 (2.0-0.8 Lf/ml) for the IgG4 assay (Table 3.5).

TABLE 3.5: COMPETITIVE INHIBITION ELISA FOR SPECIFICITY IN TETANUS TOXOID ASSAY (NO PRE-INCUBATION)

Dilutions of Tetanus Toxoid (20 Lf/ml)	% Inhibition		
	IgG	IgG1	IgG4
1/2	87	97	99
1/5	85	94	97
1:10	81	89	83
1:25	71	72	24
1:50	38	40	13
1:100	38	49	0
1:500	25	16	0.8
1:1 000	10	0.3	0
1:10 000	12	8	
1:50 000	8	4	

* **Diphtheria toxoid caused no inhibition in these assays**

TABLE 3.6: COMPETITIVE INHIBITION ELISA FOR SPECIFICITY IN TETANUS TOXOID ASSAY (PRE-INCUBATION)

Dilutions of Tetanus Toxoid (20 Lf/ml)	% Inhibition		
	IgG	IgG1	IgG4
1/2	96	95	100
1/5	79	96	100
1/10	94	94	100
1:25	88	91	100
1:50	11	23	25
1:100	4	8	9
1:500	0	3	0
1:1 000	0	3	8
1:10 000	0	4	9
1:50 000	2	7	9

* **Diphtheria toxoid caused no inhibition in these assays**

However, if the antigen was preincubated with the serum before addition to the plate, the dilutions of tetanus toxoid required to cause 50% inhibition of the signal were between 1:25 and 1:50 (0.8-0.4 Lf/ml) for the IgG tetanus toxoid, IgG1 tetanus toxoid and IgG4 tetanus toxoid assays (Table 3.6).

These results show that these assays are specific for the measurement of tetanus toxoid antibodies.

However, in the assays where the antigen was preincubated with the serum the concentration of tetanus toxoid required for 50% inhibition was the same for the IgG1 tetanus toxoid and IgG4 tetanus toxoid antibodies.

3.5 ANTIBODY AFFINITY

3.5.1 Optimal Dilution of DEA

The incorporation of 5-50 mM DEA in the serum diluent resulted in a progressive parallel shift to the left of the dose response curve for both IgG1 and IgG4 antibodies to tetanus toxoid (Figure 3.12).

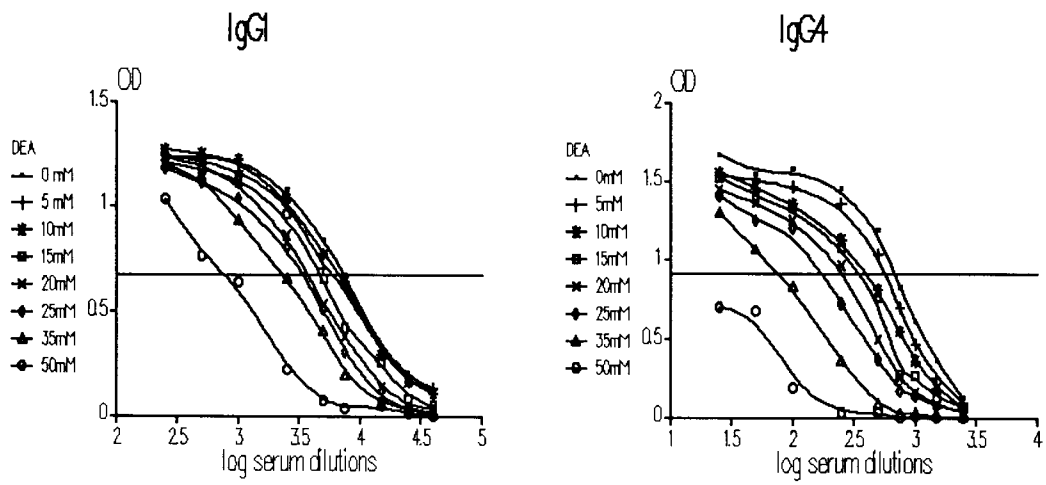


Figure 3.12: Effect of increasing concentrations of DEA (0-50 mM) on the standard curves of IgG1 and IgG4 tetanus toxoid antibodies. DEA concentrations: no DEA (•), 5 mM (+), 10 mM (*), 15 mM (▪), 20 mM (x), 25 mM (♦), 35 mM (▲) and 50 mM (o). The shift was measured at 50% of the maximum OD which is represented by the solid line drawn at 0.6 OD and 0.9 OD for the IgG1 and IgG4 tetanus toxoid ELISAs respectively

This shift of the antibody titration curves was greater in the IgG4 tetanus toxoid assays than in the IgG1 tetanus toxoid assays.

Absorbance readings in the absence of DEA represented total binding of specific antibody. With various molar concentrations of DEA the shift in the log of the serum dilution from the initial OD reading to 50% of the initial OD reading was calculated and used to determine relative affinities of anti-tetanus toxoid antibodies in sera.

Using the standard serum (hyperimmune anti-tetanus immunoglobulin), the shift for IgG4 tetanus toxoid antibody was greater than that for IgG1 tetanus toxoid antibody. This indicates that the IgG4 tetanus toxoid antibody is of lower affinity than the IgG1 tetanus toxoid antibody. With DEA concentrations of 15 to 35 mM the % difference of the IgG1 shift minus IgG4 shift was fairly constant at 60% (Table 3.7). The concentration of DEA selected for subsequent experiments with this antigen system was 25 mM.

TABLE 3.7: INCREASING CONCENTRATIONS OF DEA SHOWING THE LEFT SHIFT OF THE DOSE-RESPONSE CURVE OF IgG1 AND IgG4 TETANUS TOXOID ELISAs IN STANDARD HYPERIMMUNE ANTI-TETANUS IMMUNOGLOBULIN

DEA Concentration (mM)	Log_{10} dilution at 50% max OD		Log_{10} shift at 50% max OD		% difference in shift
	OD=0.6 IgG1	OD=0.9 IgG4	OD=0.6 IgG1	OD=0.9 IgG4	$\frac{IgG1-IgG4}{IgG1} \times 100$
0	3.94	2.62			
5	3.91	2.53	0.04	0.09	- 125
10	3.87	2.46	0.07	0.16	- 129
15	3.74	2.30	0.20	0.32	- 60
20	3.64	2.23	0.30	0.40	- 32
25	3.64	2.14	0.30	0.48	- 60
35	3.42	1.77	0.52	0.85	- 64
50	2.99	1.41	0.95	1.21	- 27

3.5.2 Effect of DEA on Solid-phase Antigen

In order to exclude the possibility that DEA altered the dose-response curves by a direct effect on the solid-phase antigen, tetanus toxoid coated plates were incubated with 25 mM DEA in the 1% casein blocker for 1 hour at 37°C and washed before the addition of sera with or without DEA in the normal DEA-ELISA. Results showed that pre-incubation with DEA had no effect on the subsequent dose-response curve (Figure 3.13). With DEA in the serum the curve was displaced to the left and in the case of IgG4 was flattened confirming the lower affinity binding capacity of the IgG4 tetanus toxoid antibodies.

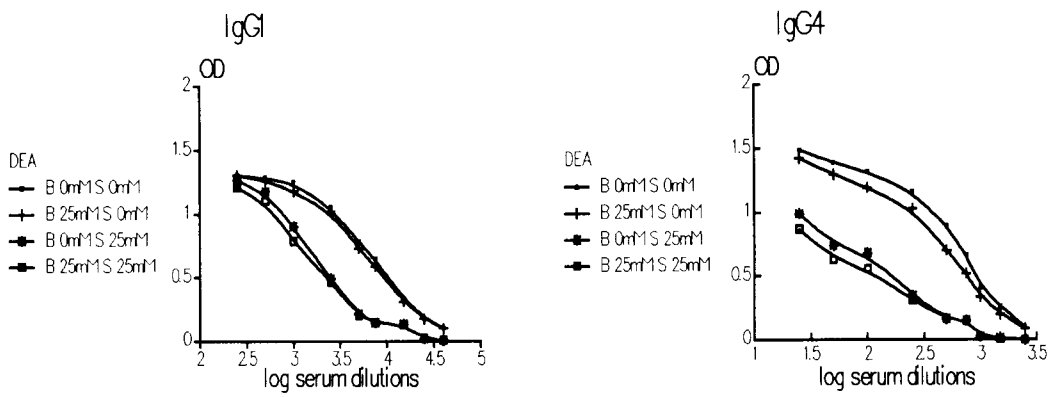


Figure 3.13: Effect of 25 mM DEA in the blocking solution (B) or serum diluent (S) on the DEA-ELISA in the IgG1 and IgG4 tetanus toxoid assays. No DEA in either the blocker or the serum diluent (▪), DEA in the blocker (+), DEA in the serum diluent (*), DEA in blocker and serum diluent (□)

3.6 SUMMARY

Tetanus toxoid is a potent immunogen and has been used to measure normal antibody responses. This response can be used as an indicator of the patient's ability to respond to an antigenic stimulus.

This chapter describes the establishment of IgG, IgG1 and IgG4 tetanus toxoid antibody ELISAs which are reproducible, sensitive and simple to perform. A number of variables were tested in order to establish suitable assays.

An important aspect of the subclass specific assays is the inclusion of sufficient background blanks to detect the degree of non-specific binding. Each tier of the ELISA must have its own blank. Using unbiotinylated monoclonal antibodies to detect subclass specific tetanus toxoid antibodies demonstrates how the exclusion of monoclonal antibody blanks can lead to inaccurate standard curves (Figure 3.7). These problems were overcome with the use of biotinylated monoclonal antibodies. There are no previous reports of IgG subclass specific tetanus toxoid ELISAs using biotinylated antibodies although it has been reported for use in IgG tetanus toxoid ELISAs (Lau 1987).

The competitive inhibition ELISAs confirmed the assays are specific for IgG, IgG1 and IgG4 tetanus toxoid antibodies.

It was found that no single dilution of serum can measure all serum levels in various individuals due to the highly variable tetanus toxoid response.

The DEA-ELISA is a simple and convenient method of assessing functional affinities of antibodies to complex antigens. Results of the DEA-ELISA suggest that the IgG4 antibody responses to tetanus toxoid were of lower affinity than the IgG1 responses, in agreement with earlier studies.

These assays were used to quantitate IgG, IgG1 and IgG4 subclass antibodies to tetanus toxoid in the patient groups (Chapter 10.5).

CHAPTER 4

HAEMOPHILUS INFLUENZAE TYPE b IgG SUBCLASS IMMUNOASSAY

This chapter describes the development of ELISAs to measure IgG, IgG1 and IgG2 subclass specific antibodies to the capsular polysaccharide, polyribose phosphate (PRP), of *H.influenzae* type b (Hib). The introduction outlines the general background and principles of these assays and includes a brief review of previously published assays. Various experiments involved in establishing optimal reagents and conditions for the IgG, IgG1 and IgG2 Hib PRP ELISAs are described. The assay methodology and experiments determining assay precision and standardization are detailed. These assays will be used to measure the immune response in children with invasive Hib infections. This chapter is sub-divided into the following sections:

4.1 INTRODUCTION

4.2 DETERMINATION OF OPTIMAL ASSAY CONDITIONS

4.3 METHODS

4.4 ASSAY STANDARDIZATION

4.5 SUMMARY

4.1 INTRODUCTION

Haemophilus influenzae type b (Hib) is the leading cause of bacterial meningitis in children younger than 2 years of age in both industrialized and developing countries (Fraser 1982; Munson et al 1989). Antibodies against the Hib capsular polysaccharide, a polymer of repeating 3-ß-D-ribose-(1-1)-D-ribitol-5-phosphate units (polyribose phosphate (PRP)) are protective in both animals and humans (Halsay et al 1983; Schreiber et al 1986).

Antibody to the Hib capsular antigen PRP has been measured by a variety of methods to study immunity to disease (Schneerson et al 1971; Anderson et al 1972) and to evaluate the immunogenicity of polysaccharide vaccines (Ward et al 1988).

Measurement of antibody responses to PRP after clinical infection requires a very sensitive assay because the post-infection antibody response of young infants is low and may be delayed by a few months (Insel and Anderson 1986a; Claesson et al 1988). Infants with a poor response are subject to a further Hib infection (Insel and Anderson 1986a).

Direct measurement of serum bactericidal activity against Hib can be used to detect complement-fixing anti-PRP antibodies but the assay also detects antibodies directed at other surface antigens of Hib and is not suitable for routine use. Passive haemagglutination with PRP sensitized erythrocytes is rapid and inexpensive and does not require the use of radioactive reagents but it is too insensitive for the detection of antibody in human infants. RIA (radioimmunoassay) and ELISA are sensitive, reproducible and simple to perform. Most anti-PRP antibody assays use RIA adapted from the method originally described by Farr (1958). Antibody binding is measured by adding dilutions of sera to radiolabelled antigen and then separating and

counting the antibody bound antigen. Because of the difficulty, instability and expense of preparing pure radio-labelled antigen, RIA is not practical or economical for assay measurement on a small scale. The slope of RIA curves (bound antigen versus serum dilution), for some children's sera does not parallel the slope of the US Food and Drug Administration (FDA) standard (Lot 1983) slope (Anderson et al 1987; Hetherington and Rutkowski 1990). An advantage of the ELISA method over RIA is that it allows measurement of the isotype and subclass of the anti-Hib PRP antibodies through the use of selective secondary antibody reagents.

Although ELISA is widely used for measuring antibodies to protein antigens the measurement of antibody to purified cell surface carbohydrates by ELISA is hindered by the failure of polysaccharides to efficiently and reproducibly adhere to a solid phase. PRP is negatively charged and binds poorly to polystyrene microtitre plates. Techniques to enhance binding of Hib PRP to polystyrene microtitre plates include the use of high concentrations (30-100 μg/ml) of PRP and extended coating times (Callahan et al 1979; Dahlberg and Branefors 1980; Barra et al 1988). Alternative procedures to adsorb polysaccharides have included pre-coating of plates with poly-L-lysine (PLL) (Leinonen and Frasch 1982) or covalent binding to poly-L-lysine (Gray 1979; Kaplan et al 1983), biotin (Schneerson et al 1980; Sutton et al 1985), tyramine (Anthony et al 1982) or albumin (Phipps et al 1990). Some of these methods have high background values and questionable specificity.

This chapter describes the development of ELISA techniques to measure IgG, IgG1 and IgG2 subclass specific Hib PRP antibody concentrations. The PRP was produced in the laboratory and linked to poly-L-lysine to allow reproducible binding to the microtitre plates.

The purpose of establishing these assays was to determine:

- The IgG, IgG1 and IgG2 specific Hib PRP antibody levels in acute and convalescent sera of children with Hib meningitis and Hib osteomyelitis/septic arthritis.
- The IgG, IgG1 and IgG2 specific Hib PRP antibody concentrations in healthy infants before and after immunization with Hib vaccine.

4.2 DETERMINATION OF OPTIMAL REAGENTS AND CONDITIONS

Various contributing technical factors and test conditions affecting assay performance needed assessment before finalised assay procedures were established (4.3)

4.2.1 Plates

The following ninety six well plastic plates were tested: Nunc Maxisorb flat-bottomed (Weil Organisation, Catalogue No 442404), Nunc Maxisorb C (certificated) flat bottomed (Catalogue No 439454), Nunc Polysorb flat bottomed (Catalogue No 475094), Nunc Polysorb C (certificated) flat bottomed (Catalogue No 449824) and Falcon PVC flat bottomed (Catalogue No 3912).

4.2.1.1 *Total IgG Hib PRP ELISA*

Although the shape of the standard curve was similar for all plates tested (Figure 4.1), OD readings varied by as much as 0.70 (32%) for a 1:100 dilution of serum. Maxisorb, Maxisorb C and Falcon plates resulted in higher OD readings than Polysorb plates. No major differences were found in negative control wells which were acceptably low in all plates (Table 4.1).

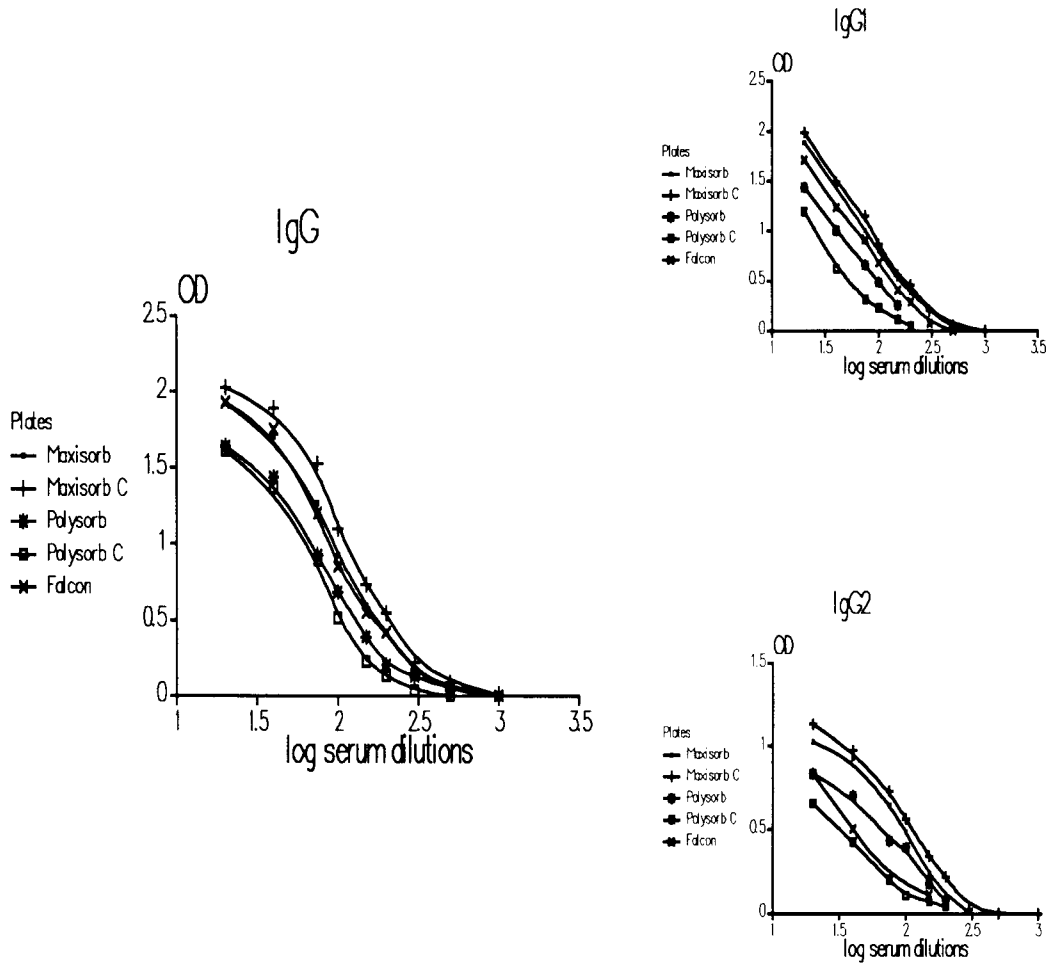


Figure 4.1: IgG, IgG1 and IgG2 Hib PRP ELISA standard curves comparing microtitre plates: Maxisorb (•); Maxisorb certificated (+); Polysorb (*); Polysorb certificated (□); and Falcon (x).

TABLE 4.1: COMPARISON OF IMMUNOPLATES IN THE IgG Hib PRP ASSAY

Plates	OD of 1:100 Serum dilution	OPTICAL DENSITY (OD) OF BACKGROUND BLANKS (% of 1:100 serum dilution)			
		Minus Antigen	Minus Serum	Minus Moab	Minus Serum and Moab
Maxisorb	1.935	0.104 (5.4%)	0.005 (0.3%)	0.126 (6.5%)	0.037 (1.9%)
Maxisorb C	2.195	0.114 (5.2%)	0.015 (0.7%)	0.092 (4.2%)	0.016 (0.7%)
Polysorb	1.709	0.106 (6.2%)	0.007 (0.4%)	0.066 (.3.9%)	0.003 (0%)
Polysorb C	1.493	0.074 (5.0%)	0.003 (0%)	0.051 (3.4%)	0.002 (0.1%)
Falcon	1.897	0.090 (4.7%)	0.005 (0%)	0.057 (3.0%)	0.006 (0%)

C = certificated plates

4.2.1.2 *IgG1 Hib PRP ELISA*

The Maxisorb and Falcon plates gave steeper standard curves than the Polysorb plates (Figure 4.1).

The OD readings were lower using the Polysorb plates, particularly Polysorb C. No significant differences in any of the background blanks were found but the minus monoclonal blanks were relatively high (± 18% of the maximum signal) (Table 4.2).

4.2.1.3 *IgG2 Hib PRP ELISA*

The Maxisorb and Maxisorb C plates gave steeper standard curves than the Polysorb or Falcon plates (Figure 4.1). The background blanks were similar to those of the IgG1 Hib assays with high minus monoclonal blanks (±20%) (Table 4.3).

4.2.1.4 *Conclusion and discussion*

These results show that very different OD readings and curve shapes were obtained with different plates. The Nunc Maxisorb C (certificated) plates were selected for use as they were found to be suitable in all 3 assay systems giving steep sigmoid shaped curves. There was no significant difference between the Maxisorb and Maxisorb C plates, the only manufacturing difference is the stringency of the quality control checks.

In previously reported ELISAs measuring IgG and IgG subclass specific Hib antibodies various plates have been used viz: Dynatech (Sutton et al 1985; Barra et al 1988), Immunolon (Barra et al 1988; Herrmann et al 1992), Costar (Shackelford et al 1987) and Nunc polysorb (Phipps et al 1990) (Table 4.4). In these assays Hib PRP had been conjugated to a variety of different proteins and it is not stated if other plates were assessed.

TABLE 4.2: COMPARISON OF IMMUNOPLATES IN THE IgG1 Hib PRP ASSAY

Plates	OD of 1:40 Serum dilution	OPTICAL DENSITY (OD) OF BACKGROUND BLANKS (% of 1:40 serum dilution)			
		Minus Antigen	Minus Serum	Minus Moab	Minus Serum and Moab
Maxisorb	1.808	0.043 (2.4%)	0.029 (1.6%)	0.285 (15.8%)	0.103 (5.7%)
Maxisorb C	1.856	0.063 (3.4%)	0.098 (5.3%)	0.300 (16.2%)	0.056 (3.0%)
Polysorb	1.308	0.032 (2.5%)	0.021 (1.6%)	0.232 (17.7%)	0.033 (2.5)
Polysorb C	0.883	0.030 (3.4%)	0.005 (0.6%)	0.186 (21.1%)	0.006 (0.7%)
Falcon	1.580	0.023 (1.5%)	0.045 (2.9%)	0.280 (17.7%)	0.030 (1.9%)

C = certificated plates

TABLE 4.3: COMPARISON OF IMMUNOPLATES IN THE IgG2 Hib PRP ASSAY

Plates	OD of 1:20 Serum dilution	OPTICAL DENSITY (OD) OF BACKGROUND BLANKS (% of 1:20 serum dilution)			
		Minus Antigen	Minus Serum	Minus Moab	Minus Serum and Moab
Maxisorb	1.387	0.110 (8.0%)	0.031 (2.5%)	0.297 (21.5%)	0.038 (2.8%)
Maxisorb C	1.544	0.130 (8.4%)	0.051 (3.3%)	0.291 (18.8%)	0.050 (3.2%)
Polysorb	1.025	0.074 (7.2%)	0.014 (1.4%)	0.215 (21.6%)	0.020 (1.9%)
Polysorb C	0.906	0.048 (5.3%)	0.005 (0.6%)	0.198 (21.9%)	0.005 (0.6%)
Falcon	1.164	0.078 (6.7%)	0.004 (0.3%)	0.255 (21.9%)	0.007 (0.6%)

C = certificated plates

TABLE 4.4: ANTIGEN, PLATES AND ANTIBODIES USED IN REPORTED Hib ELISAs

Reference	IgG	IgG1	IgG2
Sutton et al 1985	Antigen: Biotinylated PRP Plates: Dynatech coated with avidin	-	-
Granoff et al 1986	Antigen: Hib Poly-L-lysine Plates: Costar	As for IgG Moab: HG II	As for IgG polyvalent monkey anti-human
Moss et al 1987	Antigen: Hib Poly-L-lysine Plates: Dynatech	As for IgG Moab: Hybritech 912	As for IgG Moab: HP6008
Barra et al 1988	Antigen: PRP (various methods) Plates: Dynatech	As for IgG Moab: HP6012	As for IgG, Moab: HP6014:HP6008 1:3
Hetherington et al 1990	Antigen: PRP tyraminated Plates: Nunc	-	-
Borradori et al et al 1990	Antigen: PRP tyraminated Plates: Dynatech	As for IgG Moab: HP6012	As for IgG Moab: HP6014
Phipps et al 1990	Antigen: PRP - ovalbumin Plates: Nunc	-	-
Ruths et al 1991	Antigen: Hib bacteria Plates: Greiner	As for IgG Moab: MH161-1-E HP6012	As for IgG Moab: MH-161-1-E HP6008
Booy et al 1992	Farr type RIA	Antigen: PRP poly-L-lysine Plates: immunolon Moab: HP6012	As for IgG1 Moab: HP6014:HP6008
Herrmann et al 1992	Antigen: PRP poly-L-lysine Plates: Immunolon Moab: HP6017 biotinylated	As for IgG Moab: HP6069 biotinylated	As for IgG Moab: HP6002

4.2.2. Comparison of PRP Antigens

No preparation of PRP is commercially available. Purified *H.influenzae* PRP was prepared in the laboratory from an organism isolated from a patient with invasive Hib meningitis. The method of extraction and purification of PRP was adapted fron Insel and Anderson (1986a) and is described in detail in Appendix B. Various batches of PRP were prepared for use in these assays and each batch was verified against gifts of antigen obtained from the following sources:

- Purified Hib PRP (gift from Pasteur Merieux, May Baker, SA)
- Hib oligosaccharide-human serum albumin (Hbo-HA) (Praxis Biologics, Inc.)
- Hib capsular antigen (PRP, sodium salt form) gift from Dr P Anderson, University of Rochester via Lederle Laboratories.

The usual coating concentration of antigen is between 1-10 μg/l. The recommended coating concentration for the Hbo-HA was 1 μg/ml.

ELISAs were established using these antigen preparations and and the prepared PRP antigen with the US Standard human anti-*haemophilus influenzae* antibody serum (Lot 1983) as a standard antibody. The Hbo-HA was coated directly onto the plate at concentrations of 1 μg/ml and 5 μg/ml. The other antigen preparations were linked to poly-L-lysine using the method described in Appendix C prior to coating of the plates. The coating concentration of the various preparations was as follows: PRP - Merieux - 5 μg/ml; PRP - Lederle 5 μg/ml; in-house PRP 5 μg/ml and 10 μg/ml. Curve shape (i.e. parallelism), OD readings and concentrations were considered in the evaluation.

The 'in-house' antigen preparation at a coating concentration of either 5 or 10 μg/ml gave very similar shaped curves to the 'recognized' antigen preparations (Figure 4.2).

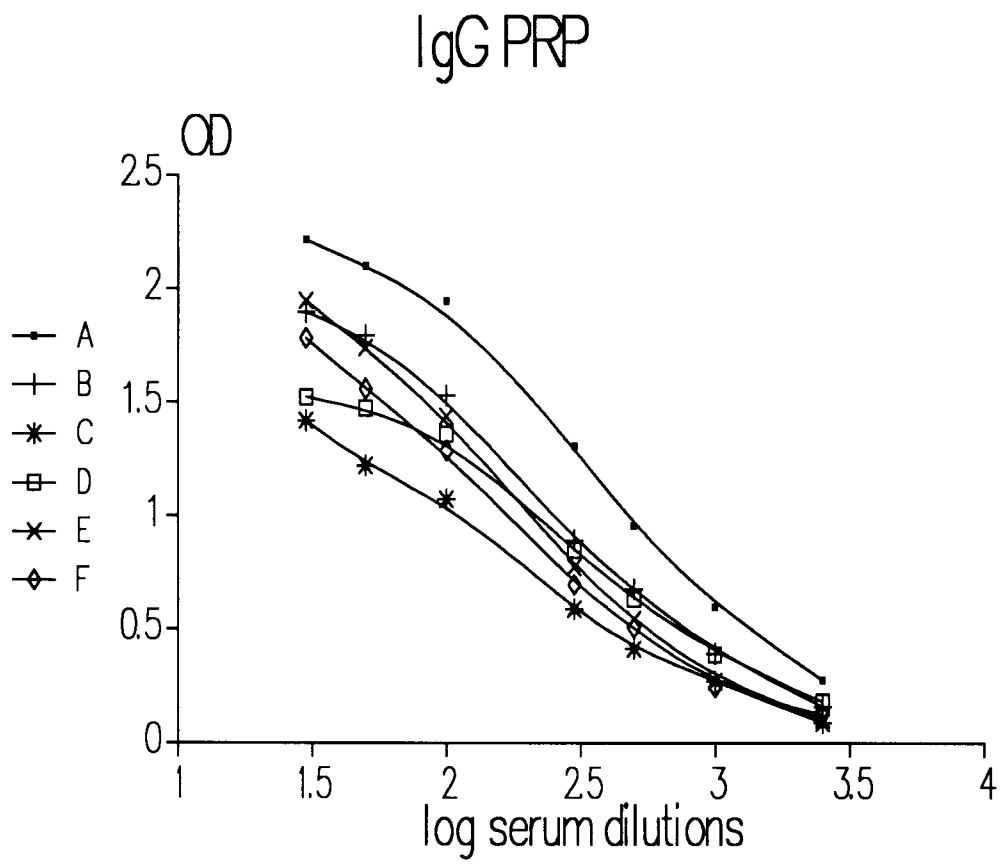


Figure 4.2: Standard curves of IgG Hib PRP ELISA assaying the reference serum with different antigen preparations: A=Merieux 5 μg/ml (▪); B=Hbo-HA 5 μg/ml (+); C=Hbo-HA 1 μg/ml (*); D=Lederle 5 μg/ml (□); E=in-house PRP 10 μg/ml (x); F=in-house PRP 5 μg/ml (◇).

Batches of PRP antigen were selected for use when they demonstrated curves that were parallel to the commercial antigen when assayed using the US reference standard (Lot 1983) and a post-vaccination serum pool (In-house antibody standard (Section 4.2.5)).

4.2.3 Antigen Coating

4.2.3.1 *IgG, IgG1 and IgG2 Hib PRP ELISAs*

Concentrations of in-house PRP antigen ranging from 1 μg/ml to 50 μg/ml was assessed using both the in-house antibody standard and US reference antibody standard. There was little difference in the shapes of the standard curves using antigen concentrations between 5 μg/ml and 50 μg/ml. When plates were coated with 1 μg/ml PRP the standard curve shifted to the left and flattened (Figure 4.3). A coating concentration of 5 μg/ml was selected for IgG, IgG1 and IgG2 Hib PRP ELISAs.

Various PRP antigen diluents were also tested to facilitate optimal binding to the plate. Phosphate buffered saline (PBS), PBS with 0.5% human serum albumin (HSA), glycine/Tris buffer (pH 7.3) and carbonate buffer (pH 9.6) were used. Carbonate buffer was selected as this diluent resulted in the lowest minus antigen blanks, highest ODs and steepest standard curve. Incubation of coating antigens for 2 hours as opposed to overnight coating was also tested, but no significant differences were noted.

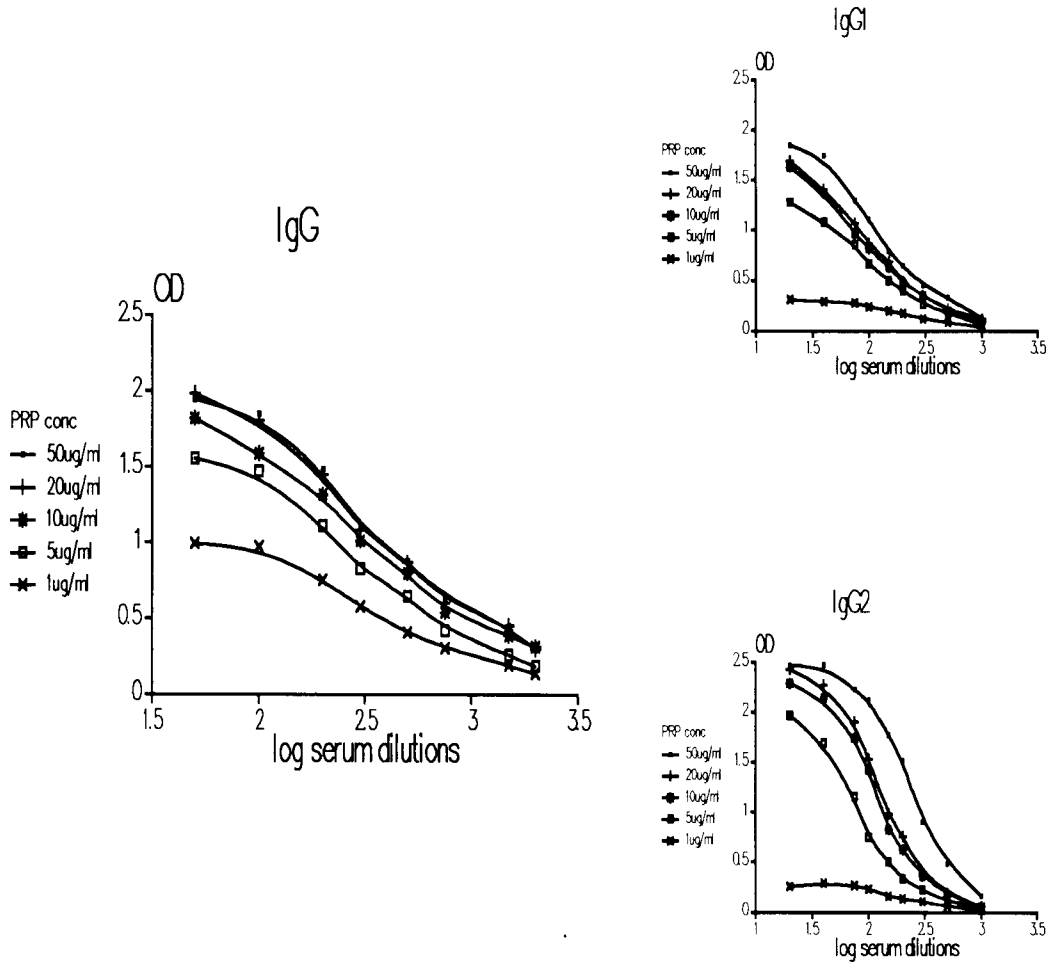


Figure 4.3: IgG, IgG1 and IgG2 Hib PRP ELISA standard curves of in-house serum standard assayed using a range of PRP concentrations: 50 μg/ml (•); 20 μg/ml (+); 10 μg/ml (*); 5 μg/ml (□); 1 μg/ml (x).

4.2.3.2 *Conclusion and discussion*

The assays performed using plates coated with either the in-house PRP antigen or 'recognised antigens' (Figure 4.2) gave parallel standard curves using the US (Lot 1983) standard serum. The control result read off these curves yielded the same values irrespective of which antigen was used.

A coating concentration of 5 μg/ml in-house PRP antigen was suitable for the IgG, IgG1 and IgG2 Hib PRP ELISAs.

In preliminary experiments when unlinked PRP antigen was coated directly onto the plates in the total IgG assay system, ODs of approximately a third of linked PRP were obtained (results not shown). This confirmed the efficacy of the poly-L-lysine-linking in facilitating antigen binding to the plate. Coating PRP coupled to poly-L-lysine has been used in several reported studies (Granoff et al 1986a; Moss et al 1987; Herrmann et al 1992).

The poly-L-lysine linked to PRP did not interfere in the assay system. In the IgG, IgG1 and IgG2 Hib PRP assays wells coated with 5-50 μg/ml of poly-L-lysine alone gave signals of less than 5%, 3.5% and 12% respectively of the signal achieved with Hib PRP linked to poly-L-lysine.

Phipps et al (1990) reported that if Hib PRP conjugated to human serum albumin was diluted in PBS made up with sterile distilled non-pyrogenic water, the minus antigen well blanks were reduced. In these experiments no difference was noted in the signal of the minus antigen wells irrespective of whether distilled water or sterile non-pyrogenic distilled water was used in the antigen-coating carbonate buffer.

4.2.4 Blocking Agents and Diluents

No major differences were seen when 1% casein was compared to 2% BSA as a blocking agent. 1% casein as a diluent for monoclonal antibody, serum and conjugate reduced the minus antigen readings by half. For the reasons of cost consideration and availability, 1% casein was selected for blocking and diluent solutions.

4.2.5 Serum Standards and Controls

4.2.5.1 *IgG, IgG1 and IgG2 Hib PRP ELISA*s

The US Standard human anti-*haemophilus influenzae* type b capsular polysaccharide serum (Lot 1983) from the Office of Biologics, FDA, Bethesda, Maryland was used as a reference antibody standard. The reference values of anti-Hib antibodies were: IgG = 60.9 μg/ml, IgG1 = 27.0 μg/ml and IgG2 = 13.4 μg/ml. Initial assays were established using this standard, but due to its limited availability an in-house antibody standard was prepared. This was done by vaccinating healthy volunteers and testing the antibody response 2, 4 and 6 weeks post vaccination. Five adult volunteers were immunized with HIB-IMMUNE (*H.influenzae* type b polysaccharide vaccine) (Lederle). Ten adult volunteers were immunized with HibTITER (*H.influenzae* tybe b polysaccharide covalently linked to non-toxic diphtheria toxin variant CRM_{197}) (Praxis Biologics, Rochester, NY). Five adult volunteers were immunized with Pedvax Hib™ (*H.influenzae* type b conjugate vaccine linked to meningococcal protein conjugate) (Merck, Sharp and Dohme). Although these vaccines were not licenced in South Africa at the time of immunization, permission for their use in adult volunteers was obtained from the Medical Control Council. All sera were initially stored individually in aliquots at -70°C. After individual testing for anti-Hib PRP antibodies, the sera from high titre vaccine responders were pooled to form an in-house standard. This variety of vaccines and number of post immunization bleeds ensured a range of antibody levels with differing isotype and subclass composition. When the in-house standard was tested against the US Standard (Lot 1983) parallel standard curves were obtained between the two. The in-house standard curve was shifted to the left indicative of lower values (Figure 4.4). The US standard serum (Lot 1983) was used to calculate anti-Hib PRP antibody

values for the in-house standard: IgG = 35.2 μg/ml, IgG1 = 18.01 μg/ml, IgG2 = 5.59 μg/ml. Subclass specific antibody levels of various test sera were calculated using both these reference antibody preparations. Similar results were obtained when the US standard and the in-house standard antibody were used to construct standard curves. The in-house pooled sera was used as the standard in routine assays and the US Standard (Lot 1983) served as a quality control serum. Dilutions of the US Standard as a control were 1:150 and 1:200 for all assays.

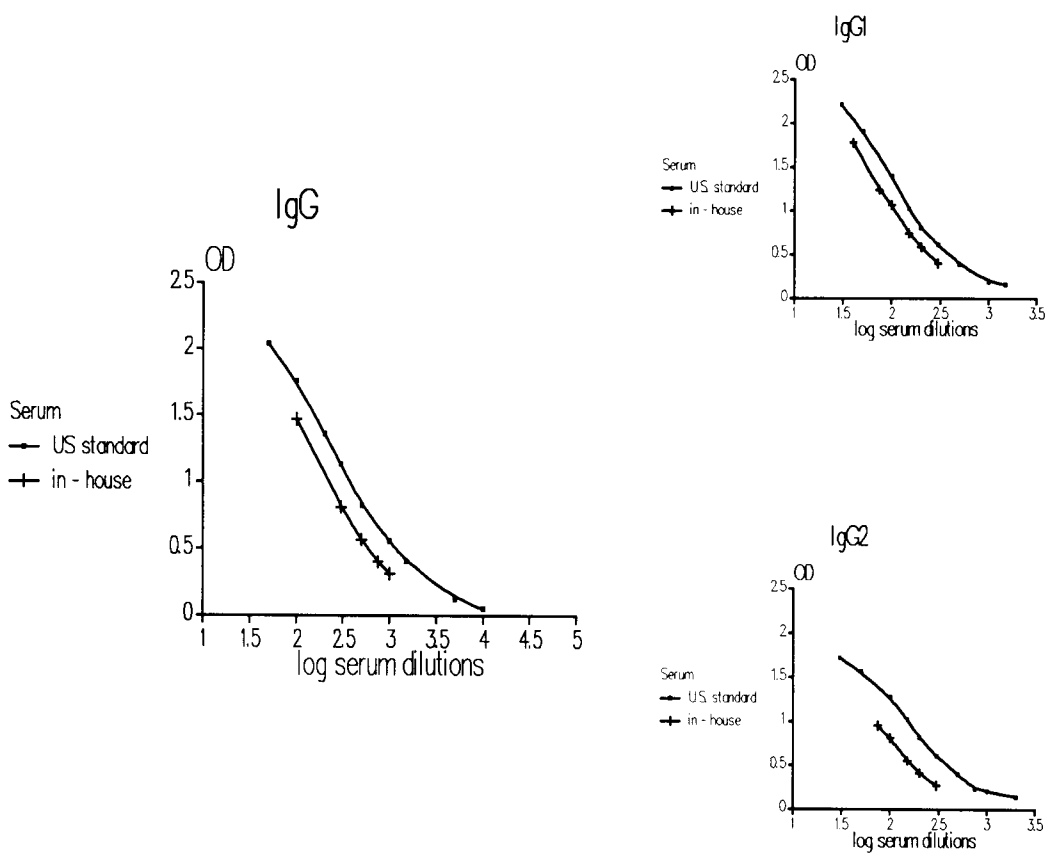


Figure 4.4: Comparison of IgG, IgG1 and IgG2 Hib PRP ELISA standard curves of in-house standard serum (+); with the US standard serum (Lot 1983) (•); demonstrating parallelism.

There was a substantial discrepancy in the reference serum (Lot 1983) between the total IgG anti-Hib antibodies and the sum of IgG1 and IgG2 antibody concentrations. A similar discrepancy was carried over into the in-house standard. In general immunological assays reflect the affinity as well as the concentration of antibody. The serum IgG antibodies to Hib PRP include several subclasses all with a potential for variation in affinity. Two markers of a qualitative IgG subclass assay are the ability of the test and reference sera to dilute in parallel and on agreement between the sum of IgG1-4 and the total IgG. The total IgG value of 60.9 μg/l in the reference standard (Madore et al 1990) may be an overestimate as Herrmann et al (1992) using a different assay and calibration system estimated the value of this reference standard to be 21.9 μg/ml. In their assay the sum of IgG1-4 anti-Hib (21.5 μg/ml) was only 1.8% less than that of the total IgG (21.9 μg/ml). Reasons for these differences were assumed to be due to inherent technical variables (in particular the monoclonal antibodies) in these analyses.

4.2.6 Incubation Period

Antigen coating times of 2 hours at room temperature vs overnight at 4°C and serum incubation times of 1 hour at 37°C vs overnight at 4°C were compared. No significant difference was found between the two incubation conditions.

4.2.7 Monoclonal Antibody Selection

4.2.7.1 *IgG Hib PRP ELISA*

The use of biotinylated monoclonal antibody (HP6017) resulted in very low OD readings and was unsuitable. ELISA formats without using a monoclonal antibody and using an anti-human conjugate or incorporating unbiotinylated monoclonal antibody (HP6064) both produced good steep

standard curves (Figure 4.5) with all blanks and control readings acceptable (Table 4.5). Unbiotinylated HP6064 was selected for use in these assays. A dilution range of 1:500 to 1:3 000 was tested and a 1:2 500 dilution was found to be satisfactory.

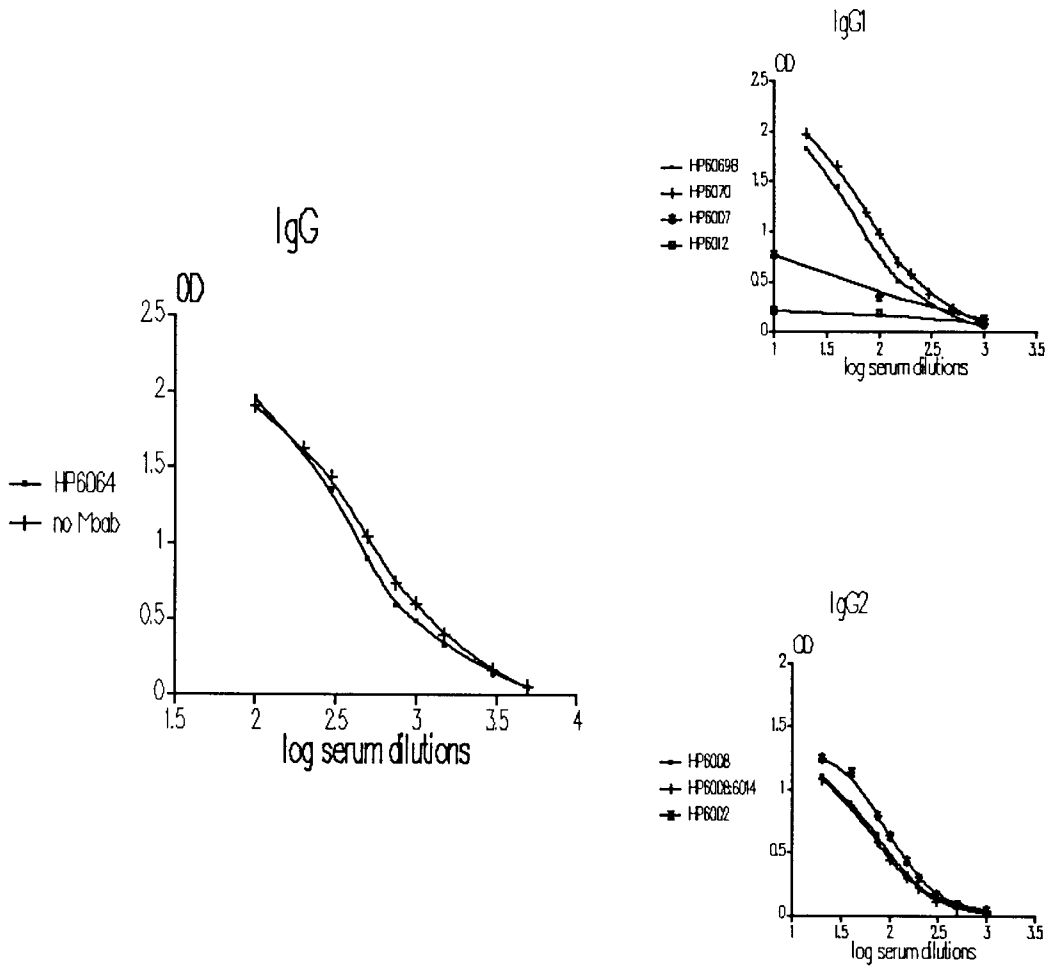


Figure 4.5: IgG, IgG1 and IgG2 Hib PRP ELISA standard curves assayed with various Moabs: IgG: HP6064 (▪), No Moab (+) (without monoclonal antibody and using an anti-human IgG conjugate); IgG1: biotinylated HP6069 B (▪), HP6070 (+), HP6007 (*), HP6012 (□); IgG2 = HP6008 (•), HP6008:6014 @ 2:1 (+), HP6002 (*)

TABLE 4.5: BACKGROUND BLANKS IN THE IgG Hib PRP ASSAY

Moab	Maximum Optical Density of Standard	OPTICAL DENSITY OF BACKGROUND BLANKS (% of the maximum signal)		
		Minus antigen	Minus serum	Minus Moab
HP 6064	1.959	0.125 (6.4)	0.010 (0.5)	0.006 (0.3)
No Moab*	1.901	0.150 (7.9)	0.017 (0.9)	-
HP6017	0.332	0.025 (7.5)	0.008 (2.4)	0.005 (1.5)

* **Without the use of a monoclonal antibody and using an anti-human IgG conjugate**

4.2.7.2 *IgG1 Hib PRP ELISA*

Virtually no difference in standard curve shape or background blanks was seen between the assays when either biotinylated (HP6069) or unbiotinylated (HP6070) monoclonal antibodies were used in the system (Figure 4.5). The HP6070 was chosen for use. The other monoclonal antibodies HP6012, HP6007, and unbiotinylated HP6069 resulted in much lower ODs when tested in the system at the same dilutions (Table 4.6).

TABLE 4.6: BACKGROUND BLANKS IN THE IgG1 Hib PRP ASSAY

Moab	Maximum Optical Density of Standard	OPTICAL DENSITY OF BACKGROUND BLANKS (% of the maximun signal)		
		Minus antigen	Minus serum	Minus Moab
HP6069 Biotiny-lated	1.827	0.031 (1.7)	0.011 (0.6)	0.015 (0.8)
HP6070	1.861	0.026 (1.4)	0.015 (0.8)	0.022 (1.2)
HP6069 Unbio-tinylated	1.557	0.122 (7.8)	0.043 (2.8)	0.039 (2.5)
HP6012	0.211	0.046 (21.8)	0.060 (28.4)	0.224 (106.0)
HP6007	0.771	0.087 (11.3)	0.111 (14.4)	0.259 (33.6)

4.2.7.3 IgG2 Hib PRP ELISA

Although the curve shapes were very similar for both monoclonal antibodies tested (HP6008 and Biotinylated HP6002) (Figure 4.5) all the blanks were significantly lower using the biotinylated HP6002 (Table 4.7). A combination of unbiotinylated monoclonal antibodies HP6008:HP6014 in a ratio of 2:1 also reduced the minus antigen and minus monoclonal antibody blanks. The biotinylated antibody HP6002 could be used at a dilution of 1:1000 (1:250 - 1:3000 tested) when compared to a dilution of 1:500 for the HP6008:HP6014 combination and was selected for use in these assays.

TABLE 4.7: BACKGROUND BLANKS IN THE IgG2 Hib PRP ASSAY

Moab	Maximum Optical Density of Standard	OPTICAL DENSITY OF BACKGROUND BLANKS (% of the maximun signal)		
		Minus Serum	Minus antigen	Minus serum Moab
HP6002 Biotiny-lated	1.621	0.053 (3.3%)	0.003 (0.2%)	0.005 (0.3%)
HP6008	1.394	0.141 (10.1%)	0.034 (2.4%)	0.297 (21.5%)
HP6008: HP6014 2:1	1.504	0.031 (2.1%)	0.023 (1.5%)	0.208 (13.8%)

4.2.7.4 *Conclusion and discussion*

The majority of published total IgG anti-Hib PRP assays have not utilised monoclonal antibodies (Table 4.4). Herrmann et al (1992) used the biotinylated HP6017 but it was found to give very low OD readings in this sytem. Unbiotinylated HP6064 gave a steep curve and strong signal.

In the reports of anti-IgG1 specific Hib PRP ELISAs (Booy et al 1992; Barra et al 1988; Borradari et al 1990) used the IgG1 specific monoclonal antibody HP6012 and Herrmann et al (1992) used the biotinylated Moab HP6069. These two monoclonal antibodies as well as the monoclonal antibodies HP6070 and unbiotinylated HP6069 were compared in this study. HP6070 and HP6069 were found to give the best results in this system.

There are only a few reported methods of anti-IgG2 specific ELISAs using commercially available monoclonal antibodies (Barra et al 1988; Borradori et al 1990; Ruths et al 1991; Herrmann et al 1992; Booy et al 1992) (Table 4.4). The most commonly used monoclonal antibodies were HP6014 and HP6008 either alone or together in various ratios. The findings in this study showed that in this system the biotinylated monoclonal antibody HP6002 gave the best results and was again similar to the report of Herrmann et al (1992).

4.2.8 Evaluation of Conjugated Antibody/Enzyme/Substrates

4.2.8.1 *IgG Hib PRP ELISA*

The detection antibodies assessed were

- Peroxidase conjugated $F(ab')_2$ fragment goat anti-mouse IgG (Fc fragment, gamma chain specific), Cappel (Catalogue No 3311-0121).
- Goat anti-mouse IgG (Fc specific) peroxidase conjugate, Sigma Immunochemicals (Catalogue No A-2554)

- Sheep anti-mouse IgG, A, M (heavy and light chains) peroxidase conjugate, Binding Site (Catalogue No PP270).

Very similar results were obtained with all 3 horseradish peroxidase conjugates. The Sigma conjugate was difficult to import and the Cappel conjugate was unsuitable for the IgG1 assay due to the high monoclonal antibody blanks (15-20% of the maximum signal). A dilution of 1:5 000 was concentrated enough to attain the required OD readings. The Binding Site conjugate, was selected as the backgrounds blanks were low (1-2% of the maximum signal).

4.2.8.2 *IgG1 Hib PRP ELISA*

The same peroxidase conjugates were tested in the IgG1 assay system with dilutions selected as follows: Cappel 1:750; Sigma 1:1 000 and Binding Site 1:1 000. Binding Site sheep anti-mouse HRP conjugate was selected for the same reasons as in the IgG Hib PRP assay.

4.2.8.3 *IgG2 Hib PRP ELISA*

As a biotinylated monoclonal antibody was used in this assay, streptavidin conjugated horseradish peroxidase was used in the detection system. Two streptavidin peroxidase conjugates were assessed viz. Zymed, (Catalogue No 43-4323) and Calbiochem (Catalogue No 189733). Both these conjugates were tested at dilutions ranging from 1:5 000 to 1:40 000. The Zymed streptavidin peroxidase conjugate (1:17 500) was selected although both conjugates were equally suitable in the assay at similar dilutions.

4.3. METHODS

The exact methods based on the experiments described above are summarised in this section.

4.3.1 IgG Hib PRP ELISA

All reagents and samples were added to the wells in 100 μl volumes except for the blocker at 250 μl and 2N H_2SO_4 at 50 μl. Flat bottomed Nunc Maxisorb microtitre plates were coated with Hib PRP at a concentration of 5 μg/ml in carbonate buffer (pH 9.6). Uncoated wells (minus Hib PRP) contained only carbonate buffer. Plates were incubated in a moist chamber for 2 hours at room temperature and blocked with 1% casein for 1 hour at 37°C. After washing, sera which had been previously diluted in 1% casein, were added to quadruplicate wells. Pooled post vaccination sera was used to obtain a standard curve by diluting from 1:30 to 1:3 500 (1:30, 1:50, 1:100, 1:200, 1:300, 1:500; 1:1 000, 1:2 500, 1:3 500) which was equivalent to 1173 - 10 ng/ml range. Two dilutions of the US standard (1983) served as a control (1:150, 1:200). Adult sera were assayed at 1:100 and 1:500 dilutions. Samples with absorbance readings which did not fall on the linear portion of the curve were repeated at appropriate dilutions. The sera of infants containing low levels of antibody had to be assayed at a 1/2 dilution which usually read off the lower slopes of the curve. Once added, serum dilutions were incubated overnight at 4°C. Wells containing only diluent (minus serum) were included as negative controls. Five washes with PBST followed before the addition of mouse anti-human IgG antibody (HP6064) at a dilution of 1:2 500 in 1% casein. The plates were incubated for 1 hour at 37°C and then washed 5 times in PBST. Sheep anti-mouse IgG, A, M conjugated to horseradish peroxidase (Binding Site, Catalogue No PP270) was added at a dilution of 1:5 000 and incubated at room temperature for 1 hour. The plates were washed five times with PBST before the addition of OPD. After 15 minutes the reaction was stopped with 2N H_2SO_4 and the plates were read at 492 nm.

4.3.2 IgG1 Hib PRP ELISA

This method was identical to the IgG assay described above except for the following:

- Standard curve dilutions ranged from 1:20 - 1:1 000 which was equivalent to 900 - 18 ng/ml
- Mouse anti-human IgG1 monoclonal antibody (HP6070) was added at 1:500 dilution.
- Sheep anti-mouse IgG, A, M peroxidase conjugate was diluted to 1:1 500

4.3.3 IgG2 Hib PRP ELISA

All the steps were similar to that of previous assays except for:

- Standard curve dilutions ranged from 1:20 - 1:1 000 which is equivalent to 279.5 - 5.6 ng/ml
- Mouse anti-human IgG2 biotinylated monoclonal antibody (HP6002) was added at a dilution of 1:1 000.
- Streptavidin conjugated horseradish peroxidase (Zymed, Catalogue No 43-4323) diluted 1:17 500 in 1%casein was then added.

4.3.4 *Calculation of results*

Standard dose curves were constructed from OD readings and concentrations that had been entered into a curve fitting four parameter logistic programme based on the mathematical model described elsewhere (Chapter 1.12). These standard curves were used to calculate the Hib PRP antibody concentrations.

4.4 ASSAY STANDARDIZATION

4.4.1 Standard Curves

The in-house pooled serum standard, quantitated against a US Reference serum (1983) (4.2.5) was used to construct standard curves for IgG, IgG1 and IgG2 specific Hib antibodies relating absorbance to antibody concentration (Figure 4.4). Each standard curve was constructed from nine different triplicate dilutions of standard serum on each plate. The titration curves of the in-house standard and US Reference serum were parallel in all three assays (Figure 4.4). The immunoglobulin concentrations of the standard curves range from: 1173 to 10 ng/ml for IgG, from 900 to 18 ng/ml for IgG1 and 279.5 to 5.6 ng/ml for IgG2. Individual post vaccination sera gave titration curves that demonstrated linearity and parallelism with the in-house standard curve (Figure 4.6).

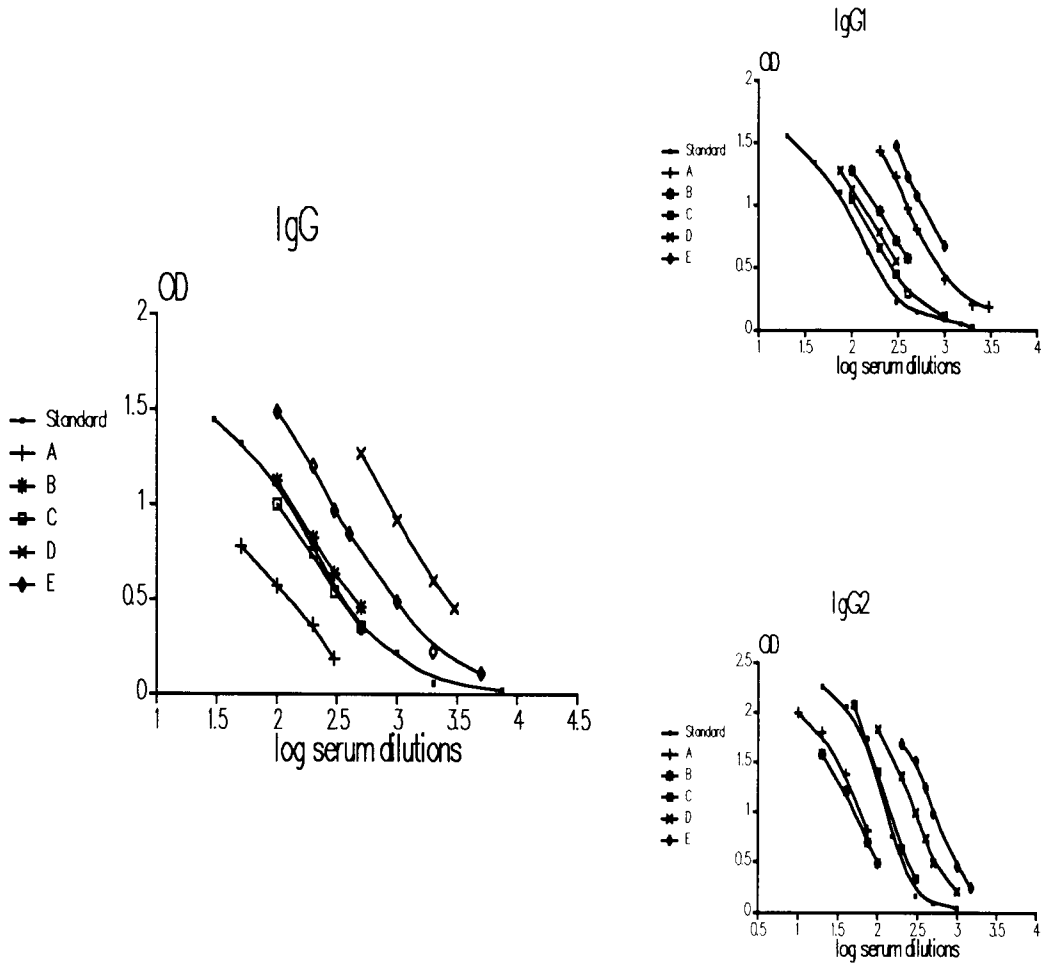


Figure 4.6: Curves of individual post-vaccination responders serum, A (+), B (*), C (□), D (x) and E (◇) for IgG, IgG1 and IgG2 Hib PRP and compared to the in-house serum standard curve (▪) for linearity and parallelism.

4.4.2 Precision and Sensitivity

To determine assay precision intra-plate, intra-assay, inter-assay and inter-dilutional CVs were calculated (Table 4.8).

TABLE 4.8: EVALUATION OF Hib PRP ELISA ASSSAY PRECISION

	IgG	IgG1	IgG2
Intra-plate CV (%)	2.1	2.3	1.7
Intra-assay CV (%)	4.3	2.7	2.1
Inter-assay CV (%)	9.9	8.8	18.0
Inter-dilutional CV (%)	7.2	6.7	11.4

CV = coefficient of variation

The lowest level of sensitivity in these assays was 0.011 μg/ml for IgG, 0.012 μg/ml for IgG1 and 0.006 μg/ml for IgG2. The background minus antigen blanks were always less than 7% of the standard OD reading at the lowest dilution.

Assay results in which the control value was more than 2 SD from the known mean value of the control were repeated.

4.4.3 Inter-laboratory Comparison

It has been assumed that antibody levels measured in different laboratories by different methods are similar because all assays used a US (FDA) serum standard for quantitation of antibody binding. There is evidence that quantitation of antibody in various laboratories using different assay methods differs significantly (Ward et al 1988; Edwards et al 1987; Greenberg et al 1987).

Hib PRP was measured in 5 adult samples pre- and two weeks post-HibTITER immunization and 5 samples from children with Hib meningitis using the ELISAs described here and also and by Dr P Shackelford, Department of Pediatrics, Washington School of Medicine, St Louis, Missouri using their ELISA and RIA (FARR) methods. Although the results are largely in agreement some variability is present (Table 4.9).

TABLE 4.9: INTER-LABORATORY COMPARISON OF HIB PRP ANTIBODY MEASUREMENT (μg/ml)

	1ST SPECIMEN			2ND SPECIMEN		
Specimen	ELISA*	RIA*	RXH** ELISA	ELISA*	RIA*	RXH** ELISA
A	2.9	4.0	1.94	972	475	572.7
B	0.7	3.4	0.22	38	30	11.0
C	≤0.3	3.3	0.037	73	125	61.4
D	5.6	26.0	18.10	41	45	39.6
E	4.0	8.6	5.02	313	350	386.9
F	≤0.3	0.10	0.000			
G	≤0.3	0.08	0.063			
H	≤0.3	0.08	0.061			
I	≤0.3	0.42	0.044			
J	≤0.3	0.12	0.043			

1st specimen = Initial specimen taken prior to HibTITER immunization or at the time of dignosis of Hib meningitis
2nd specimen = Specimen taken 2 weeks post-immunization with HibTITER
* = Hib PRP antibody measured by Dr Shackelford, St Louis, Missouri by ELISA + radioimmunoassay (RIA) methods
** = IgG Hib PRP measured by the ELISA developed in this study

4.4.4 Specificity

The specificity of the IgG, IgG1 and IgG2 Hib ELISA assays were assessed by a competitive inhibition ELISA using soluble antigen inhibition with either Hib PRP, or non-specific carbohydrate antigen *S.aureus* teichoic acid (Appendix D) and pneumococcal polysaccharide (Pneumovax).

These ELISAs were carried out as described in section 4.3.1, 4.3.2 and 4.3.3 except that the competitive antigens were pre-incubated with standard serum for 2 hours at room temperature before the addition of the mixture to the PRP coated wells.

Unlinked PRP was mixed with serum in concentrations ranging from 50 to 0.01 μg/ml. *S.aureus* teichoic acid was added to the serum in dilutions ranging from 50 to 0.01 μg/ml, and pneumococcal polysaccharide (Pneumovax Merck, Sharpe and Dohme) from 10 μg/ml to 0.01 μg/ml. A dilution of the standard serum falling on the linear section of the sigmoid curves for these assays was selected viz 1:150. Binding of the Hib PRP antibody to wells coated with Hib PRP could be inhibited by incubation with unlinked Hib PRP antigen but not with the non-specific carbohydrate antigens *S.aureus* teichoic acid or pneumococcal polysaccharide (Table 4.10). These results show that these assays are specific for the measurement of Hib PRP antibodies.

TABLE 4.10: COMPETITIVE INHIBITION ELISAs FOR SPECIFICITY IN Hib PRP ASSAYS

PRP μg/ml	% INHIBITION IgG	IgG1	IgG2
50	100	99	95
20	96	97	97
10	81	84	94
5	79	82	92
1	63	69	84
0.75	65	78	86
0.5	46	64	76
0.25	33	50	63
0.1	23	33	45
0.05	18	25	31
0.025	3	10	7
0.01	0	3	12

* *S.aureus* teichoic acid or pneumococcal polysaccharide (Pneumovax) (50-0.01 μg/mL) caused no inhibition in these assays

4.4.5 Antibody Affinity

4.4.5.1 *DEA-ELISA for IgG1 and IgG2 Hib PRP*

These assays were based on the method described by Devey et al (1988), using DEA as the chaotropic agent. The details of the ELISAs are identical to those described earlier (4.3) except for the addition of DEA to the serum diluent.

Serial dilutions of sera were made in 1% casein in the presence or absence of various concentrations of DEA (5-50 mM). OD was plotted against serum dilution and the left shift in the dose response curve with DEA measured at 50% of the maximum OD of the curve without DEA. The absorbance readings in the absence of DEA represented total binding of specific antibody. The shift in the log of the serum dilution from the initial OD reading to 50% of the initial OD reading was calculated with the various molar concentrations of DEA. Results were expressed as the $\log_{10}$ of the shift.

The addition of increasing concentrations of DEA from 5-50 mM in the serum diluent resulted in an increasing parallel shift to the left of the dose response curve for both IgG1 and IgG2 Hib PRP antibodies (Figure 4.7). Calculation of these antibody shifts at 50% of the maximum OD showed that when using the in-house standard serum, the shifts were similar for IgG1 and IgG2 Hib PRP ELISAs (Table 4.11). These results suggest that the IgG1 and IgG2 Hib PRP antibody responses are of similar affinity.

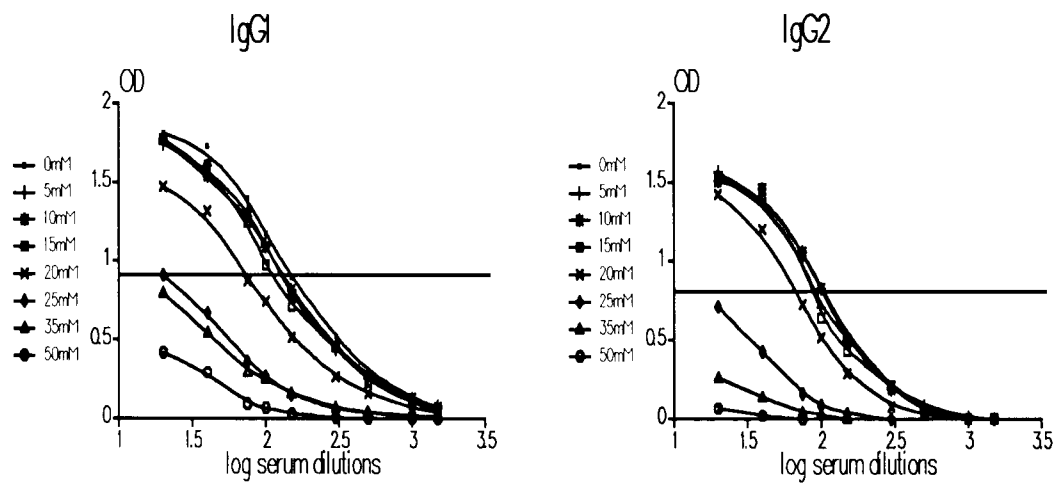


Figure 4.7: Effect of increasing concentrations of DEA (0-50 mM) on the standard curves of IgG1 and IgG2 Hib PRP antibodies. DEA concentrations: 0 mM (•), 5 mM (+), 10 mM (*), 15 mM (□), 20 mM (x), 25 mM (◇), 35 mM (Δ) and 50 mM (o). The shift was measured at 50% of the maximum OD with no DEA which is represented by the solid line drawn at 0.9 and 0.8 OD for the IgG1 and IgG2 Hib PRP ELISAs respectively

TABLE 4.11: EFFECT OF INCREASING CONCENTRATIONS OF DEA ON THE LEFT SHIFT OF THE DOSE-RESPONSE CURVE OF IgG1 AND IgG2 HIB PRP ANTIBODIES

DEA Concentration (mM)	Log_{10} dilution at 50% max OD		Log_{10} shift at 50% max OD		% shift $\frac{Log_{10}\text{-shift} \times 100}{Log_{10}\text{ dilution}}$	
	OD=0.9 IgG1	OD=0.8 IgG2	OD=0.9 IgG1	OD=0.8 IgG2	IgG1	IgG2
0	2.176	2.038	-	-	-	-
5	2.141	1.997	0.035	0.041	1.6	2.0
10	2.130	1.982	0.046	0.056	2.2	2.8
15	2.078	1.974	0.098	0.064	4.7	3.2
20	1.888	1.845	0.288	0.193	15.2	10.5
25	1.324	1.240	0.852	0.798	64.3	64.3
35	1.065	0.548	1.111	1.490	104	272

4.5 SUMMARY

Few of the studies reporting measurement of human IgG antibodies to Hib PRP have used the same reagents or methods and thus it is difficult to compare reported IgG antibody levels and subclass patterns from these studies. Most studies have used Farr type radioimmunoassays to measure total anti-PRP antibodies and ELISA to measure IgG subclass specific antibodies to PRP.

ELISAs are described for the quantitation of total IgG, IgG1 and IgG2 anti-Hib PRP antibodies that employ commercially available HP series monoclonal antibodies. The coating of polysaccharide directly to polystyrene plates was problematical. In these ELISAs Hib PRP was prepared and covalently linked to poly-L-lysine to facilitate binding of the

Hib PRP to microtitre plates whilst still retaining its specific antigenicity. Precision, specificity, sensitivity and parallelism in the assays have been evaluated.

These assays were standardized using US Reference standard (Lot 1983) and inter-laboratory analysis total Hib PRP antibody levels gave comparable results.

The antibody concentration sufficient for protection is not definitely known but is estimated to be between 0.06 and 1.0 μg/ml of total Hib PRP antibody as measured by Farr-type antigen-binding assays (Robbins et al 1973; Käyhty et al 1983a). The assays developed in this study are sensitive enough to accurately detect these low antibody levels.

These ELISAs provided a simple and reproducible method which were applied to evaluate Hib antibody responses in children after natural infections with Hib meningitis and Hib osteomyelitis/septic arthritis.

CHAPTER 5

STAPHYLOCOCCAL AUREUS

IgG SUBCLASS IMMUNOASSAYS

This chapter discusses the previous methods used to measure *S.aureus* teichoic acid (TA) antibodies and the rationale for setting up the assays. The development of ELISAs to measure IgG, IgG1 and IgG2 subclass specific *S.aureus* TA antibodies and experiments involved in establishing optimal reagents and conditions for these assays are described. The ELISA methods used to measure IgG, IgG1 and IgG2 *S.aureus* subclass specific antibodies are detailed and results of experiments relating to the precision and standardization of the assays are discussed. Finally the merits of the assays and their application are mentioned. This chapter is divided into a number of sections as follows:

5.1 INTRODUCTION

5.2 DETERMINATION OF OPTIMAL ASSAY CONDITIONS

5.3 METHODS

5.4 ASSAY STANDARDIZATION

5.5 SUMMARY

5.1 INTRODUCTION

S.aureus is a gram positive coccus which occurs in pairs, short chains or clusters. There are several immunodominant antigens of *S.aureus* reported e.g. teichoic acid, peptidoglycan (PG), protein A and leukocidin (Cohen 1986). The principle component of the cell wall is peptidoglycan. This substance consists of chains of alternating links of N-acetyl glycosamine and N-actyl muramic acid moieties connected by ß 1, 4 glycosidic bonds. A second important component of the cell wall is ribitol teichoic acid. A third major cell wall component is protein A which interacts non-specifically with IgG.

Teichoic acids are major cell wall components comprising 40% of the staphylococcal cell wall. Teichoic acids of *S.aureus* consist of polymers of ribitol connected by phosphate diester linkage, with side chains of D-alanine and varying proportions of N-acetylglucosamine in either α or ß glycosidic linkage. Teichoic acids are in themselves poor immunogens, but when bound to the peptidoglycan they incite specific antibody formation. Most human pathogenic staphylococci contain both the α and ß forms of ribitol teichoic acid (Nagel et al 1977). Most strains of *S.epidermidis* contain only glycerol teichoic acid which is immunologically distinct from the ribitol teichoic acid of *S.aureus*. Some strains of *S.epidermidis* contain ribitol teichoic acid as minor antigen determinants. Most people produce teichoic acid antibodies following staphylococcal infections (Crowder and White 1972; Tuazon and Sheagren 1976; Wheat et al 1978; Tenenbaum and Archer 1980).

Demonstration of teichoic acid antibodies in serum has been reported to be of diagnostic value in patients with endocarditis and osteomyelitis (Tenenbaum and Archer 1980). Since Crowder and White (1972) reported a

strong correlation between the presence of serum antibodies to staphylococcal teichoic acid and the diagnosis of staphylococcal endocarditis, methods to measure such antibodies have been of interest. The gel diffusion method was found to be specific but relatively insensitive, whereas counter-immunoelectrophoresis was more sensitive but less specific (Nagel et al 1975). RIA has been found to be more sensitive than counter-immunoelectrophoresis for detecting teichoic acid antibodies (Wheat et al 1978). ELISA has been reported to be a sensitive, specific and rapid method of quantifying antibody to *S.aureus* teichoic acid in adults with staphylococcal disease (Verbrugh et al 1981; Granström et al 1983; Yamada et al 1983).

Osteomyelitis/septic arthritis (OM/SA) are serious infections most commonly caused by *S.aureus* and sometimes by *S.pyogenes* or *H.influenzae*. Immune defences involve opsonization by complement and/or antibodies and killing by phagocytic cells (Adlam and Easmon 1983). Serum IgA, IgG and IgM levels have been measured in patients with osteomyelitis but there are few reports on the measurement of IgG subclasses in these patients. Osteomyelitis has been reported in an IgG2 (Gottsegen 1987) and an IgG4 deficient patient (Heiner et al 1988).

IgG subclass deficiencies may occur even in the presence of normal total serum IgG levels and may be an important indicator of impaired antibody production to certain types of antigens.

The purpose of establishing these assays was to assess whether children who have *S.aureus* osteomyelitis/septic arthritis develop a specific antibody response to the infecting organism. IgG, IgG1 and IgG2 *S.aureus* subclass specific ELISAs were developed in order to do this.

5.2 DETERMINATION OF OPTIMAL ASSAY CONDITIONS

Factors affecting the performance of the assays were assessed to determine the optimal conditions for the ELISAs and are described in the following section.

5.2.1 Plates

Experience from establishing previous ELISAs had shown that Nunc 96 well flat bottomed microtitre plates were suitable for this type of ELISA. They are readily available, cheap and easy to load without splashing, giving good reproducibility. Since the background blanks were in acceptable ranges, other sources and types of plates were not assessed.

5.2.2 Coating Antigen

Cell wall teichoic acid of the Lafferty strain of *S.aureus* was used as a standard antigen. It has previously been found that antibody titres obtained with Lafferty strain antigen were the same as titres obtained with antigen from individual blood stream isolates.

Most studies have used preparations of crude extracts of the Lafferty strain of *S.aureus* (Crowder and White 1972; Thisyakorn et al 1984; Wheat et al 1984; Leung et al 1988). Antigen preparations from the Lafferty strain have been as sensitive and specific as preparations of infecting *S.aureus* isolated from a patient (Sheargren et al 1981). However other studies have used Wood 46 (Julander et al 1983; Granström et al 1983; Thisyakorn et al 1984; Monteil et al 1990) or Copenhagen (Larinkari et al 1977; Thisyakorn et al 1984) strains of *S.aureus*. Several methods have been used to partially purify antigen such as ultrasonic disruption (Crowder and White 1972; Wheat et al 1984), disruption with Lysostaphin or other enzymes (Rydén et

al 1990), acid extraction, ethanol precipitation (Tenenbaum and Archer 1980) and phenylhydrazine extraction (Archibald and Baddiley 1965). The Lafferty and Copenhagen strains contain both α and ß N-acetylglucosaminylribitol teichoic acid. The Wood 46 strain contains only ß-N-acetylglucosaminylribitol teichoic acid which is present in 96-100% of human isolates of *S.aureus*. Thisyakorn et al (1984) found no difference in detection of teichoic acid antibody in ELISAs using teichoic acid prepared from Lafferty, Copenhagen or Wood 46 strains of *S.aureus*.

Lysostaphin is a lytic factor which shows specific activity against staphlococci. The lysis is caused mainly by action of the peptidase which attacks glycyl-glycine linkages in the cross bridges of the staphylococcal cell wall peptidoglycan. All staphylococci possess this bridge which is exclusive to these types of bacteria (Gutierrez et al 1981). Lysostaphin susceptibility is a property directly related to the composition of the cell wall peptidoglycan and it seems to lyse staphylococci with low guanine and cytosine content.

It is possible to get a non-specific reaction to protein A (present in most coagulase positive staphylococci) with the Fc fragment of IgG. Protein A can be eliminated by treating the antigen preparation with trypsin.

The method for extraction and purification of teichoic acid from the Lafferty strain of *S.aureus* is described in detail in Appendix D and included trypsin treatment.

The purified teichoic acid product obtained in this laboratory elicited a single line of identity in gel diffusion with commercial teichoic acid antibody (Meridian Diagnostics).

5.2.2.1 *Comparison of teichoic acid antigens*

The only commercially available source of the teichoic acid was part of the 'Endo-Staph' Ouchterlony kit (Meridian Diagnostics) used for semiquantitative measurement of antibodies to teichoic acid. Details of this antigen and its preparation are not available. It is supplied at low concentration (30 μg/ml protein), is relatively expensive and of limited availability. Several batches of teichoic acid were prepared in the laboratory for use in these assays and each batch was verified against the commercial preparation of Staphylococcal ribitol teichoic acid antibody (Meridian Diagnostics, Catalogue No 290202).

Using both radioimmunodiffusion (RID) and ELISA the prepared teichoic acid antigen was compared to the commercial teichoic acid using normal pooled human immunoglobulin and the commercial anti-staphylococcal ribitol teichoic acid (Meridian Diagnostics, Catalogue No 290302).

5.2.2.2 *Comparison of commercial and in-house teichoic acid antigen by RID*

The method for immunodiffusion was adapted from the package insert of the Endostaph immunodiffusion assay (Appendix E). Briefly 1% agarose in PBS (pH 7.4) containing 3.8% polyethylene glycol, 0.7% sodium chloride and 0.02% sodium azide, was coated onto gelbond (10 cm x7.5 cm) (10 ml/piece) and left to set. When set, 3 rows of wells (3 mm in diameter with an interval centre to centre distance of 5 mm) were cut. 5 μl of the purified teichoic acid antigen were added to the wells of the centre row and 5 μl of various dilutions of anti-teichoic acid antibody or serum were added to the wells of the top and bottom rows. The gel was incubated for 48 hours at 4°C in a moist chamber. It was then soaked in 0.9% saline for 15 minutes, distilled water for 15 minutes, dried, fixed with 2% acetic acid for 10

minutes and stained with amido black stain (0.5% in methanol/glacial acetic acid 9:1). It was destained with methanol/glacial acetic acid 9:1.

The undiluted commercial teichoic acid antigen did not give a definite precipitation line with either pooled normal immunoglobulin or the commercial anti-teichoic acid antibody whereas the in-house teichoic acid preparation did. This was observed with several batches of commercial teichoic acid. Only minor precipitation lines were observed between undiluted commercial teichoic acid antibody and a 1:4 dilution of pooled normal immunoglobulin (Figure 5.1).

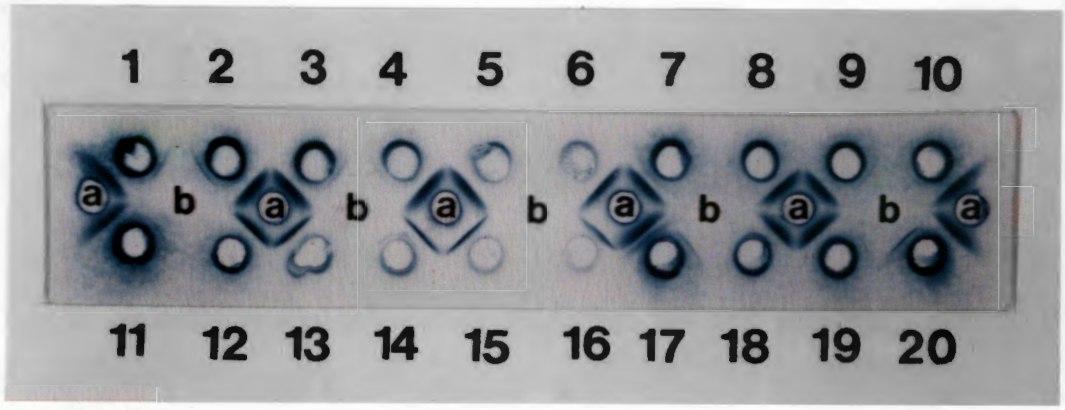


Figure 5.1: Precipitin lines in agar gel showing a line of identity between the in-house teichoic acid preparation (wells a) and both normal human immunoglobulin (wells 1-10) and commercial teichoic acid antibody (wells 11-20). Faint precipitation lines were seen with the commercial teichoic acid antigen (wells b) and normal human immunoglobulin but not with the commercial teichoic acid preparation. Wells of the top row contain serial dilutions from undiluted to 1:32 of normal human immunoglobulin. The middle row contains the teichoic acid antigen: a = in-house preparation; b = commercial preparation. Wells of the bottom row contain commercial teichoic acid antibody in serial dilutions from undiluted to 1:32

A 1:8 dilution of the in-house teichoic acid showed optimal precipitation lines with undiluted antibody and a 1:32 dilution of pooled normal immunoglobulin.

5.2.2.3 *Comparison of commercial and in-house S.aureus teichoic acid antigen by ELISA*

The ELISA method is described in detail in this chapter under section 5.3.2.

A 1:25 and 1:500 dilution of commercial teichoic acid gave similar shaped curves to 1:2 500-1:25 000 dilutions of the in-house teichoic acid antigen for IgG, IgG1 and IgG2 teichoic acid ELISAs (Figure 5.2). The standard curves obtained with the commercial antigen were not as steep and shifted to the left compared to the in-house teichoic acid antigen (Figure 5.2).

5.2.2.4 *Conclusion and discussion*

The commercial teichoic acid antigen did not give a definite precipitation line with commercial teichoic acid antibody whereas the in-house teichoic acid antigen did. In the teichoic acid ELISA the commercial and in-house teichoic acid antigen gave parallel standard curves at different concentrations. This demonstrated that the in-house product was similar to the commercial teichoic acid but was far more concentrated.

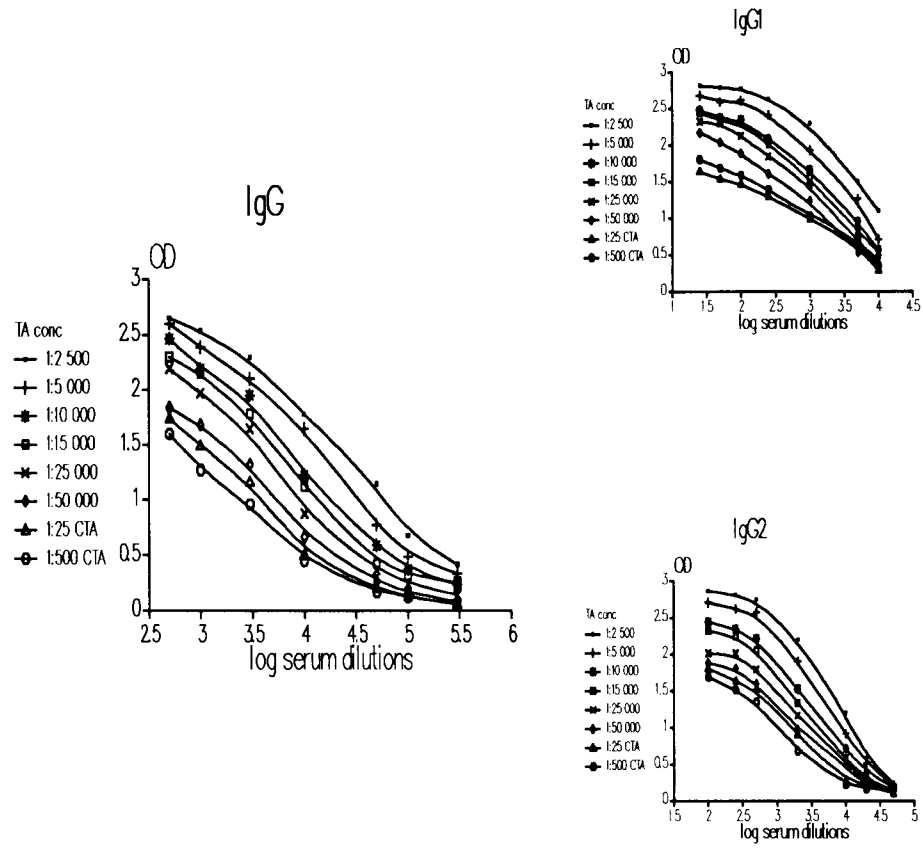


Figure 5.2: IgG, IgG1 and IgG2 *S.aureus* teichoic acid ELISA standard curves assayed using laboratory prepared teichoic acid in a range of dilutions from 1:2 500 - 1:50 000: 1:2 500 (▪) 1:5 000 (+), 1:10 000 (*), 1:15 000 (□), 1:25 000 (x) and 1:50 000 (♦) and compared to the commercial teichoic acid antigen (CTA) at 1:25 (Δ) and 1:500 (o)

5.2.3 Antigen Coating

5.2.3.1 *IgG, IgG1 and IgG2 teichoic acid ELISAs*

Preliminary checkerboard titrations were done to determine optimum coating dilutions of teichoic acid. The microtitre plates were coated with 100 μl of teichoic acid dilutions ranging from 1:1 000 to 1:500 000 diluted in carbonate buffer pH 9.6. Plates were incubated overnight in a moist chamber at 4°C. A coating dilution of 1:15 000 was selected for the IgG

assay, 1:5 000 for the IgG1 assay and 1:1 000 for the IgG2 assay (Figure 5.2). All plates were coated just prior to use.

Pooled human immunoglobulin was used (by back titration against newly prepared batches of antigen) to standardize the antigen coating concentration for construction of standard curves in these assays.

5.2.4 Blocking Agents

5.2.4.1 *IgG, IgG1 and IgG2 teichoic acid ELISAs*

Casein 1% (Centrolab, Saarchem, Catalogue No 1528140), BSA 2% (Albumin Fraction V, Boehringer Mannheim, Catalogue No 735086) and 2% denatured goat serum (for the IgG ELISA) were assessed as blockers in these assays. 1% casein gave low background readings for all the blanks and was selected as the blocker and serum diluent in all three assays (Table 5.1).

5.2.4.2 *Conclusion and discussion*

There are only a few reports of teichoic acid ELISA methods and in the most quoted method of Granström et al (1983) a blocking step is not mentioned. Where a blocking step is included BSA (0.5-2.0%) was used (Thisyakorn et al 1984; Rydén et al 1990). In this system 1% casein gave the best results.

TABLE 5.1: EFFECT OF BLOCKING AGENTS ON BACKGROUND BLANK VALUES OF IgG, IgG1, IgG2 TEICHOIC ACID SPECIFIC ASSAYS

			OPTICAL DENSITY (OD) OF BACKGROUND BLANKS (% of maximum signal)		
Assay	**Blocker**	**OD Maximum Signal**	**Minus Antigen**	**Minus Serum**	**Minus Moab**
IgG TA	2% BSA	1.904	1.354 (71.1)	0.039 (2.1)	0.050 (2.6)
	1% Casein	2.145	0.139 (6.5)	0.007 (0.3)	0.047 (2.2)
	Denatured Goat serum 2%	1.517	1.083 (71.4)	0.028 (1.9)	0.170 (1.1)
IgG1 TA	2% BSA	2.173	1.135 (52.2)	0.023 (1.1)	0.009 (0.4)
	1% Casein	2.240	0.147 (6.6)	0.012 (0.5)	0.006 (0.3)
IgG2 TA	2% BSA	2.198	0.135 (6.1)	0.122 (5.6)	0.104 (4.7)
	1% Casein	2.226	0.089 (3.9)	0.059 (2.6)	0.062 (2.7)

5.2.5 Serum Standards and Controls

5.2.5.1 *IgG, IgG1 and IgG2 teichoic acid ELISAs*

Normal pooled human immunoglobulin (NBTS), pooled AB serum and commercial anti-staphylococcal ribitol teichoic acid (Meridian Diagnostics, Catalogue No 290302) were compared as antibody standards and showed parallel curves (Figure 5.3).

Normal pooled human immunoglobulin shifted the curve to the right compared to the AB serum and commercial teichoic acid antibody preparation (Figure 5.3). The normal pooled human immunoglobulin was

selected as the calibrator for the standard curve as it could be used in greater dilutions which resulted in lower uncoated (minus antigen) well blank readings. It was also commercially available at low cost whereas the commercial anti-teichoic acid antibody preparation was difficult to obtain, was more expensive and the stock of pooled AB serum was limited.

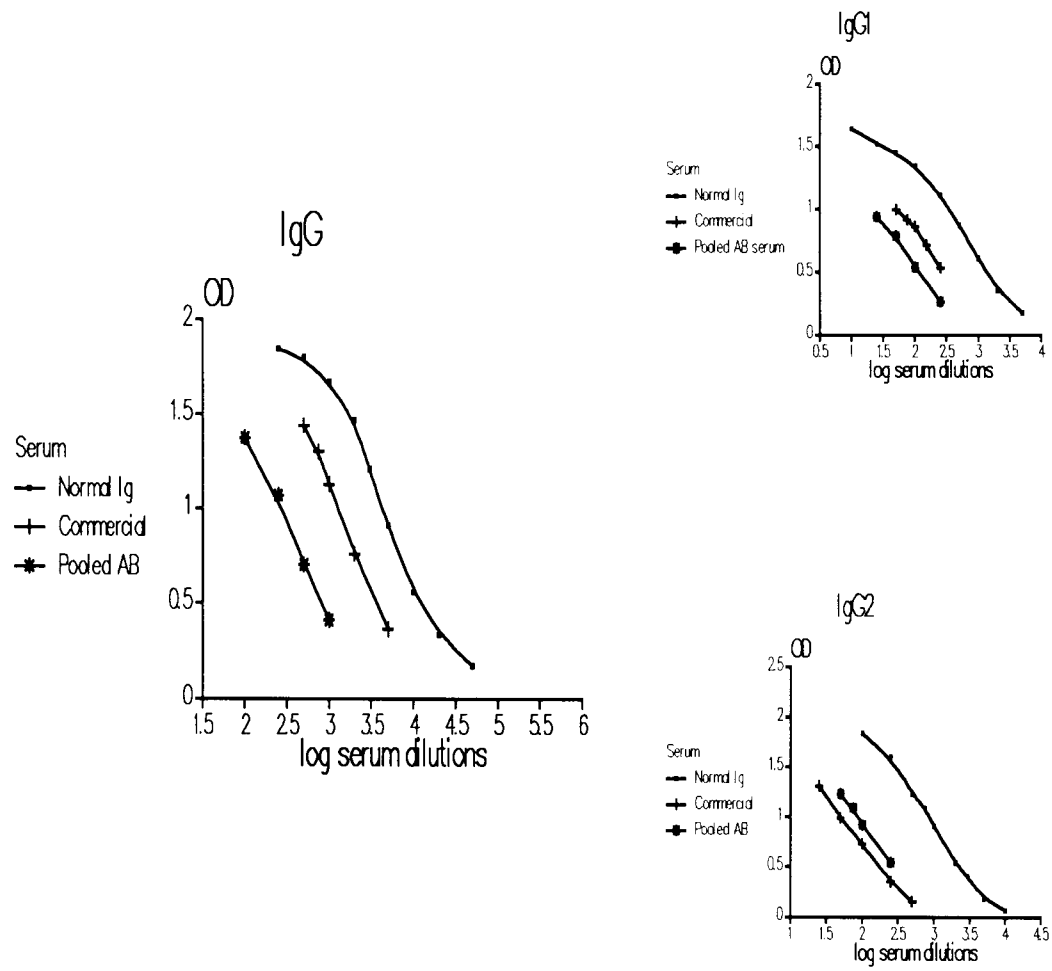


Figure 5.3: Comparison of IgG, IgG1 and IgG2 *S.aureus* teichoic acid ELISA standard curves of normal pooled immunoglobulin (▪), with the commercial anti-teichoic acid antibody serum (+) and pooled AB serum (*) demonstrating parallelism

The commercial anti-staphylococcal ribitol teichoic acid and pooled AB serum were used as positive controls in the assay. Pooled batches of the standard and positive control were aliquotted and stored at -70°C until use. Various dilutions of the control and samples were compared to assess reproducibility of results. Calculated antibody concentrations were similar for dilutions read off the linear section of the curve. In the IgG teichoic acid assay the AB serum positive control was diluted 1:100 and the commercial anti-teichoic acid antibody was diluted 1:100 and 1:500.

In the IgG1 teichoic acid ELISA the AB serum was diluted to 1:75, the commercial anti-teichoic acid antibody was diluted to 1:75 and 1:100.

In the IgG2 teichoic acid ELISA the AB serum was diluted to 1:50 and commercial anti-teichoic acid antibody was diluted to 1:750 and 1:1 000.

5.2.6 Incubation Periods

No significant differences were found in the standard curve or sample data when the serum was incubated for either 1 hour at 37°C or overnight at 4°C.

5.2.7 Monoclonal Antibody - Selection

Details of the various IgG subclass specific monoclonal antibodies evaluated for these assays can be found in Appendix A.

5.2.7.1 *IgG teichoic acid ELISA*

In the total IgG teichoic acid ELISA the background blanks were acceptably low using the unbiotinylated antibody HP6064 (Table 5.2). The concentration of antibody was tested in dilutions ranging from 1:250-1:10 000 and the optimal dilution of 1:4 000 was selected. The biotinylated

antibody HP6017, gave low minus antigen, minus monoclonal and minus serum blanks (5.0%, 0.6% and 0.9% respectively of the maximum standard signal). However, this antibody had to be used at a dilution of 1:500 or less whereas the unbiotinylated antibody HP6064 could be used in dilutions of 1:4 000 and was thus far more economical. The curve was shifted to the right with HP6064, the most concentrated standard dilution being 1:500 as opposed to 1:20 with the biotinylated antibody HP6017 (Figure 5.4).

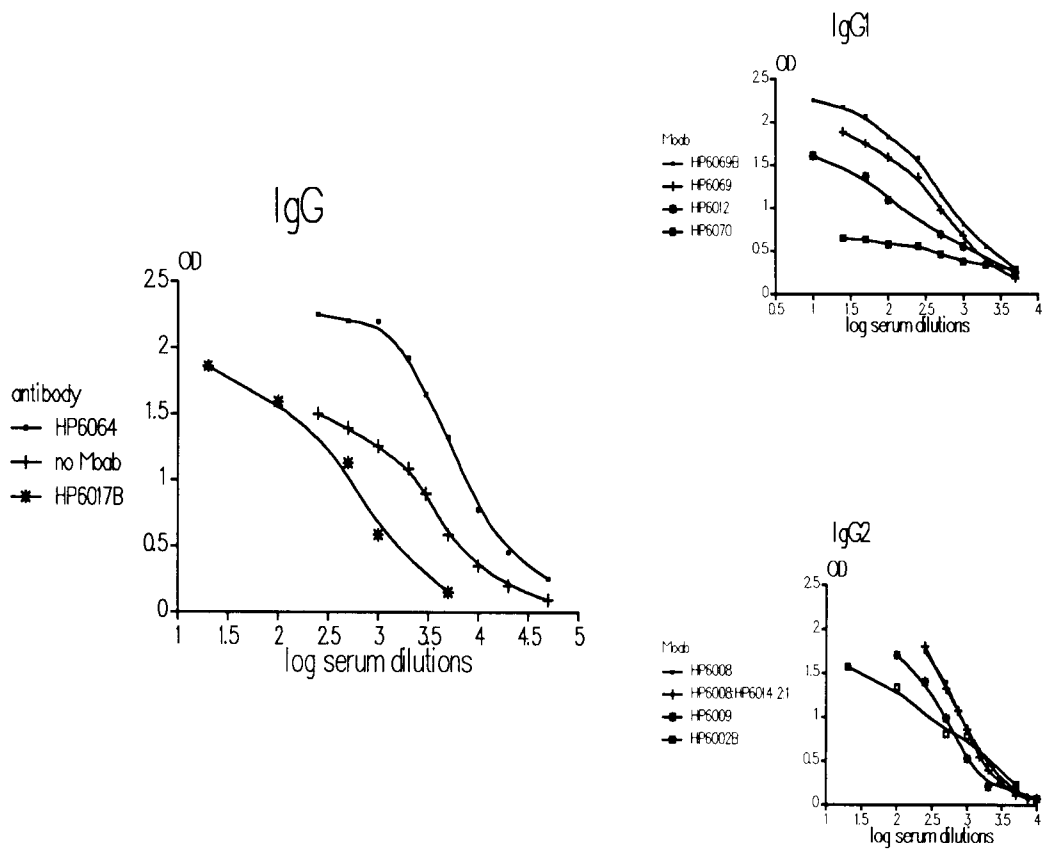


Figure 5.4: IgG, IgG1 and IgG2 *S.aureus* teichoic acid ELISA standard curves assayed with various Moabs: IgG = HP6064 (•); No Moab (+) (without monoclonal antibody and using an anti-human IgG conjugate); biotinylated HP6017 (HP6017B) (*). IgG1 = biotinylated HP6069 (HP6069B) (•), HP6069 (+), HP6012 (*), HP6070 (▪). IgG2 = HP6008 (•), HP6008:HP6014 (2:1) (+), HP6009 (*), biotinylated HP6002 (HP6002B) (▪)

TABLE 5.2: BACKGROUND BLANKS IN THE IgG *S.AUREUS* TEICHOIC ACID ASSAY

Moab	OD Maximum Optical Signal	OPTICAL DENSITY (OD) OF BACKGROUND BLANKS (% of maximum signal)		
		Minus Antigen	Minus Serum	Minus Moab
HP6064	1.784	0.089 (5.0)	0.019 (1.1)	0.028 (1.6)
HP6017 Biotinylated	1.588	0.080 (5.0)	0.010 (0.6)	0.014 (0.9)
No Moab	1.534	0.099 (6.5)	0.018 (1.3)	0.010 (0.7)

These assays were also assessed by using a polyclonal anti-human conjugated detecting antibody and omitting the intermediate monoclonal antibody. The shapes of the standard curves using these two formats are seen in Figure 5.4. The curve with the HP6064 monoclonal antibody was steeper than that with the polyclonal antibody although the value of the unknown samples read off both curves was similar. The assay format using the monoclonal antibody HP6064 was selected for use in these assays as it had a steeper slope which allowed a wider range of sample dilutions to be read off it. It was also more convenient as it was the format used in the

IgG1 teichoic acid and IgG2 teichoic acid assays and allowed synchronization of incubation steps.

5.2.7.2 *IgG1 teichoic acid ELISA*

Although the shape of the standard curves were similar when using biotinylated HP6069 or unbiotinylated HP6069 monoclonal antibodies (Figure 5.4), the latter had higher background blanks (Table 5.3). The biotinylated HP6069 antibody was selected in preference to the antibody HP6012 as it gave a steeper standard curve (Figure 5.4). Minus antigen blanks were very high using the antibody HP6070 (Table 5.3) and the standard curve was very flat once these background blanks had been subtracted (Figure 5.3). The antibody HP6007 was initially tried but it gave a very poor signal and was not studied further.

TABLE 5.3: EFFECT OF VARIOUS ANTI-IgG1 MONOCLONAL ANTIBODIES ON BACKGROUND BLANKS IN THE IgG1 TEICHOIC ACID ELISA

		OPTICAL DENSITY (OD) OF BACKGROUND BLANKS (% of maximum signal)		
Monoclonal Antibody 1:1 000	**OD Maximum Signal**	**Minus Antigen**	**Minus Serum**	**Minus Moab**
HP6069 Biotinylated	1.953	0.105 (5.4)	0.014 (0.7)	0.035 (1.8)
HP6069 Unbiotinylated	1.441	0.188 (13.1)	0.143 (9.9)	0.042 (2.9)
HP6012	2.328	0.436 (18.7)	0.346 (14.8)	0.173 (7.4)
HP6070	1.369	0.736 (53.8)	0.056 (4.1)	0.165 (12.1)

Checkerboard titrations of the biotinylated HP6069 were done to find the optimal working dilution of this antibody. It was tested in a range from 1:500 to 1:2 000 dilution and a dilution of 1:1 000 was selected.

5.2.7.3 *IgG2 teichoic acid ELISA*

In the IgG2 teichoic acid ELISA there was no real difference in the shape of the standard curves produced using either HP6008, `a5.4.1

HP6009 or a combination of HP6008:HP6014 (2:1) (Figure 5.4). In the assays the background blanks using these antibodies were similar and were less than 7% of the maximum signal. The HP6008 antibody was selected for use in these assays as it could be used at a greater dilution than the HP6009 antibody and was thus more economical. HP6008 was tested in dilutions ranging from 1:250 to 1:2 000 and an optimal working dilution of 1:1 000 was selected.

The biotinylated antibody HP6002 had to be used at a low dilution (1:250) and produced a flat standard curve (Figure 5.4).

5.2.7.4 *Conclusion and discussion*

There are a few reports of the IgG subclass distribution of antibodies against *S.aureus* teichoic acid but the monoclonal antibodies used to detect the subclass specific teichoic acid antibodies are not detailed (Hammarström et al 1984, Hammarström and Smith 1986). In a report of the subclass distribution of antibodies against the whole staphylococcal organism (Monteil et al 1990) the mouse anti-human monoclonal antibodies used were: IgG1 (HP6012), IgG2 (HP6014), IgG3 (HP6050) and IgG4 (HP6011 and HP6013) all at 1:1 000 dilution.

Although the IgG1 antibody HP6012 could have been used in the assays described in this thesis, HP6069 was selected due to its lower background values.

Anti-IgG2 HP6014 is known to preferentially bind lambda over kappa bearing IgG2 protein (Hamilton 1987; Madassery et al 1988; Aucouturier et al 1992a) and is therefore not recommended for use on its own in subclass ELISAs. In these assays there was no advantage to using this antibody in combination with HP6008 over HP6008 alone.

5.2.8 Evaluation of the Enzyme Detector System

5.2.8.1 *IgG teichoic acid ELISA*

The following conjugated antibodies were evaluated:

- non-affinity purified adsorbed HRPO sheep anti-mouse IgG (gamma chain) (Binding Site, Catalogue No PP272).
- non-affinity purified adsorbed HRPO sheep anti-mouse IgG, A, M (Binding Site, Catalogue No PP270).
- affinity purified $F(ab')_2$ goat anti-human gamma chain specific HRPO (Cappel, Catalogue No 3601-0121).

The Binding Site sheep anti-mouse IgG, A, M HRPO conjugate was selected as it gave the lowest background blanks (Table 5.4). It was diluted 1:2 500 in 1% casein.

TABLE 5.4: EFFECT OF CONJUGATES ON BACKGROUND BLANKS IN THE IgG TEICHOIC ACID ELISA

		OPTICAL DENSITY (OD) OF BACKGROUND BLANKS (% of maximum signal)			
Conjugate HRPO	**OD Maximum Signal**	**Minus Antigen**	**Minus Moab**	**Minus Serum**	**Minus Moab Minus Antigen**
Binding Site gamma chain specific 1:750	1.503	0.316 (21.0)	0.461 (30.7)	0.510 (33.9)	0.466 (31.0)
Binding Site IgG,A,M 1:2 500	1.505	0.053 (3.5)	0.018 (1.2)	0.016 (1.1)	0.021 (1.4)
Cappel $F(ab')_2$ gamma chain specific 1:1 000	1.895	0.477 (25.2)	0.049 (2.6)	0.238 (12.6)	0.057 (3.0)

5.2.8.2 *IgG1 teichoic acid ELISA*

The detection antibody was streptavidin conjugated HRPO. The streptavidin peroxidase conjugates from Zymed (Catalogue No 43-4323) and Calbiochem (Catalogue No 189733) were evaluated. There was no difference in the shape of the standard curve obtained using either of these conjugates. As it

was more readily available, streptavidin peroxidase (Zymed) at 1:1 500 dilution in 1%casein was selected for these assays.

5.2.8.3 *IgG2 teichoic acid ELISA*

The conjugated antibodies detailed in 5.2.8.1 were also evaluated for use in this ELISA. The Binding Site sheep anti-mouse IgG, A, M HRPO conjugate was selected for use in these assays. It was diluted 1:1 000 in 1% casein.

5.2.8.4 *Conclusion and discussion*

Checkerboard titrations of each new batch of conjugate were done to find the optimum working dilution of the conjugate. Previous studies (Herzog et al 1984) have added protein A to the conjugate to reduce background levels when anti-human IgG conjugates were used. This was not found to be necessary in the present ELISAs.

5.3 METHODS

Optimum reaction conditions and reagents were determined in preliminary experiments detailed in Section 5.2. All reagents and samples were added to the wells in 100 μl volumes except for the 2N H_2SO_4 of which 50 μl volumes were used. After each step in the procedure plates were hand washed five times with PBST unless otherwise stated.

5.3.1 IgG teichoic acid ELISA

Flat bottomed Nunc Maxisorb uncertificated microtitre plates (Weil, Catalogue No 4-4206) were coated with staphylococcal ribitol teichoic acid diluted 1:15 000 in carbonate buffer pH 9.6. Plates were incubated for 2 hours at room temperature. Non-specific protein binding sites were blocked with 250 μl 1% casein (Unilab) for 30 minutes at 37°C. Plates were

washed once with PBS and serial dilutions of sera diluted in 1% casein were added to quadruplicate wells. Pooled normal human immunoglobulin in 9 dilutions ranging from 1:500 - 1:200 000 (1:500, 1:1 000, 1:2 000, 1:5 000, 1:10 000, 1:20 000, 1:50 000, 1:100 000, 1:200 000) was used as the standards. Positive controls (commercial anti-staphylococcal ribitol teichoic acid) were added in two dilutions of 1:1 500 and 1:2 000. The sera from the patients was diluted 1:100, 1:200 and 1:400. The samples were diluted so as to be read on the linear section of the curve. Samples with absorbance readings which did not fall on the linear section of the curve were repeated at appropriate dilutions. The samples were incubated overnight at 4°C.

Buffer as well as a control serum with no detectable antibody were included in each assay as negative controls.

The mouse anti-human monoclonal antibody HP6064, diluted 1:4 000 in 1% casein was added to the wells and incubated at room temperature for 1 hour.

The plates were washed with PBST and then sheep anti-mouse IgG, A, M horseradish peroxidase conjugated antibody (Binding Site) diluted 1:2 500 in 1% casein was added to the wells and incubated for 1 hour at room temperature. The plates were again washed with PBST. OPD substrate buffer was added and incubated for 15 minutes at room temperature in the dark. The reaction was stopped with 50 μl of 2N H_2SO_4. The optical density was read at 492 nm in an SLT ELISA reader.

5.3.2 IgG1 teichoic acid ELISA

This was similar to the method mentioned above for the IgG teichoic acid ELISA except for the following dilution and antibody differences:

- The coating dilution of teichoic acid was 1:5 000
- The standard curve dilutions of the normal pooled human immunoglobulin ranged from 1:25 to 1:20 000 (1:25, 1:100, 1:250, 1:500, 1:1 000, 1:2 000, 1:5 000, 10 000, 1:20 000)
- Positive control serum (commercial anti-staphyloccal ribitol teichoic acid) was added in dilutions of 1:100 and 1:150
- Patient sera were added in dilutions 1:100, 1:400 and 1:750
- The biotinylated mouse anti-human IgG subclass specific antibody to IgG1 (HP6069) was diluted in 1% casein to 1:750
- Streptavidin peroxidase HRPO conjugate was diluted 1:1 500 in 1% casein

5.3.3 IgG2 teichoic acid ELISA

This was similar to the method mentioned above for the IgG1 teichoic acid ELISA except for the following dilution and antibody differences:

- The coating dilution of the ribitol teichoic acid was 1:10 000
- The standard curve dilutions of the pooled normal human immunoglobulin ranged from 1:100 to 1:50 000 (1:100, 1:250, 1:500, 1:1 000, 1:2 000, 1:5 000, 1:10 000, 1:20 000, 1:50 000).
- Positive control sera was added in dilutions of 1:500 and 1:750
- Patient sera were initially screened at a 1/5 dilution and then re-assayed at appropriate dilutions if necessary
- The mouse anti-human IgG2 subclass specific antibody HP6008 was diluted to 1:1 000 in 1% casein
- Sheep anti-mouse IgG,A,M specific horseradish peroxidase conjugate (Binding Site)was diluted 1:1 000 in 1% casein.

5.3.4 Design of Plate

Every assay plate included 9 dilutions of the standard normal human immunoglobulin for the calibration curves, background reaction wells (minus serum), uncoated (minus antigen) wells, minus monoclonal antibody wells (where applicable) and dilutions of the control staphylococcal ribitol teichoic acid antibody for monitoring reproducibility. All reactions were performed in triplicate. For the routine analysis of unknown samples the design of the plate is shown in Chapter 1, Figure 1.1.

For all the assays the background values obtained from wells with antigen and monoclonal antibody but no serum (serum blanks) were very similar to those with antigen and serum (monoclonal antibody blanks) and those with antigen but no serum or monoclonal antibody (background blanks). Initially these blanks were carried out in triplicate but as they were consistently low they were only included as single blanks in routine runs to allow more unknown samples to be included in each run.

5.3.5 Calculation of Results

A value of 100 units/ml for IgG, IgG1 and IgG2 anti-teichoic acid antibody was arbitrarily assigned to the standard to allow the specific anti-teichoic acid antibodies to be quantitated. Levels of IgG, IgG1 and IgG2 antibodies were calculated relative to these references.

As previously described (Chapter 1.12) standard dose curves were constructed from average absorbance values and concentrations that had been entered into a curve fitting four parameter logistic programme. The concentration of each analyte in each sample was obtained by interpolating the average absorbance values from the standard curve and multiplying the

result by the dilution factor. Only values from the linear sections of the curves were considered valid.

5.4 ASSAY STANDARDIZATION

5.4.1 Standard Curves

Standard curves for the IgG, IgG1 and IgG2 teichoic acid ELISAs using the pooled normal human immunoglobulin, pooled AB serum and commercial teichoic acid antibody preparation were parallel (Figure 5.5). Thus the pooled normal human immunoglobulin preparation was used to construct standard curves for the teichoic acid ELISAs. Nine triplicate dilutions of the standard were used to construct curves for these ELISAs.

For all 3 assays titration curves were constructed from patient samples in order to demonstrate parallelism to the standard curve at various dilutions. In the IgG and IgG1 *S.aureus* teichoic acid ELISA test serum samples showed parallelism to the standard curves (Figure 5.4). In the IgG2 teichoic acid ELISA samples with high IgG2 teichoic acid antibody concentrations demonstrated parallelism to the standard curve, but this disappeared as the antibody concentration decreased.

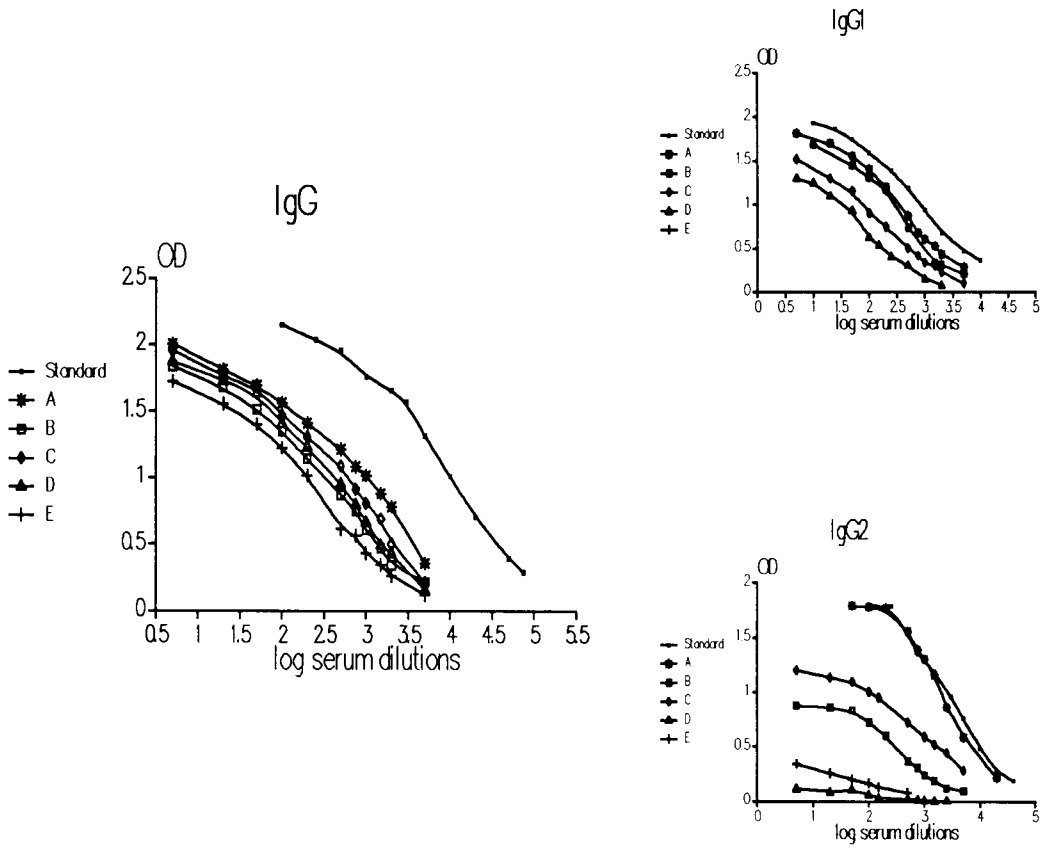


Figure 5.5: Curves of individual serum samples, A (*), B (□), C (♦), D (▲) and E (+) for IgG, IgG1 and IgG2 *S.aureus* teichoic acid and compared to the standard curve (•) demonstrating linearity and parallelism

5.4.2 Precision and Sensitivity

Two dilutions of the commercial teichoic acid preparation and of the pooled AB serum were inserted in every run. Running means were calculated from these controls inserted in each run and experiments were repeated if the values fell outside the 2 SD range. Assay precision was assessed using intra- and inter-assay and interdilutional coefficients of variation (Table 5.5).

TABLE 5.5: EVALUATION OF *S.AUREUS* ELISA PRECISION

	IgG	IgG1	IgG2
Intra-assay CV%	7.1	8.2	6.0
Inter-assay CV%	10.6	11.1	14.6
Inter-dilutional CV%	13.2	14.9	11.8

The intra-assay variation was obtained by assaying the same sample 14 times in one run. This was done on 3 occasions and the mean CV was used. The inter-assay variation was assessed with 2 samples assayed in triplicate at 4 dilutions in 18 different runs. The inter-dilutional variation was obtained by assaying 5 dilutions of the same sample on a plate. This was done on 18 different samples over 6 weeks and the mean CV was used.

The lower limit of quantitation for each assay was 0.001 units for IgG teichoic acid antibody, 0.005 units for IgG1 teichoic acid and IgG2 teichoic acid antibodies. These lower detection limits in each assay were at least 2-3 times the background absorbance of the highest blank. The OD of these lower limits correlated with the OD of the 1:100 000 standard dilution of the IgG teichoic acid, the 1:10 000 dilution of the IgG1 teichoic acid and the 1:5 000 dilution of the IgG2 teichoic acid. For practical purposes samples were called negative if the OD was less than the OD of the above mentioned standard dilutions in that run.

5.4.3 Specificity

The specificities of the IgG, IgG1 and IgG2 teichoic acid ELISA assays were assessed by a competitive inhibition ELISA using either teichoic acid, or the non-specific antigens Hib PRP polysaccharide or pneumococcal polysaccharide (Pneumovax-Merck, Sharpe and Dohme).

These ELISAs were carried out as described in Section 5.3.1, 5.3.2 and 5.3.3 except that the competititve antigens were preincubated with an equal volume of standard serum for 2 hours at room temperature before the addition of the mixture to teichoic acid coated wells. Dilutions of the serum reading on the linear section of the sigmoid curves for each assay were selected. In the IgG teichoic acid ELISA the dilution was 1:500, in the IgG1 teichoic acid ELISA 1:750 and in the IgG2 teichoic acid ELISA 1:1 000. The competitive antigens were mixed with the serum in concentrations ranging from 300 to 0.01 μg/ml of teichoic acid, 20 to 0.01 μg/ml of pneumococcal polysaccharide and 20 to 0.01 μg/ml of Hib PRP polysaccharide.

Binding of the teichoic acid antibody to wells coated with teichoic acid could be inhibited by incubation with teichoic acid antigen but not with non-specific carbohydrate antigens - Hib PRP and pneumococcal polysaccharide (Table 5.6)

These results show that the assays are specific for the measurement of teichoic acid antibodies.

TABLE 5.6: COMPETITIVE INHIBITION ELISAs FOR SPECIFICITY IN TEICHOIC ACID ASSAYS

	% INHIBITION		
TA μg/ml	IgG	IgG1	IgG2
200	85.3	85.3	
100		77.7	
20	89.8	60.4	99.8
10	84.3	50.3	91.3
5	82.2	43.8	92.7
1	79.6	18.3	80.4
0.75	66.9	13.9	72.3
0.5	59.7	0.5	70.1
0.25	55.8	0	68
0.10	43.1	1	51.4
0.05	34.5	5	36.8
0.01	13.7	0	24.3

* **Hib PRP or pneumococcal polysacchairde (Pneumovax) (0.01-20 μg/ml) caused no inhibition in these assays**

5.4.4 Antibody Affinity

5.4.4.1 *DEA-ELISA for IgG1 and IgG2 teichoic acid*

These assays were based on the method of Devey et al (1988) using DEA as a chaotropic agent and are similar to the DEA-ELISAs for TT and Hib PRP antibodies detailed in Chapter 3.3.6, 3.5 and Chapter 4.4.5.

The incorporation of 5-50mM DEA in the serum diluent resulted in an increasing shift to the left of the dose response curve for both IgG1 and IgG2 teichoic acid antibodies (Figure 5.6). The shift of the dose response curve was greater in the IgG2 teichoic acid assays than in the IgG1 teichoic acid assays.

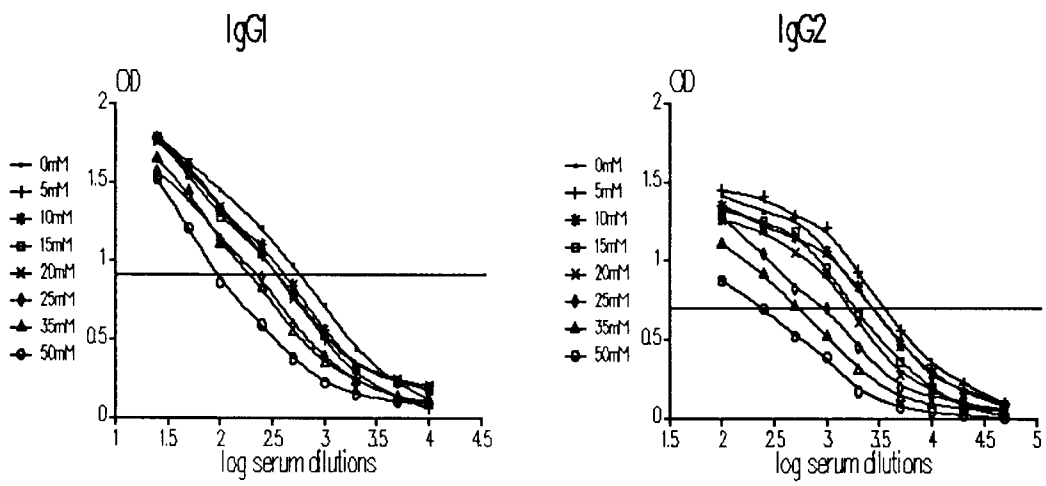


Figure 5.6: Effect of increasing concentrations of DEA on the standard curves of IgG1 and IgG2 teichoic acid antibodies. DEA concentration: 0 mM (•), 5 mM (+), 10 mM (*), 15 mM (▪), 20 mM (x), 25 mM (♦), 35 mM (▲) and 50 mM (o) DEA. The shift was measured at 50% of the maximum OD with no DEA which is represented by the solid line drawn at 0.90 and 0.70 for the IgG1 and IgG2 teichoic acid ELISAs respectively.

The absorbance readings in the absence of DEA represented total binding of specific antibody. The shift in the log of the serum dilution from the initial OD reading to 50% of the initial OD reading was calculated with various molar concentrations of DEA.

Calculations of this shift at 50% of the maximum ODs showed that in this serum (normal human immunoglobulin), the shift for IgG2 teichoic acid antibody tended to be greater than that for IgG1 teichoic acid antibody at higher DEA concentrations (Table 5.7). This suggests that the IgG1 teichoic acid antibody response is of higher affinity than the IgG2 response.

TABLE 5.7: EFFECT OF INCREASING CONCENTRATIONS OF DEA ON THE LEFT SHIFT OF THE DOSE-RESPONSE CURVE OF IgG1 AND IgG2 TEICHOIC ACID ANTIBODIES IN NORMAL HUMAN IMMUNOGLOBULIN

DEA Concentration (mM)	Log_{10} dilution at 50% max OD		Log_{10} shift at 50% max OD		% Shift $\frac{IgG1-IgG2}{IgG1} \times 100$	
	OD=0.6 IgG1	OD=0.9 IgG2	OD=0.6 IgG1	OD=0.9 IgG2	IgG1	IgG2
0	3.099	3.462	-	-	-	-
5	2.911	3.528	0.188	0.066	7.52	-
10	2.876	3.325	0.233	0.137	6.07	3.96
15	2.848	3.368	0.251	0.094	8.10	2.73
20	2.859	3.265	0.240	0.197	7.74	5.69
25	2.677	3.011	0.422	0.451	13.62	13.03
35	2.616	2.761	0.483	0.701	15.59	20.25
50	2.330	2.254	0.769	1.208	24.81	34.89

5.5 SUMMARY

This chapter describes the development of IgG, IgG1 and IgG2 subclass specific ELISAs to *S.aureus* teichoic acid antibodies. An in-house teichoic acid antigen preparation was used which had been compared to a commercially available 'standard' antigen.

The detection of teichoic acid antibody by ELISA has been reported to be more sensitive than the gel diffusion method (Thisyakorn et al 1984). The only other reports of subclass specific antibodies to teichoic acid are from Hammarström et al 1984. The major disadvantage of the *S.aureus* teichoic acid assays, is that there is no standardized single antigen and different laboratories prepare and use their own antigen preparations. Sensitivity and specificity have varied widely making it difficult to compare results obtained by different groups. The assays described are simple, sensitive and reproducible. However, due to the non-parallelism between test sera with low concentrations of IgG2 teichoic acid specific antibodies and the standard, low levels of IgG2 teichoic acid antibodies cannot be accurately quantitated. Linearity between test samples and the standard is assumed when unknown concentrations of the sample are calculated off the standard curve. The assays were used to study the isotypic IgG, IgG1 and IgG2 teichoic acid specific antibody responses in children with *S.aureus* osteomyelitis/septic arthritis.

CHAPTER 6

G2m(n) and G1m(f) IMMUNOASSAYS

In 1976 at a WHO meeting (WHO 1976) it was agreed to accept both alphameric and numeric nomenclature for immunoglobulin allotypes (Appendix F). In this study the alphameric nomenclature is used. Initially it was thought that immunoglobulin allotypes were of no clinical relevance except for the study of population genetics. Recently however, there have been associations described between allotype markers and susceptibility to Hib disease and response to Hib vaccination (Ambrosino et al 1985; Sarvas et al 1990). Although the haemagglutination inhibition test is the most widely used method for immunoglobulin allotyping it is semi-quantitative and cumbersome. ELISAs permit quick and simple, quantitative determination of allotypes. This chapter reviews the methods used to measure allotypes and the reasons for establishing these assays. The experiments done to establish optimal reagents and conditions for these ELISAs, the methods finally used for the assays and the results of experiments relating to the precision and standardization of the assays are described.

6.1 INTRODUCTION

6.2 DETERMINATION OF OPTIMAL ASSAY CONDITIONS

6.3 METHODS

6.4 ASSAY STANDARDIZATION

6.5 SUMMARY

6.1 INTRODUCTION

Allotypic antigens of human immunoglobulins were first described in 1956 (Grubb 1956; Grubb and Laurell 1956). The test system used then, based on haemagglutination-inhibition is still widely used. This method is based on inhibition of agglutination of antigen coated red blood cells. The antibodies specifically directed against the antigen are complexed by the antigen if it is present in the sample tested, inhibiting the agglutination of the target-coated RBC. Although this method is qualitative, semi-quantitative estimates of the concentration of the antigenic determinants can be obtained by assaying serial dilutions of test samples.

Allotyping by classical immunodiffusion methods is used, widely in mice and rabbits but has rarely been possible due to the paucity of reagents available for humans studies (Kunkel 1966). However, with the availability of monocloncal antibodies to some of the allotypic markers this is now possible. Recent reports from Rautonen et al (1989) and Sarvas et al (1989) describe a double diffusion assay for genotyping G2m(n) with a monoclonal antibody and its differentiation into homozygous positive, homozygous negative and heterozygote patterns.

Although radioimmunoassay (RIA) is one of the most convenient, sensitive and quantitative methods for measuring allotypes of mouse, rabbit and chicken immunoglobulins, there are only few reports of human typing by RIA (Reisner 1976, Salier et al 1979). This is largely a consequence of the difficulties experienced in retaining antibody activity after radiolabelling the anti-allotype antisera.

Good typing reagents (antisera) for Gm allotyping are difficult to produce and not readily available. The reagents originally used for Gm typing were

obtained from sera of patients with rheumatoid arthritis. The generation of antibodies in non-rheumatoid donors occurs as a result of immunisation in recipients of multiple transfusions, multiparous women alloimmunized against paternal determinants and immunization of the fetus and newborn by genetically incompatible maternal IgG. Without further stimulation antibodies formed to incompatible maternal factors disappear between 5-10 years of age. However, 1-2% of the adult population have antibodies to allotypic determinants (Schanfield and van Loghem 1986).

The antisera obtained fortuitously from the above mentioned sources often required considerable processing to yield monospecific reagents needed for Gm typing. Antisera to particular Gm specifications are often limited in supply and may be impossible to replace once exhausted. Certain Gm allotypes have been lost when supplies of an antisera have become depleted e.g. Gm(r) and Gm(p). The development of typing antisera in animals has been partially successful (Kunkel 1966).

The classic haemagglutination inhibition technique is still the most widely used method for routine immunoglobulin allotyping. Both human and polyclonal animal antisera are useful as typing reagents in haemagglutination inhibition (HAI) assays. However, this method presents several problems: (1) the shelf-life of coated red cells is relatively short and thus must be replaced at frequent intervals; (2) anti-Gm sera are scarce and of low titre; (3) the occasional occurence in serum samples of antibodies to IgG and/or red cell antigens invalidates the test unless they are adsorbed by a time consuming procedure; (4) the test cannot be used for the quantitative analysis of Gm allotypes. With the recent development and availability of monoclonal antibodies to several of the Gm allotypes serological methods have become more widely used. The monoclonal antibodies have several

advantages over the polyclonal anti-allotypic antisera in that they are monospecific, unlimited in supply, relatively cheap to produce and give reproducible results. In ELISA or RIA, these antibodies can be used at high dilutions with very small amounts of test sera.

Recently, monoclonal antibodies to G2m(n) and G1m(f) allotypes have become commercially available. This chapter describes the development of a new method to measure G2m(n) and G1m(f) allotypes by ELISA using these monoclonal antibodies in a direct coating ELISA assay.

The G2m(n) allotype is frequently found in whites but is rarely found in African Blacks (Shackelford et al 1985b; de Lange 1991). Although studies on Black Americans have shown the occasional presence of the G2m(n) allotype (Shackelford et al 1985b) this probably reflected the influence of racial admixture. G1m(f) is said never to have been found in Blacks (de Lange 1991).

The purpose of establishing these assays was to determine:

- The frequency of G2m(n) and G1m(f) positive alleles in our local population
- Whether the G2m(n) allotype influences levels of total IgG2 and IgG2 polysaccharide specific antibody
- Whether the G1m(f) allotype influences levels of total IgG1 and IgG1 polysaccharide specific antibody
- Whether children with Hib meningitis, Hib osteomyelitis/septic arthritis and *S.aureus* osteomyelitis/septic arthritis have the same frequency of the G2m(n)+ve or G1m(f)+ve allele as the control group.

6.2 DETERMINATION OF OPTIMAL REAGENTS AND CONDITIONS

This section describes various experimental conditions and reagents assessed in establishing the methods for the G2m(n) and G1m(f) ELISAs. The positive (R1110, R1114, R1117) and negative (R1113, R1118) G2m(n) and G1m(f) reference sera used in these assays were obtained from the central laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB).

6.2.1 Plates

Experience from establishing IgG subclass and subclass specific tetanus toxoid ELISAs had shown that Nunc 96 well flat bottom microtitre plates were suitable for all ELISA systems tested, readily available, cheap and easy to load without splashing, therefore giving good reproducibility. Since the background blanks were in acceptable ranges using these plates, other sources and types of plates were not assessed.

6.2.2 Coating - G2m(n) ELISA

Assays for determination of the G2m(n) allotype using a direct (coating with sera) and monoclonal capture (coating with G2m(n) antibody) ELISAs were compared. The monoclonal antibody used in these assays was mouse anti-human IgG2 [G2m(n)] Clone SH-21 (Sigma, Catalogue No I-7010).

6.2.2.1 *Direct ELISA*

Preliminary checkerboard titrations were done to determine optimum coating sera dilutions, G2m(n) monoclonal antibody dilutions and conjugate dilutions. Pooled AB serum was used for coating the ELISA plates in dilutions ranging from 1:250 to 1:500 000. Various dilutions of the monoclonal antibody were evaluated ranging in dilutions from 1:500 to 1:100 000 (Figure 6.1). Nine dilutions of AB sera ranging between 1:2 000 and 1:50 000 were selected for construction of the standard curve. A drop

off in signal was only noticed at dilutions greater than 1:20 000. The optimum concentration of the G2m(n) monoclonal antibody in the direct ELISA was 1:10 000 (Figure 6.1).

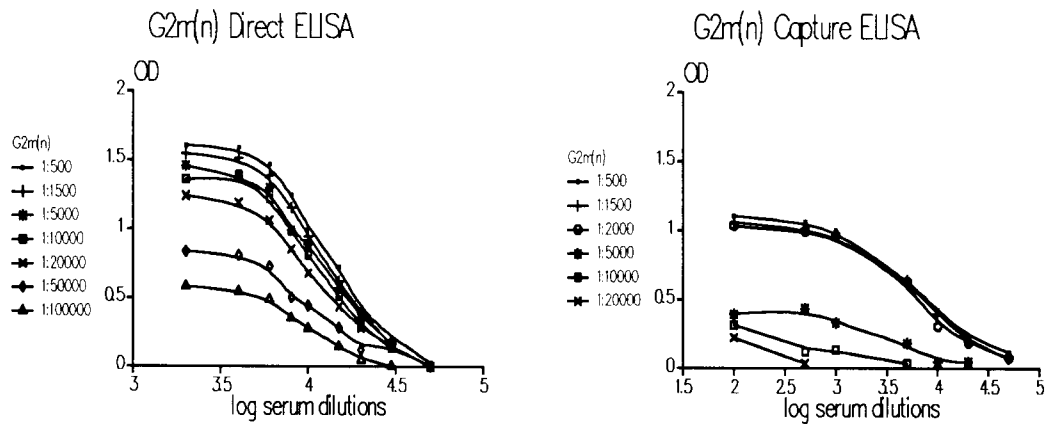


Figure 6.1: Comparison of standard curves obtained using G2m(n) direct ELISA or G2m(n) capture ELISA. The monoclonal antibody G2m(n) was tested at various dilutions: 1:500 (•); 1:1 500 (+); 1:2 000 (o); 1:5 000 (*); 1:10 000 (□); 1:20 000 (x); 1:50 000 (♦), and 1:100 000 (▲)

6.2.2.2 *Monoclonal capture ELISA*

The microtitre plates were coated with 100 μl of a purified monoclonal anti-G2m(n) antibody in dilutions ranging from 1:500 to 1:20 000 in glycine/Tris buffered saline for 30 minutes at room temperature followed by incubation with 200 μl of 2% BSA for 30 minutes at room temperature. A coating concentration of 1:500 was selected (Figure 6.1).

Samples of positive (R1110) and negative (R1113) control sera were diluted in 0.2% BSA/TBS/0.005% Tween 20 (1:10 blocker solution) and 100 μl of the dilutions 1:100 - 1:500 000 were added to the wells, and incubated for 30 minutes at room temperature.

The conjugates assessed were goat anti-human gamma chain specific IgG conjugated to horseradish peroxidase (Cappel, Catalogue No 3001-0121 and Zymed, Catalogue No 628420) both diluted to 1:1 000 in 1:10 blocker solution.

The substrate used was OPD and the reaction was stopped with 50 μl 2N H_2SO_4. After each incubation the wells were washed 4 times with TBST.

6.2.2.3 *Conclusion and discussion*

The standard curve obtained using the direct (serum coating) ELISA was steeper than that of the capture ELISA (monoclonal antibody coating) and was shifted to the right (Figure 6.1). The negative control (R1113) was negligible in both assays (Table 6.1).

The capture ELISA also had significantly higher blanks than the direct ELISA (Table 6.1). For these reasons the direct ELISA was selected.

TABLE 6.1: EFFECT ON BLANKS IN THE G2m(n) AND G1m(f) ELISAs USING DIRECT OR CAPTURE ASSAYS

ELISA	Maximum Optimal Density of Signal		Optical Density (OD) of Background Blanks (% of maximum signal)		Negative Control R1113 % of +ve control
	Dilution	OD	Minus serum	Minus Moab	
Direct G2m(n) 1:10 000	1:2 000	1.495	0.041 (2.74)	0.098 (6.6)	0%
Capture G2m(n) 1:2 000	1:100	2.141	1.420 (66.32)	0.251 (11.7)	0%
Direct G1m(f) 1:1 500	1:500	2.144	0.093 (4.34)	0.172 (8.02)	0%
Capture G1m(f) 1:5 000	1:100	2.104	0.796 (37.8)	0.426 (20.25)	0%

6.2.3 Coating - G1m(f) ELISA

A similar comparison of direct (coating with sera) and capture (coating with G1m(f) antibody) ELISAs was undertaken. The monoclonal antibody used in these assays was mouse anti-human IgG1 (Fab specific) [G1m(f)], Clone SG-16 (Sigma, Catalogue No I-5385).

6.2.3.1 *Direct ELISA*

Preliminary checkerboard titrations were performed to determine optimal dilutions of coating sera, G1m(f) monoclonal antibody and conjugate. The optimum dilution of the G1m(f) monoclonal antibody in the direct ELISA was 1:1 500 (Figure 6.2).

6.2.3.2 *Monoclonal capture ELISA*

This method was similar to the G2m(n) capture ELISA except for the following dilution and antibody differences:

- The plates were coated with 100 μl of a purified monoclonal anti-G1m(f) antibody solution ranging in dilution of 1:100 to 1:100 000 (10-0.001 μg/ml) diluted in 1:10 blocker solution.
- The conjugate used was a goat anti-human gamma chain specific IgG conjugated to horseradish peroxidase diluted to 1:1 000 in 1/10 blocker solution (Cappel, Catalogue No 3001-0121).

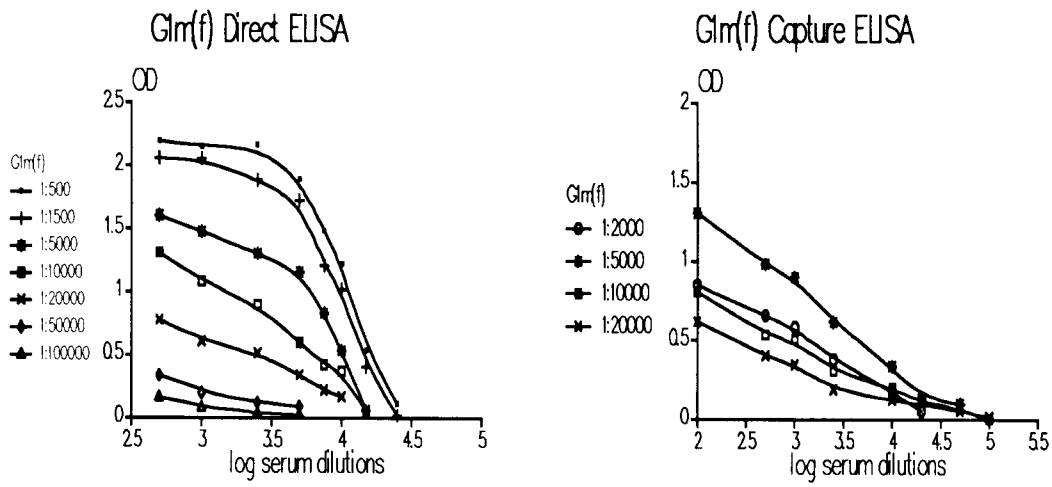


Figure 6.2: Comparison of standard curves obtained using G1m(f) direct ELISA or G1m(f) capture ELISA. The monoclonal antibody G1m(f) was tested at various dilutions: 1:500 (•); 1:1 500 (+); 1:2 000 (o); 1:5 000 (*); 1:10 000 (□); 1:20 000(x); 1:50 000 (♦) and 1:100 000 (▲)

Preliminary checkerboards had shown that the optimum G1m(f) monoclonal antibody concentration in the direct ELISA system was 1:1 500 and for the capture ELISA system 1:5 000 (Figure 6.2). Using the G1m(f) monoclonal antibody in the capture assay at dilutions 1:100 - 1:2 000 the minus serum blanks ranged from 95.8% to 72.7% respectively. At a 1:5 000 dilution the minus serum blank was 37.8%.

In both the direct and capture G1m(f) ELISA assays there was a clear discrimination of the positive control R1110 and the negative control R1113 (Table 6.1). However in the direct system the blanks (minus monoclonal blanks and minus serum blanks) were much lower than in the capture ELISA (Table 6.1).

Thus the direct ELISA method was also selected for the G1m(f) ELISA.

6.2.3.3 *Conclusion and discussion*

There are a few published reports of ELISAs to measure the G2m(n) allotype. Ota et al (1991) reported an inhibition ELISA using a G2m(n) myeloma protein (gift from Dr de Lange) as the coating antigen and adding samples that had been preincubated with a rabbit anti-Gm antiserum (made in their laboratory). These reagents are not readily available.

A capture ELISA system has an advantage over the direct immobilization ELISA in being more sensitive. It has a disadvantage in being more complex and expensive as plates need to be coated with purified anti-allotypes or anti-IgG subclass monoclonal antibodies as capture antibodies. There are several reports showing that a given monoclonal antibody may show assay restriction (Bird et al 1984; Jefferis et al 1985; de Lange et al 1989; Nelson et al 1990) which appeared to be dependent on the means of antigen presentation. Thus antibodies must be assessed in the system they are to be used in.

The CLB Netherlands recommend the capture ELISA for the determination of Gm allotypes G1m(z), G1m(a), G1m(f), G3m(g) using antibodies raised in their laboratory. However, an anti-G1m(f) monoclonal antibody (5F10)

can be used in both direct or capture ELISAs (de Lange et al 1989). There are no published reports using this method for the G2m(n) ELISA.

Tamaki et al (1990) also described direct and indirect ELISAs to measure anti-G1m(f) antibodies using a monoclonal antibody they had produced. They commented that low dilutions (1:10 dilutions) of the serum sample should not be used because unrelated serum proteins compete with IgG in the sample for binding sites on the solid phase. High dilutions of sample gave reliable results and eliminated the need for removal of the competing proteins with DEAE-cellulose. In the direct ELISA described here, coating concentrations of samples were 1:10 000 and dilutions of the standard curve ranged from 1:500 to 1:50 000.

6.2.4 Antibody

6.2.4.1 *G2m(n) ELISA*

Both a monoclonal and polyclonal rabbit antibody to human G2m(n) were assessed for usefulness in this system.

6.2.4.1.1 *Polyclonal rabbit anti-G2m(n) antibody*

The rabbit anti-human G2m(n) polyclonal-antibody was obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB) (CLB Product No R1072). This polyclonal antibody was tested at various dilutions from 1:250 - 1:16 000. Two detection conjugate antibodies were used:

- Goat anti-rabbit IgG, A, M conjugated to horseradish peroxidase. Cappel (Catalogue No 32120231)
- Goat anti-rabbit IgG affinity purified antibody conjugated to horse radish peroxidase. Kirkegaard and Perry (Catalogue No 14-15-16).

In this system the minus serum blanks were very low ($\leq$ 4% of signal) but the minus polyclonal antibody blanks gave very high background signals using either of the above mentioned conjugate antibodies (Table 6.2). This system could not discriminate between the positive (R1110) and negative (R1113) controls whose standard curves created were virtually superimposable (Figure 6.3).

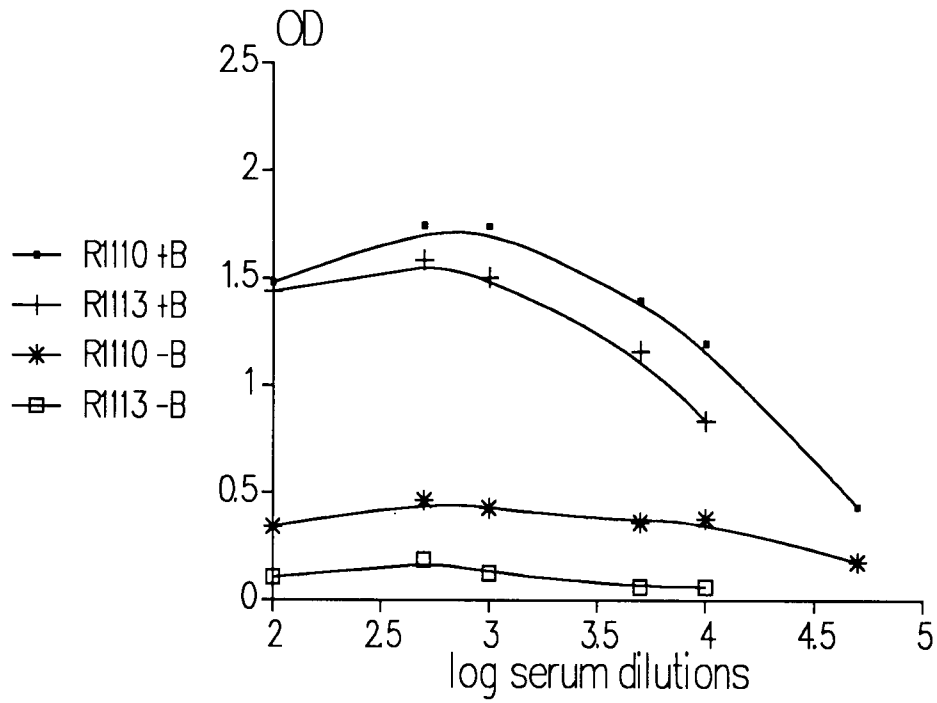


Figure 6.3: Comparison of dose response curves with the G2m(n) positive reference serum R1110 and the G2m(n) negative reference serum R1113 in the direct G2m(n) ELISA using polyclonal antiserum. Curves are drawn without (+B) and with (-B) the background blanks subtracted

TABLE 6.2: EFFECT ON THE BLANKS OF USING POLYCLONAL OR MONOCLONAL ANTI-G2m(n) ANTIBODIES IN THE ELISA

ANTIBODY	OPTICAL DENSITY (OD) OF BACKGROUND BLANKS (% of maximum signal)	
	Minus Serum	Minus Antibody
Polyclonal anti-G2m(n)	4.1%	77.0%
Monoclonal anti-G2m(n)	2.7%	6.6%

6.2.4.1.2 *Monoclonal anti-G2m(n) antibody*

Checkerboard titrations of mouse anti-human IgG2 (G2m(n)) monoclonal antibody Clone SH-21 ranging in dilutions from 1:500 to 1:100 000 were done to find the optimum monoclonal antibody dilution to use. There was little difference in the shape of the curve using monoclonal antibody dilutions 1:500 to 1:20 000. There was a significant drop-off in signal and the curve became flatter using dilutions greater than 1:50 000 (Figure 6.1). The monoclonal antibody dilution of 1:10 000 was selected.

With the monoclonal anti-G2m(n) antibody the minus serum blanks were low ($\leq$ 3% of maximum signal) as were the minus monoclonal antibody blanks (6.6%) (Table 6.2).

There was clear discrimination between the positive (R1110) and the negative (R1113) control sera, the latter having no detectable signal in the assay. The monoclonal antibody to G2m(n) was clearly preferable to the polyclonal antibody.

6.2.4.2 *G1m(f) ELISA*

The monoclonal anti-G1m(f) antibody Clone SG-16 was assessed for use in this system. Checkerboard titrations of monoclonal antibody in dilutions ranging from 1:500 to 1:100 000 were done to find the optimum monoclonal antibody dilution to use. There was little difference using a dilution of 1:500 or 1:1 500 but the curve changed shape and became flatter using dilutions of 1:1 500 or greater (Figure 6.2) and a dilution 1:1 500 was selected. This resulted in minus serum and minus monoclonal blanks that were low (4.3% and 8.0% of the maximum signal respectively) (Table 6.1) and there was also a clear discrimination between the positive (R1110) and negative (R1113) controls.

6.2.4.5 *Conclusion and discussion*

The rabbit polyclonal anti-human G2m(n) antibody tested in the direct ELISA showed poor allotype detection whereas the monoclonal antibody SH-21 showed specificity.

Several monoclonal antibodies have been raised in various laboratories to G1m(f) (Bird et al 1984; de Lange et al 1989; Tamaki et al 1990; Ota et al 1991) and to G2m(n) (Ota et al 1991) which have been reported to be suitable for use in ELISA. To date there are three commercially available monoclonal antibodies to G1m(f); Clone SG-16 (Sigma, Biomakor) and Clone 5F10 (CBL, Netherlands) and Clone TM14 (HP6027) (Zymed). There have been no reports of the comparison of these three antibodies in ELISA assays. 5F10 has been shown to be useful in both a direct and capture ELISA system (de Lange et al 1989) and TM14 showed anti-allotypic activity in direct and inhibition ELISAs but not in the capture ELISA (Nelson et al 1990).

6.2.5 Conjugates

6.2.5.1 *G2m(n) ELISA*

The various anti-mouse IgG antibodies coupled to horseradish peroxidase were evaluated:

- Non-affinity purified horseradish peroxidase goat anti-mouse IgG (gamma chain). (Cappel, Catalogue No 3301-0121)
- Horseradish peroxidase conjugated goat anti-mouse IgG (H and L) (Oxoid Catalogue No A81032).
- Non-affinity purified adsorbed horseradish peroxidase sheep anti-mouse IgG, A, M (Binding Site, Catalogue No PP270).
- Non-affinity purified, adsorbed horseradish peroxidase sheep anti-mouse IgG (gamma chain) (Binding Site, Catalogue No PP272).

The Binding Site sheep anti-mouse IgG, A, M horseradish peroxidase conjugate was selected as it gave the lowest blank readings in both the minus serum and minus monoclonal blanks (Table 6.3). It was diluted 1:1 000 in 1:10 blocker solution.

TABLE 6.3: EFFECT OF CONJUGATED ANTIBODIES ON BLANKS IN THE G2m(n) ELISA

		Optical Density (OD) of Background Blanks (% of maximum signal)	
Conjugate Dilution	**Maximum OD of Standard**	**Minus Serum**	**Minus Moab**
Oxoid GαM* H+L chain 1:500	0.714	0.026 (3.6)	0.153 (21.4)
Cappel GαM* γ chain 1:1 500	1.268	0.052 (4.1)	0.186 (14.6)
Binding site SαM** GAM 1:1 000	1.326	0.033 (2.5)	0.074 (5.6)
Binding site SαM** γ chain 1:2 000	1.029	0.077 (7.5)	0.151 (14.7)

* GαM = goat anti mouse
** SαM = sheep anti mouse

6.2.5.2 *G1m(f) ELISA*

The same anti-mouse horseradish peroxidase antibodies were evaluated in the G1m(f) ELISA. There was little difference in the blanks between the Cappel goat anti-mouse conjugate and the Binding Site sheep anti-mouse IgG, A, M conjugate (Table 6.4). The blanks of both were significantly lower than the blanks of the Binding Site gamma chain specific conjugate.

The Binding Site IgG, A, M conjugate was selected for these ELISAs as it was also the conjugate best suited to the G2m(n) ELISA.

TABLE 6.4: EFFECT OF CONJUGATED ANTIBODIES ON BLANKS IN THE G1m(f) ELISA

		Optical Density (OD) of Background Blanks (% of maximum signal)	
Conjugate Dilution	**Maximum OD of Standard**	**Minus Serum**	**Minus Moab**
Cappel GαM γ chain 1:2 500	1.697	0.018 (0.8)	0.130 (7.7)
Binding site SαM IgG, A, M 1:1 000	1.852	0.025 (1.3)	0.094 (5.2)
Binding site SαM γ chain 1:500	1.472	0.405 (27.5)	0.361 (24.5)

6.3 METHOD

6.3.1 G2m(n) ELISA

All reagents and samples were added to the wells in 100 μl volumes and all incubation periods were for 30 minutes at room temperature unless otherwise stated. After each step in the procedure plates were hand washed with TBST (20 mM TBS).

96 well Nunc maxisorb uncertificated flat bottomed plates (Weil, Catalogue No 4-4204) were coated with serum diluted in glycine/Tris buffer pH 7.0 - 7.5. A standard curve was constructed from nine dilutions of an in-house standard of pooled AB sera. The dilutions used for G2m(n) assay were 1:2 000, 1:4 000, 1:6 000, 1:8 000, 1:10 000, 1:15 000, 1:20 000, 1:30 000 and 1:50 000. Two dilutions (1:15 000 and 1:20 000) of the positive (R1110) and negative (R1113) reference sera were included in each plate. Positive and negative reference controls were obtained from the CLB (Netherlands). Reference sera R1110 was G2m(n)+ve and G1m(f)+ve whereas sera R1113 was G2m(n)-ve and G1m(f)-ve. Patient sera were diluted 1:15 000 for the G2m(n) assays. All sera were diluted in glycine/Tris buffer and were assayed in triplicate. The plates were washed once and then blocked with 2% BSA in TBST. Plates were washed twice before the addition of the mouse anti-human IgG2 (G2m(n)) monoclonal antibody Clone SH-21. Monoclonal antibody SH-21 was diluted 1:10 000 in 1:10 blocker solution and then added to the wells. After a 30 minute incubation the plates were washed four times with TBST. The Binding Site IgG, A, M conjugate diluted 1:1 000 in 1:10 blocker solution was then added to the wells. After incubation and 5 washes the enzyme substrate OPD was added. The reaction was stopped after 15 minutes with the addition of 2N H_2SO_4 and the plate was read on the ELISA reader, at 492 nm.

6.3.2 G1m(f) ELISA

This method was similar to the method mentioned above except for the following dilution and antibody differences.

- The standard curve dilutions of the 'in-house' standard were 1:500, 1:1 000, 1:2 500, 1:5 000, 1:7 500, 1:10 000, 1:15 000, 1:25 000 and 1:50 000.

- Dilutions of positive (R1110) and negative (R1113) controls were 1:10 000 and 1:15 000 and test sera 1:10 000
- The monoclonal mouse anti-human IgG1 (Fab specific) [G1m(f)] Clone SG-16 was diluted to 1:1 500 in 1:10 blocker solution.

6.3.3 Design of the plate

Every assay plate included 9 dilutions of the standard AB serum for the calibration curves, background reaction wells (minus monoclonal antibody), uncoated (minus monoclonal antigen wells) and dilutions of the positive and negative reference sera for monitoring reproducibility. All reactions were performed in triplicate. The design of the plate is similar for the G1m(f) and G2m(n) ELISAs and is illustrated in Chapter 1 (Figure 1.1). The blanks in column 10 in the Gm allotype ELISAs are:

Row A, B C: Minus coating antigen (serum), plus monoclonal antibody

Row D Minus monoclonal antibody, minus serum

6.3.4 Calculation of results

The blanks were subtracted from the mean of the triplicates of the standard sample dilutions. Standard curves relating absorbance to the concentration of IgG1 G1m(f) and IgG2 G2m(n) subclass specific antibody were constructed. These standard curves were used to calculate the concentration of allotype specific immunoglobulin present in the sample. The pooled AB serum had been calibrated against the WHO standard 67/97 with assigned values for IgG1 and IgG2 subclasses (Chapter 2.2.7). Standard dose curves were constructed from average absorbance values and concentrations that had been entered into a curve fitting four parameter logistic programme based on the mathematical model described in Chapter 1.12. The concentration of analyte in each sample was obtained by interpolating the

average absorbance values for the standard curve and multiplying the result by the dilution factor. Only values from the linear portions of the curves were considered valid. This allowed quantitative evaluation of the G1m(f) and G2m(n) allotype in each sample. The total IgG1 and IgG2 level of each sample measured by the ELISAs described in Chapter 2.3, was compared to the G1m(f) IgG1 and G2m(n) IgG2 measured in the ELISAs described here. In this way homo- and hetero-zygosity of the allotypes was determined (Chapter 9.3.2). If the subclass value of the sample measured in the allotype specific assay was 75% or greater of the total subclass value, the sample was considered homozygous positive for the allotype. Samples with negative values in the allotype specific assays were homozygous negative. Samples of allotypic subclass with a value less than 75% of the total subclass value were considered heterozygous for the allotype.

6.4 ASSAY STANDARDISATION

6.4.1 Standard Curves

6.4.1.1 *G2m(n) and G1m(f)*

Standard curves for the G2m(n) and G1m(f) ELISAs using the 'in-house' pooled AB serum standard and the positive reference control R1110 were parallel (Figure 6.4). Nine triplicate dilutions of pooled human AB serum were therefore used to construct the routine standard curves for both ELISAs.

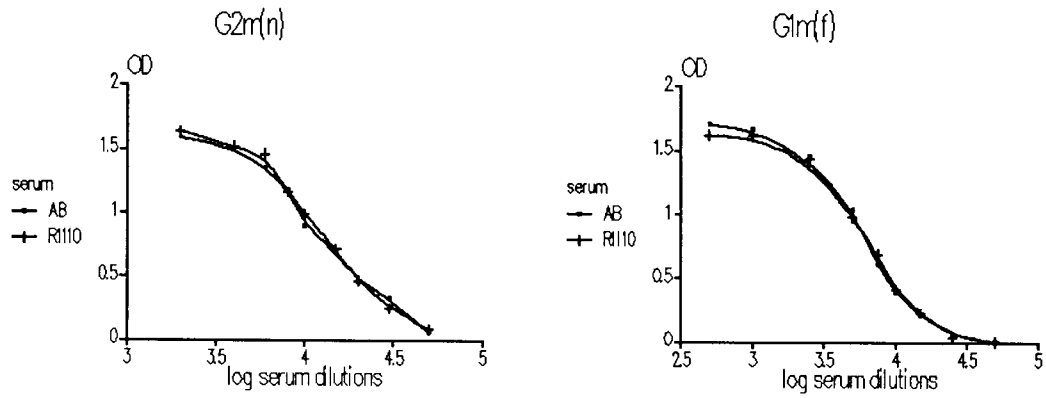


Figure 6.4: Comparison of G2m(n) and G1m(f) standard curves of AB serum (•) with the reference standard R1110 (+)

6.4.2 PRECISION

6.4.2.1 *G2m(n)*

Two dilutions of the positive (R1110) and negative (R1113) reference serum were inserted in every assay. Running means were calculated from the positive reference control inserted in each run and experiments were repeated if the values fell outside the 2 SD range. Assay precision was assessed using inter- and intra-assay coefficient of variation (CV). The inter-assay CV was 13.57% calculated from values obtained in 40 runs over 20 consecutive weeks. The intra-assay CV was 6.9% obtained by assaying the same sample 12 times in one run on 3 occasions and using the mean CV.

6.4.2.2 *G1m(f)*

Running means were calculated from controls inserted in each run as described for G2m(n) ELISA and experiments were repeated if the values fell outside the 2 SD range.

The inter- and intra-assay CV's were calculated as described above for the G2m(n) ELISA. The inter-assay CV was 11.6% and the intra-assay CV was 3.1%.

6.4.3 Specificity

The specificity of the assays were assessed by competitive inhibition ELISAs by double diffusion in gels (G2m(n) only) and by haemagluttination inhibition.

6.4.3.1 *G2m(n) Competitive inhibition ELISA*

This ELISA was carried out as described in 6.3.1 except that various dilutions of G2m(n)+ve sera (1:10-1:200 000) were mixed with the anti-G2m(n) monoclonal antibody and preincubated for 30 minutes at room temperature before addition to the coated ELISA plates. Preincubation of the monoclonal antibody with dilutions of G2m(n)+ve sera of up to 1:2 000 caused 100% inhibition in the G2m(n) ELISA. A 50% inhibition of the assay was caused with dilutions of positive sera in the range of 1:2 000 to 1:5 000 (Table 6.5).

Negative control sera at dilutions of greater than 1:30 cauused no significant inhibition (Table 6.5). Results were identical with several different G2m(n)+ve sera tested (data not shown).

6.4.3.2 *G1m(f) Competitive inhibition ELISA*

This ELISA was carried out as described in 6.3.2 except that dilutions of G1m(f)+ve sera (1/10-1:200 000) were mixed with the anti-G1m(f) monoclonal antibody and pre-incubated for 30 minutes before addition to the coated plates. Dilutions up to 1:1 000 caused 100% inhibition. The negative control sera did not cause significant inhibition at dilutions greater than 1:20 (Table 6.5). Results were similar with several different G1m(f)+ve sera tested (results not shown).

TABLE 6.5: G2m(n) AND G1m(f) COMPETITIVE INHIBITION ELISAs

Dilution of Serum	% INHIBITION BY ALLOTYPIC POSITIVE SERA			
	G2m(n)+ve Serum	G2m(n)-ve Serum	G1m(f)+ve Serum	G1m(f)-ve Serum
1/10	100	53	100	44
1:20	100	37	100	37
1:30	100	7	100	14
1:40	100	0	100	1
1:50	100	0	100	2
1:100	100	0	100	4
1:200	100	0	100	0
1:500	100	0	100	0
1:1 000	100	0	100	0
1:2 000	88		81	
1:5 000	39		42	
1:10 000	20		32	
1:20 000	18		27	
1:40 000	11		16	
1:100 000	19		15	
1:200 000	20		10	

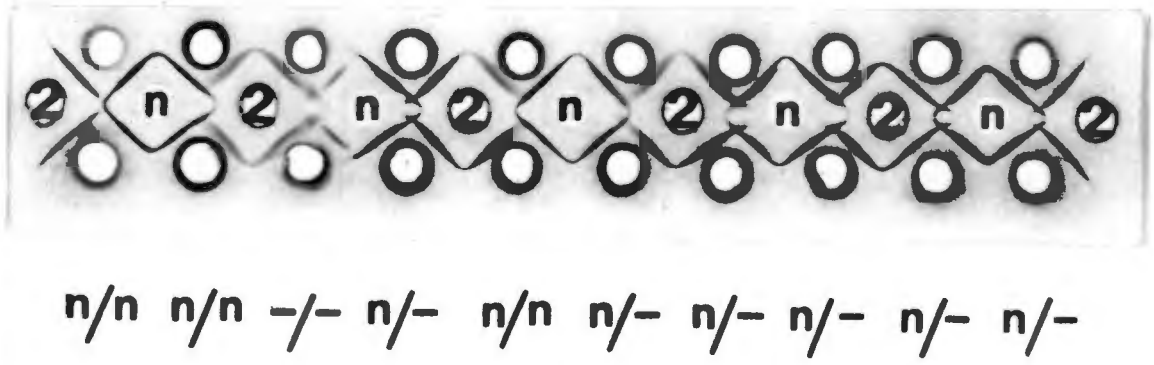
6.4.3.3 Double diffusion assay for genotyping of G2m(n)

Gels for the double diffusion test contained 1% agarose, 3.8% polyethylene glycol 6 000, 0.9% sodium chloride and 0.02% sodium azide in 0.05 M phosphate buffer pH 7.4. Agarose was coated onto gelbond (10 cm x 7.5 cm) (10 ml/piece) and allowed to set. Three rows of wells (3 mm in diameter) were punched in the agarose. Each serum sample was simultaneously tested against anti-G2m(n) (SH-21 Sigma) and anti-IgG2 (HP6014). 2 μl of anti-G2m(n) (SH-21) or 4 μl anti-IgG2 (HP6014) monoclonal antibody were added to alternate wells of the centre row (Figure 6.5). 4 μl of test serum was added to the wells of the top row and 6 μl of test serum to the wells of the bottom row. The gelbond was incubated overnight at 4°C in a moist chamber to prevent drying out. It was then soaked in 0.9% saline for 15 minutes, distilled water for 15 minutes, pressed between No 1 chromotography paper for 30 minutes and then left to dry overnight at 37°C. The gelbond was fixed with 2% acetic acid for 10 minutes and then stained for 15 minutes in amido black stain (0.5% in methanol/glacial acetic acid 9:1). It was destained with methanol/glacial acetic acid 9:1.

Specimens including positive and negative G2m(n) samples were measured with this technique and there was complete concordance with results obtained using the ELISA method.

The three precipitation patterns I, X and T of G2m(n) homozygous negative, heterozygous and homozygous positive individuals described by Rautonen et al (1989) were duplicated in this study using identical antibodies and similar conditions. These precipitation patterns can be seen in Figure 6.5, when the test samples used were the positive (R1110, R1114 and R1117) and negative

(R1113, R1118) G2m(n) reference sera and 1:1 mixtures of the positive and negative reference sera.



n/n n/n -/- n/- n/n n/- n/- n/- n/- n/-

Figure 6.5: Precipitation patterns detected with the three well double diffusion assay. Wells in the top contained 4 μl of sample and wells in the bottom row 6 μl of the same sample. Wells in the middle row alternately contained 2 μl anti-G2m(n) = (n) or anti-IgG2 = (2). The interpretation of the resultant precipitation bands was: x = heterozygote n/-, T = homozygous positive n/n and I = homozygous negative -/-

There was 95% agreement in reading the patterns of the double diffusion gels between two independent observers. There was 96% agreement between the G2m(n) ELISA and the Ouchterlony on the presence or absence of the allotype in known reference specimens. The only sample in disagreement was the negative reference standard (R1118) which was negative in the ELISA but had a small but definite precipitation line in the Ouchterlony. The other negative reference standard (R1113) was negative in both assays. There was a 90% correlation between these two methods in determining the homo- or heterozygous state of positive reference specimens. With unknown samples there was a 75% correlation between these two methods.

Rautonen et al (1989) noted that monoclonal antibody SH-21 bound to IgG2 molecules (Fc fragments) but binding was much weaker to G2m(n)-ve than G2m(n)+ve Fc regions. In gel G2m(n)+ve sera precipitated with SH-21 and thus they used this monoclonal antibody as a typing reagent. G2m(n)+ve sera developed stronger precipitation bands with SH-21 than with anti-IgG2 HP6014. In the double diffusion assay testing each serum simultaneously against SH-21 and HP6014, three types of precipitation patterns developed. Samples of homozygous negative (-/-) individuals produced pattern I, heterozygous (+/-) sera generated pattern X and homozygous positive (+/+) sera developed pattern T. The explanation for these patterns was that if the anti-G2m(n) precipitate emerged first, IgG2 molecules of a +/+ homozygote would be prevented from crossing the line and pattern T would develop. The G2m(n)-ve molecules of a heterozygote could partially cross the anti-G2m(n)+ve precipitate and pattern X would arise. Homozygous negative sera would give the pattern I as a precipitation line would only develop with anti-IgG2 and not with the SH-21 (anti-G2m(n)) +ve antibody.

Rautonen, Sarvas and colleagues (Department of Bacteriology and Immunology, University of Helsinki, Finland) are the only group to have published data relating to the determination of heterozygous G2m(n) positive samples using the double diffusion method. In reproducing their method it was found that the precipitation lines were much clearer using 4 or 6 μl of sample rather than 2 or 4 μl originally described.

There is no reference standard for G2m(n) heterozygous positive measurement. There was 75-90% correlation between the homo- or heterozygous positive samples measured in the G2m(n) ELISA and the double diffusion Ouchterlony. It was not possible, but would have been of interest, to have correlated samples typed by G2m(n) ELISA and Ouchterlony in this laboratory to those typed by the double diffusion method in Dr Rautonen's laboratory.

6.4.3.4 Haemagglutination inhibition

G2m(n) and G1m(f) allotyping was undertaken by Dr G de Lange at CLB, Netherlands on 10 samples. Results obtained by this method were the same as those obtained with their ELISA method.

6.5 SUMMARY

The G2m(n) determinations for many of the studies worldwide have been assayed by haemagglutination inhibition (Ambrosino et al 1985; Petersen et al 1987; Sjöholm et al 1987).

Direct, capture and inhibition ELISA assays have all been described for various Gm allotypes using polyclonal and monoclonal reagents (Bird et al 1984; de Lange et al 1989; Tamaki et al 1990; Ota et al 1991; Oxelius and

Carlsson 1993). Despite the commercial availability for several years of anti-G2m(n) (SH-21) and anti-G1m(f) (SG-16) monoclonal antibodies there are no reports of ELISA systems utilizing them. The monoclonal antibody SH-21 has been reported to be specific for G2m(n) in both haemagglutination inhibition assays and precipitation after double diffusion in agar (Jefferis et al 1985; Rautonen et al 1989). The monoclonal antibody SG-16 has been reported to be specific for the G1m(f) allotype in ELISA (Jefferis et al 1985). In the monoclonal capture G2m(n) assay the background values of non-specific binding were greater than in the direct ELISA (Table 6.1). Since both the G2m(n) and G1m(f) were sensitive and specific using the direct ELISA system this was selected for convenience.

Groups from Finland and Sweden (Rautonen et al 1989; Sarvas et al 1989, 1990; Oxelius 1990a; Takala et al 1991) have published extensively on G2m(n) allotyping using the commercial monoclonal antibody SH-21 in a double diffusion system.

This study is the first detailed report using commercial, readily available monoclonal antibodies G2m(n) (SH-21) and G1m(f) (SG-16) in ELISA. The direct ELISAs described are simple and practical in that they require only 2-3 hours of bench work and no specific coating antigens. The assays are specific, reproducible and quantitative and are sensitive enough for routine allotyping, being capable of detecting G2m(n) and G1m(f) allotypes in the nanogram range. In both assays there was a clear discrimination between the positive and negative controls. G2m(n) and G1m(f) typing by direct ELISA with these antibodies can reliably separate sera into positive and negative allotypes. This is confirmed by the results of samples kindly typed by Dr de Lange's Laboratory (CLB, Netherlands). It is more difficult to evaluate the reliability of the 'genotyping' as although the precipitation

patterns of the double diffusion method of Rautonen (1989) could be duplicated, there was not 100% agreement between samples tested in both methods. The final evaluation of the method must await the availability of other methodology.

These assays will be used to study the 'gene dosage effect' in healthy children and in assessing the clinical significance of Gm allotypes in disease.

CHAPTER 7

Km(3) IMMUNOASSAY

The typing of light chain kappa allotypes is commonly done by the classical agglutination inhibition test. However ELISA is now a practical alternative method as anti-Km(3) antibody raised in rabbits is commercially available. This ELISA system would not be suitable for the measurement of Km(1) antibodies as only polyclonal human anti-Km(1) antibody is available. The chapter is divided into a number of sections discussing previous methods for Km(3) measurement and reasons for establishing the ELISA. The method and development of a direct ELISA to measure Km(3) allotypes is detailed. Finally results of experiments relating to the precision of the assay and applications of the assay are described.

7.1. INTRODUCTION

7.2. DETERMINATION OF OPTIMAL ASSAY CONDITIONS

7.3 METHODS

7.4 ASSAY STANDARDIZATION

7.5 SUMMARY

7.1 INTRODUCTION

The typing of kappa markers is commonly done by the classical inhibition of agglutination technique where the test serum inhibits the agglutination of antigen coated erythrocytes by anti-allotypic sera. The control sera, anti-allotypic sera and the method (Appendix G) are obtainable from the Central Laboratory of the Netherlands Blood Transfusion Service (CLB). Problems encountered with this method were the difficulty in obtaining a regular source of blood group OR_2R_2 erythrocytes, non-specific binding of uncoated erythrocytes with some of the control sera, and difficulty with scoring the results.

Young et al (1989) reported a modification of this method using 2 μl of reagent volumes (not 25 μl volumes) which enabled considerable savings of the valuable reagents. This method was attempted (Appendix H) but again problems were encountered with supply of OR_2R_2 erythrocytes and non-specific binding of uncoated erythrocytes. The plates were read microscopically and the agglutination results could be graded easily.

Moxley and Gibbs (1992) have recently reported a novel polymerase chain reaction-based genotyping for Km allotypic markers. This involves amplification of the constant segment of the kappa chain by polymerase chain reaction (PCR) followed by restriction enzyme digestion. Restriction sites in this constant region PCR product correlate with allotypic differences among Km(1), Km(1,2) and Km(3) alleles.

The commercial availability of an anti-Km(3) antibody raised in rabbits made the ELISA a possible alternative. The ideal ELISA requires monoclonal antibodies which are currently not available for Km(1) or Km(3).

The purpose of establishing this ELISA was to determine:

- The frequency of Km(3) positive allotypes in our local population
- Whether children with Hib meningitis, Hib osteomyelitis/septic arthritis and *S.aureus* oesteomyelitis/septic arthritis have the same frequency of the Km(3) allele as the control group.
- Whether the presence of the Km(3) allele has any influence on IgG subclass or subclass specific antibody responses.

7.2 DETERMINATION OF OPTIMAL REAGENTS AND CONDITIONS

7.2.1 Coating

Preliminary checkerboard titrations were done to determine the optimum coating concentration of the sera. Serial dilutions from 1:1 000 to 1:32 000, of the positive control serum giving the lowest signal (R1110), the negative control with the highest signal (R1118) and a 1:1 mixture of R1110:R1118 were used. A plateau effect was noted: there was no difference in signal with sera dilutions of 1:1 000 or 1:2 000 but the signal started to drop off with dilutions of 1:5 000 and was significantly reduced at dilutions between 1:10 000 to 1:32 000 (Figure 7.1). A coating dilution of 1:8 000 was selected, which was on the linear portion of the curve.

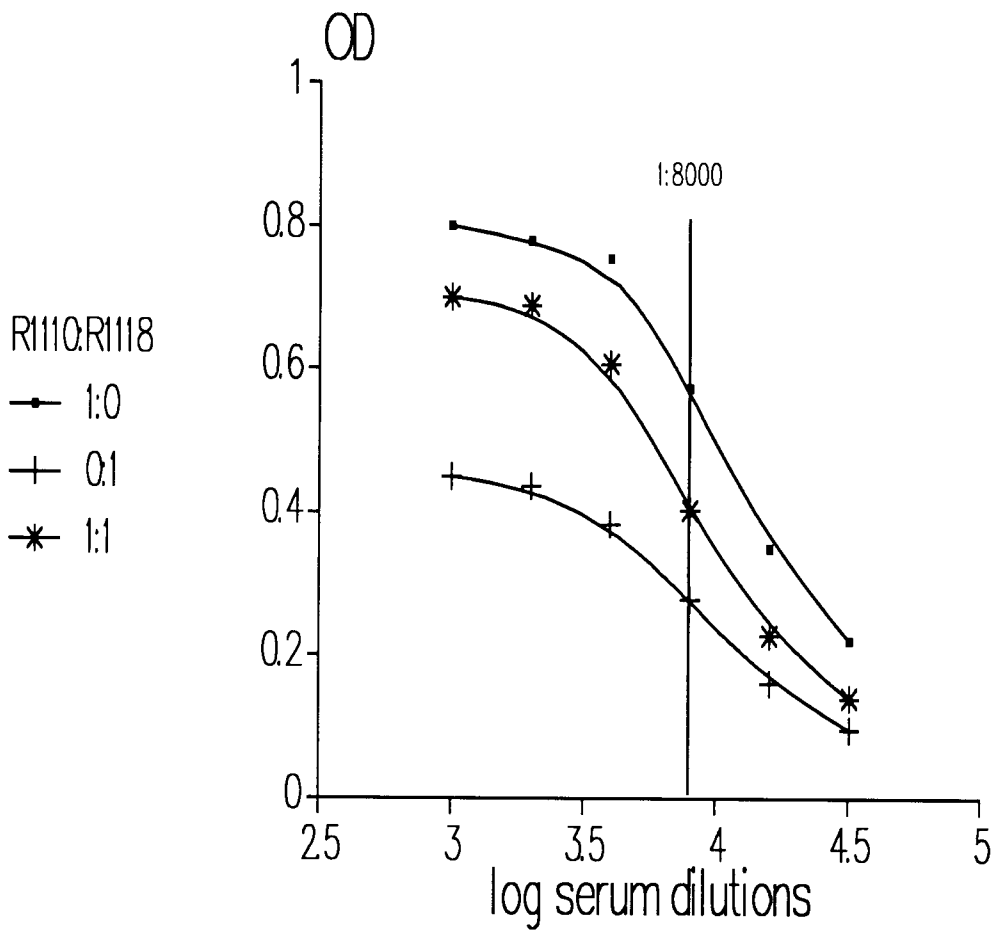


Figure 7.1: Km(3) ELISA titration curves with the positive standard R1110 (▪), the negative standard R1118 (+) and a 1:1 mixture of R1110 and R1118 (*) using coating dilutions of sera ranging from 1:1 000 to 1:32 000. A coating dilution of 1:8 000 was selected

7.2.2 Antibody

Checkerboard titrations were done to determine optimum dilution of the rabbit anti-human Km(3) antibody (CLB, Product No R1081). It was tested in doubling dilutions from 1:125 to 1:32 000. At dilutions of 1:125 and 1:250 there was a clear distinction between the positive and negative controls and the ratio of positive (R1110): negative (R1118) sera was greater

than 2:1. At dilutions of greater than 1:1 000 the ratio remained constant at 1.4:1. A dilution of 1:250 was selected for the assays.

7.2.3 Conjugates

The detection antibody used was anti-rabbit IgG antibody coupled to horseradish peroxidase. The following conjugated antibodies were evaluated:

- Horseradish peroxidase goat anti-rabbit IgG (H and L) Kirkegaard and Perry Laboratories Inc, Catalogue No 14-15-16
- Horseradish peroxidase goat anti-rabbit G,A and M (H and L), Cappel, Catalogue No 3212-0231
- Affinity purified horseradish peroxidase goat anti-rabbit IgG (H and L), Zymed, Catalogue No 62-6120.

The Zymed affinity purified goat anti-rabbit (H and L) horseradish peroxidase conjugate was selected as it gave the lowest uncoated well blanks (Table 7.1). The minus serum blanks were similar for all 3 conjugates evaluated.

TABLE 7.1: EFFECTS OF VARIOUS CONJUGATE ANTIBODIES ON BACKGROUND BLANKS IN THE Km(3) ELISA

HORSERADISH* PEROXIDASE CONJUGATES	OPTICAL DENSITY OF POSITIVE CONTROL R1110 (1:1 000)	OPTICAL DENSITY (OD) OF BACKGROUND BLANKS (% of R1110)	
		Minus Serum	Minus Antibody
KP 1:2 000	1.426	0.139 (9.8%)	0.442 (31.0%)
Zymed 1:1 000	1.097	0.110 (10.0%)	0.080 (7.4%)
Cappel 1:1 000	1.305	0.183 (14.0%)	0.990 (75.9%)

* = See text for details (7.2.3)

7.3 METHOD

7.3.1 Km(3) direct ELISA

All reagents and samples were added to the wells in 100 µl volumes and all incubation periods were for 30 minutes at room temperature unless otherwise stated. After each step in the procedure plates were hand washed with Tris buffered saline (TBS) (20 mM) containing 0.01% Tween 20 (TBST). Nunc flat bottomed microtitre plates (non certificated) (Nunc 4-42404), were coated with serum diluted to 1:8 000 in glycine/Tris buffer pH 7.0-7.5. An initial serum dilution of 1:100 was made and this was then further diluted 1:80. One positive control (R1110), one negative control (R1118) and mixtures of 1:4, 1:19 and 1:99 of R1110 and R1118 reference sera were included in each plate. All samples were run in triplicate. The plates were washed once and then blocked with 2% BSA, 0.05% Tween 20 and 1% goat sera diluted in TBS. Plates were washed twice before the addition of the polyclonal rabbit anti-Km(3) antibody (Catalogue No R1081, CLB) diluted 1:250 in 1/10 blocker solution to the wells. After washing the plates four times, Zymed affinity purified horseradish peroxidase conjugated goat anti-rabbit IgG antibody diluted 1:1 000 in 1/10 blocker with 1% goat serum was added. After incubation and 6 washes the enzyme substrate OPD was added. The reaction was stopped after 15 minutes by the addition of 100 µl of 2N H_2SO_4 and the optical density was read at 492 nm and 620 nm on the Anthos Labtec ELISA reader.

7.3.2 Design of plate

The plate format used in the Km(3) ELISA is different to that used in all other ELISAs in this study and is shown in Figure 7.2. Each plate includes one Km(3) positive standard (R1110), one Km(3) negative standard (R1118), three control mixtures made of a 1:4 (20%); 1:19 (5%) and 1:99

(1%) ratios of R1110:R1118, minus serum (uncoated wells) and minus polyclonal antibody well blanks. All reactions were performed in triplicate. The 'minus serum' blanks and the 'minus antibody' blanks were ≤ 10% of the positive standard. The 'minus serum' and individual 'minus antibody' blanks were subtracted from the mean of the triplicates of their respective serum readings.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	B1		CONTROLS					UNKNOWN SAMPLES				
C												
D			MINUS ANTIBODY									
E												
F			UNKNOWN SAMPLES									
G												
H			MINUS ANTIBODY									

B1 = minus serum blanks

Figure 7.2: ELISA template

7.3.3 Interpretation of results

The OD ratio of the average of the positive control to the average of the negative control was calculated and varied from 2.0:1.0 to 2.5:1.0. The Km(3) positive standard R1113 gave a higher signal than the R1110 positive standard.

The on-board software of the Anthos Labtec ELISA Reader was used to make a qualitative evaluation of the OD using programmed cut-offs to generate positive, negative or indeterminate results. Initially all unknown samples that gave an average OD that was greater than that of R1110 were interpreted as being positive for the Km(3) marker and samples were considered negative only if the average OD of the sample was below the highest single reading of the negative control R1118.

A titration of R1110/R1118 mixture was performed in an attempt to type samples whose ODs fell inbetween the positive and negative control values. The results of a titration of mixtures from 1% to 100% positive is shown in Figure 7.3.

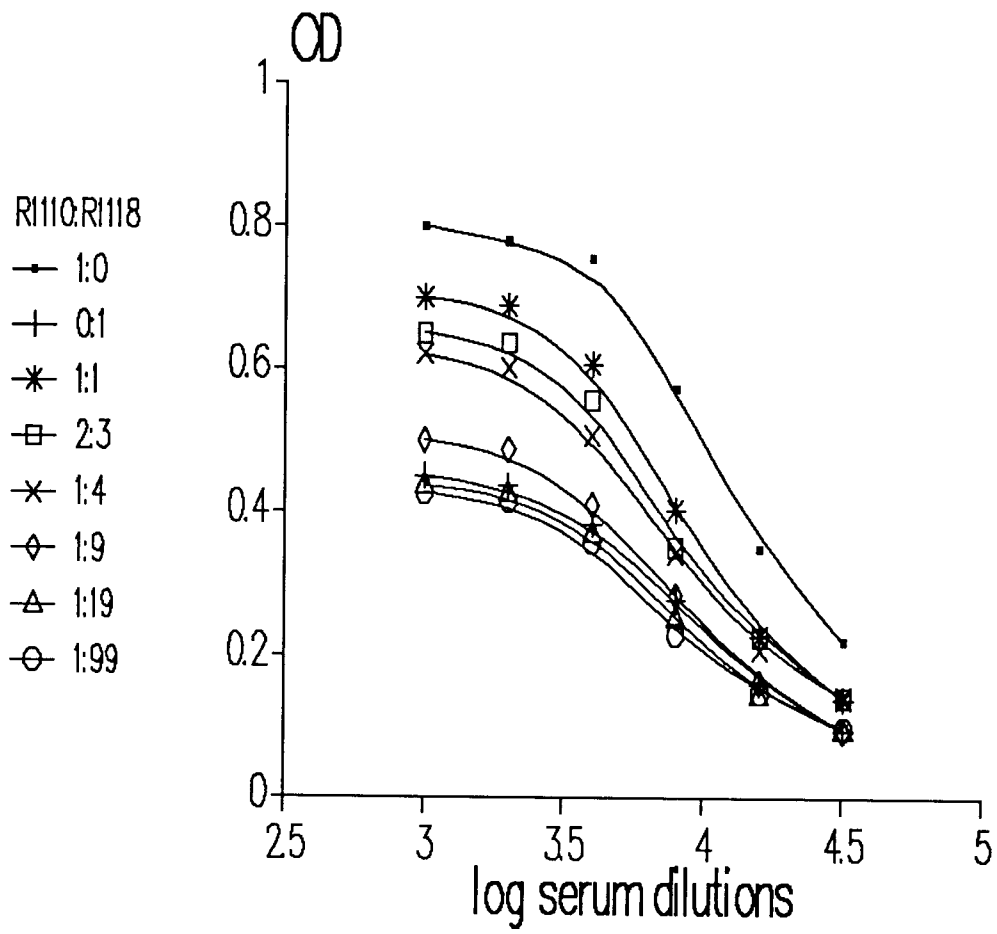


Figure 7.3: Km(3) ELISA titration curves of the positive standard R1110 (▪), the negative standard R1118 (+) and mixtures of the positive:negative controls ranging from 50% to 1%: 1:1 (*); 2:3 (□); 1:4 (x); 1:9 (◇); 1:19 (Δ) and 1:99 (o)

The mean OD, SD, 2SD range and inter-assay CV were calculated from 10 runs done over four weeks on the 1%, 5% and 20% control mixtures, 10 child control samples whose ODs fell between the positive and negative control values and 10 negative samples. From this data three clearcut groups could be distinguished (Figure 7.4). Samples with ODs below that of the 5% control mixture were considered negative for the marker;

samples with ODs greater than that of the 20% control mixture were considered positive for the marker and those samples with ODs falling in between the 5% and 20% control mixture were classified as non-typable. Thus additional controls of a 5% and 20% mixture of R1110:R1118 were included in each run. All samples falling into the negative or non-typable groups were repeated and if they remained in these groups they were classified as such. Of 411 healthy controls assayed for the Km(3) allotype only 3.4% were non-typable and 3.2% Km(3) negative using the above criteria.

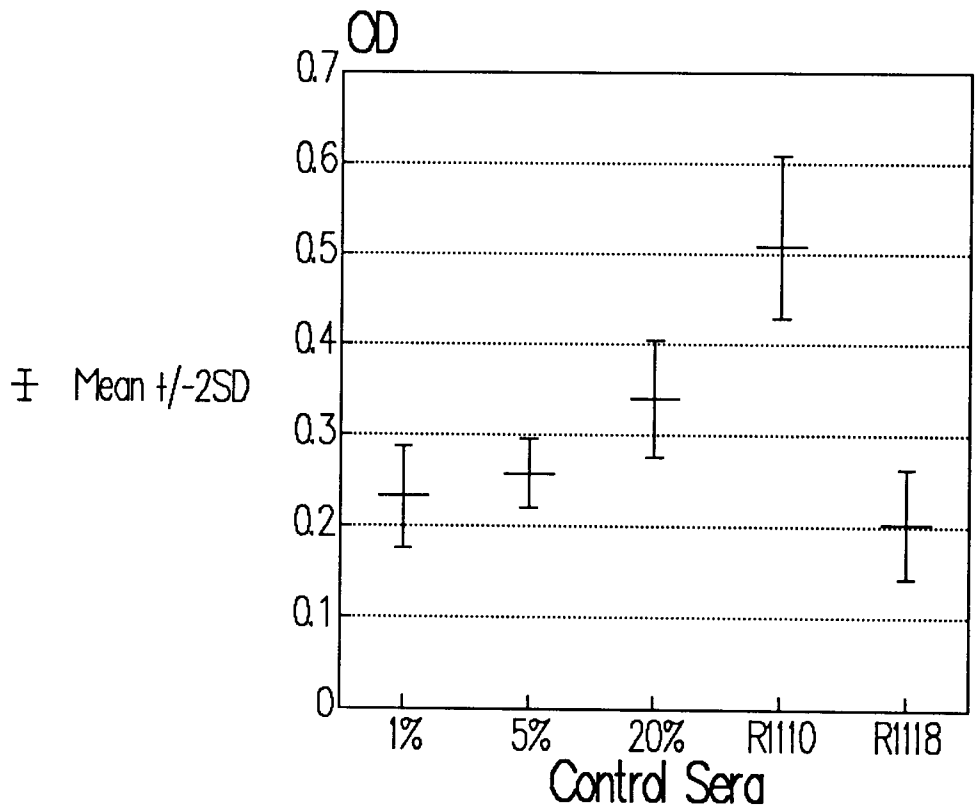


Figure 7.4: Mean +/- 2 standard deviations (SD) of optical density of the positive control R1110, the negative control R1118 and 1%, 5% and 20% mixtures of the positive and negative controls

7.4 ASSAY STANDARDISATION

7.4.1 Precision

The inter-assay coefficient of variation was 5.95%. This was calculated from the ratio of the positive to negative control over 23 consecutive runs.

If the ratio of the positive to negative control (R1110:R1118) was not in the range of 2.1-2.5:1 the run was repeated.

The results of ten specimens (2 negative, 8 positive) measured in the Km(3) ELISA correlated with the results obtained from the haemagglutination inhibition method done in Dr G de Lange's Laboratory, CLB Netherlands.

7.4.2 Specificity

The specificity of the assay was further assessed by an inhibition ELISA comparing the Km(3) positive reference serum R1113 against the Km(3) negative reference serum R1117 and Km(3)+ve light chain protein isolated from Bence-Jones Protein from the urine of a patient with multiple myeloma whose serum tested positive for Km(3) with the rabbit anti-human Km(3) antibody.

7.4.2.1 *Purification of light chains*

The light chains were separated by Fast Performance Liquid Chromatography (FPLC) using a Superose 6 (Pharmacia 17-0489-01) column. Alternating fractions #20-42 of the first peak emerging from the column were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% discontinuous gel according to the method of Laemmli (1970). Samples were diluted 1/2 in 0.0625M Tris-HCL (pH 6.8), 5% 2-mercaptoethanol, 2.5% SDS, 20% glycerol and 0.001% bromophenol blue. The proteins were then denatured by boiling

for 5 minutes. Separation of the samples (2-8 μg of protein per slot), was performed at constant voltage for 4 hours. The gel was stained with Coomassie Blue R250 and decolourised with glacial acetic acid/ethanol and dried using a Hoeffer Gel Dryer (Hoeffer SE S40). Low molecular weight markers (Pharmacia, Catalogue No 17-0446-61) were included and the molecular weight of the bands calculated. A band of 27 KD molecular weight corresponding to that of immunoglobulin light chains was evident from fractions #30 to #42 (Figure 7.5). Purified light chains in fraction #42 were used in the inhibition ELISA.

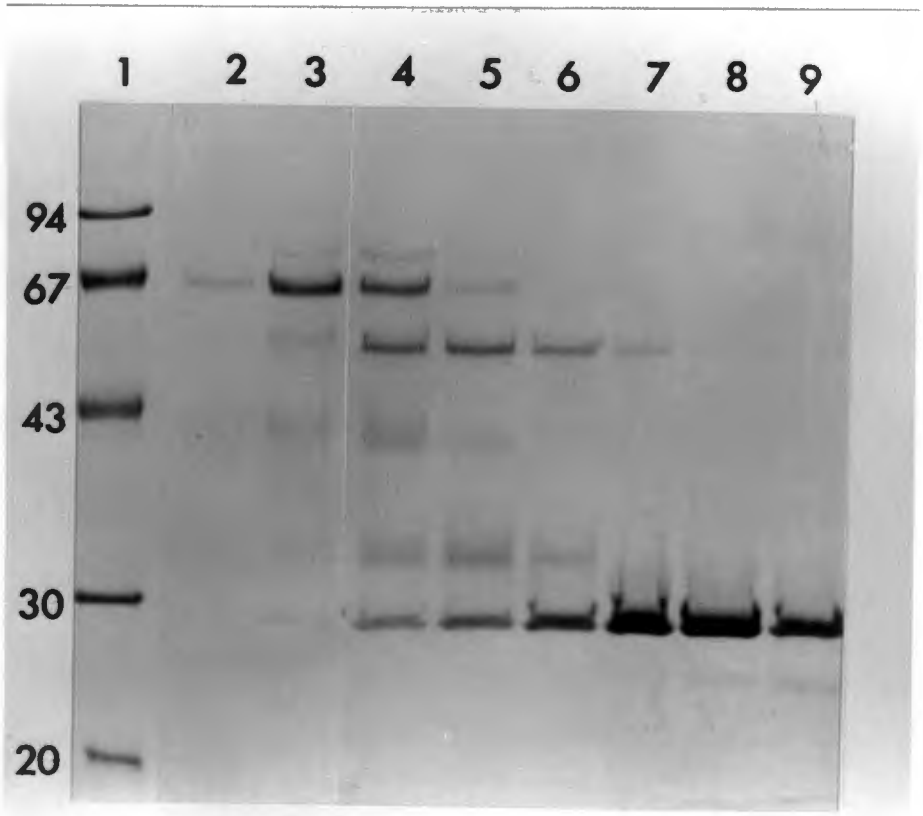


Figure 7.5: SDS-PAGE 10% discontinuous gel electrophoresis of Km(3) light chain proteins isolated from Bence Jones protein. Lane 1: low molecular markers; Lanes 2-9: (#28,30,32,34,36,38,40 and 42) (2-8 μg protein/slot)

7.4.2.2 *Competitive inhibition ELISA*

Binding of the Km(3) antibody to wells coated with both the positive control R1113 or purified light chains at a coating concentration of (1.25 μg/ml) could be inhibited by absorption with the isolated light chain protein (Figure 7.6) or (Table 7.2). This coating concentration of light chains was selected as it gave a signal equivalent to the mean of the positive controls. The negative control R1117 was unaffected in the inhibition ELISA. The competitive inhibition ELISA was carried out as described in 7.3.1 except that the Km(3)+ve light chains in concentrations ranging from 5 μg/ml - 0.08 μg/ml were mixed with rabbit Km(3) antibody (1:250) and added immediately to the plate.

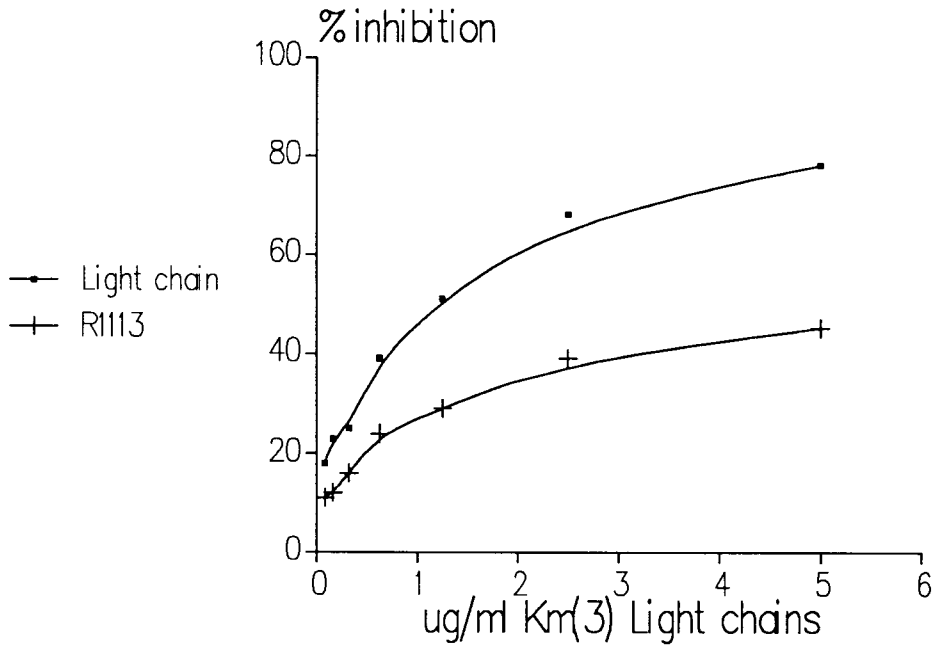


Figure 7.6: Competitive inhibition of Km(3) antibody with Km(3) positive light chains (▪) and Km(3) positive sera R1113 (+)

TABLE 7.2: COMPETITIVE INHIBITION OF Km(3) ANTIBODY

Purified Light Chains	% INHIBITION OF UNABSORBED READING	
	R1113 Positive Control	Purified Light Chains
5 μg/ml	45	78
2.5 μg/ml	39	68
1.25 μg/ml	29	51
0.64 μg/ml	24	39
0.32 μg/ml	16	25
0.16 μg/ml	12	23
0.08 μg/ml	11	18

7.5 SUMMARY

Most population studies have included only Km(1) allotyping due to the scarcity of sera with anti-Km(2) and anti-Km(3) activity (Steinberg 1969). The most commonly used method of Km(3) typing is the haemagglutination inhibition method although Lima and Newall (1990) reported an ELISA to identify Km(3) from dried blood stains. This assay has been successfully used to determine the presence or absence of the Km(3) allotype in human sera. The principle of the ELISA described by Lima and Newall (1990) is similar to the method described in this chapter with the test samples and controls coated on to the plates and revealed with a polyclonal antibody. The concentration of antigen coated onto the plate can not be compared since the sample was extracted from blood stained material and a 1:300 dilution made. The incubation time for coating was also short - 30 minutes. In both assays the ratio of the positive to negative controls was 2:1. 2% BSA with 0.05% Tween was used in this assay whereas 1% BSA with

0.005% Tween was used Lima's assay. Blocking could be achieved at 37°C for 45 minutes instead of 4°C overnight. Lima and Newall (1990) used the same antibody as in this assay but at a dilution of 1:100. The assay described here could be performed in two and a half hours compared to 2 days for the reported assay.

CHAPTER 8

LITERATURE REVIEW

The following review is divided into three sections. The first will cover the diseases Hib meningitis, Hib osteomyelitis/septic arthritis and *Staphylococcus aureus* osteomyelitis/septic arthritis (OM/SA). The epidemiology of the disease, the organisms and the immune response to these bacteria will be discussed. The second section reviews IgG subclasses, IgG subclass deficiency and regulation. Lastly, genetic susceptibility with particular reference to Gm and Km allotypes, C4B complotypes and mannose binding protein will be discussed.

8.1 ORGANISMS/DISEASE

8.1.1 *Haemophilus influenzae* type b (Hib) meningitis

8.1.1.1 *Epidemiology/incidence*

- Hib meningitis

  Hib, *Streptococcus pneumonia* and *Neisseria meningitidis* are the major aetiological pathogens in septic meningitis. There has been little change in the incidence of infection with each of these organisms over the past few decades and although mortality rates have declined, significant morbidity still occurs.

  Hib meningitis is a significant cause of morbidity and mortality in developed and developing countries (Ward and Cochi 1988). The reported incidence of Hib meningitis in children less than 5 years of age varies from 20-30 cases/100 000/year in England, the Scandinavian countries and the United States (Mäkelä et al 1992) to 150 - 450/100 000/year among native American, Alaskan or Australian populations. A recent study from South Africa (Hussey et al 1994) indicates that the overall incidence is similar to the USA and

Europe. However, the incidence is higher in Blacks and is similar to that from Gambia (300 cases/100 000/year in children less than 1 year) (Table 8.1). The disease is more common in a poor socio-economic setting.

TABLE 8.1: INCIDENCE RATE PER 100 000 POPULATION FOR CHILDREN WITH Hib MENINGITIS IN DEVELOPED AND DEVELOPING AREAS OF THE WORLD

LOCATION	ANNUAL INCIDENCE/ 100 000 POPULATION		REFERENCE
	< 1 year	< 5 years	
South Africa			Hussey et al 1994
Overall	112	34	
Black	210	50	
Coloured	113	27	
White	103	25	
Sweden		31	Bijlmer 1991
UK - Oxford		25	Booy et al 1993
USA	120-130	19-67	Bijlmer 1991
Alaskan Eskimos	871	782	Ward et al 1986
Najovo Indians	81	152	Bijlmer 1991
Gambia	297	60	Bijlmer et al 1990
Australia		450	Bijlmer et al 1990
Aborigines			

- Age and Sex

  Age is the most important risk factor for invasive Hib disease. The peak incidence of disease caused by Hib occurs between the age of 6 months and 12 months in both the developed (Madore et al 1990) and developing world (Hussey et al 1994). In the developed world 50% of Hib infections occur in children under 1 year of age whereas in developing countries more than 80% of cases occur in this age group.

  Early occurrence of Hib disease occurs in the same populations that have a high incidence of the disease. Hib meningitis and septic arthritis are the most serious manifestations. Epiglottitis is a serious complication of Hib disease that usually occurs after 2 years of age. Epiglottitis in older children is rare in areas where almost all Hib disease is seen in children $\leq$ 2 years of age.

  The sex distribution of invasive Hib disease shows a male predominance (Cochi et al 1985; Takala et al 1989; Gervaix and Suter 1991).

- Race

  The incidence of Hib disease is higher among Blacks than Whites in the USA and South Africa (Ward and Cochi 1988; Hussey et al 1994). The incidence of Hib meningitis in Coloured children in Cape Town is similar to that of White children in Cape Town and White children in the USA and Europe prior to immunization (Hussey et al 1994). Other ethnic groups at increased risk include Hispanics, Apache and Navajo Indians and Alaskan Eskimos (Hetherington and Lepow 1987; Ward and Cochi 1988).

- Socioeconomic

  Risk factors for primary invasive Hib disease include factors that increase exposure to Hib (day care attendance, presence of siblings, over crowded households and poverty) and factors that increase an individual's susceptibility to Hib infections (short duration of breast feeding, parental smoking and frequent infections in general) (Takala and Clements 1992).

- Nutrition

  Nutritional status has not been associated with an increase risk of invasive disease. Rosen and Davis (1980) found no association between meningitis and malnutrition and this was confirmed by Mulla et al (1984) who found that malnourished children are not any more prone to develop pyogenic meningitis from *H.influenzae*, pneumococcus or meningococcus than well nourished children.

8.1.1.2 *Organism*

H.influenzae is a gram negative pleomorphic coccobacillus which was first isolated by Pfeiffer in 1892. Man is the only natural host. In the 1930's Margaret Pittman defined two major groups of *H.influenzae*: encapsulated (typable) and unencapsulated (non-typable). Encapsulated isolates are surrounded by polysaccharide capsules which are distinguished serologically and are classified into six types, designated a to f depending on the basic sugar found in the capsule. Type b (Hib) is the most important pathogen and is responsible for more than 95% of invasive childhood haemophilus infections (Table 8.2).

TABLE 8.2: SPECTRUM OF *H.INFLUENZAE* DISEASE

Invasive disease: (predominantly type b (95%))

Meningitis	Cellulitis	Septic arthritis
Epiglottitis	Bacteraemia	Osteomyelitis
Pericarditis	Empyema	Pneumonia

Non-Invasive disease: (predominantly due to nontypable or non-type b strains)

Bronchitis	Otitis media
Sinusitis	Urinary tract infection

The number of cases of other forms of invasive Hib disease (such as pneumonia, epiglottitis, cellulitis, arthritis, pericarditis) appears to be similar to the number of cases of Hib meningitis (Hill 1983).

8.1.1.3 *Immunity*

Natural immunity to Hib is multifactorial and involves the functioning and integration of many components of the immune system including: i) mucosal immune factors ii) humoral antibodies iii) complement mediated activation of inflammatory responses and opsonization iv) phagocytosis and killing by macrophages and polymorphonuclear cells and v) T-cell mediated immune functions. The mechanisms which determine the natural acquisition of anti-PRP antibodies is not completely understood. Most individuals acquire protective immunity in the first few years of life without ever having developed Hib disease. This naturally acquired immunity develops as a result of pharyngeal colonization with Hib as well as asymptomatic colonization of the gut with normal enteric flora that are antigenically cross reactive with Hib (e.g. E.coli K100) (Schneerson and Robbins 1975). *H.influenzae* has at least 3 kinds of immunogenic surface components i.e. a polysaccharide capsule (PRP), outer membrane proteins (OMP) and

lipopolysaccharide (LPS). Although humans exposed to Hib may respond with serum antibody production to all three kinds of surface components, antibody to PRP appears to be most important in protection from Hib infection (Musher et al 1986). Antibody to Hib PRP is opsonic and bactericidal in the presence of complement and protective in humans and animals (Schreiber et al 1986). This polysaccharide capsule is composed of a linear polymer of ribose ribitol and phosphate [(-3) ß-D-ribose (1→1) ribitol (5-phosphate -)] abbreviated PRP.

The protective role of antibody to LPS or OMP is incompletely understood (Granoff and Rockwell 1978; Marks et al 1982; Kimura et al 1985).

The importance of antibodies in providing protection against invasive Hib disease is suggested by the inverse relation between the age specific risk of Hib infection and the levels of naturally acquired Hib antibodies observed during the early years of life (Hall et al 1987). Very few children between the ages of 3 months and 18 months acquire Hib PRP antibody (Käyhty et al 1981; Ward et al 1981; Claesson et al 1988). Newborns usually acquire transplacental bactericidal IgG antibodies but these antibodies wane in the early months of life and their loss is coincident with the marked increase in the risk of Hib disease. Production of PRP antibody is influenced by a variety of factors including age of the individual, and the type and duration of antigen exposure. The serum concentration of PRP antibody necessary for protection is not precisely known but is estimated to be 0.15-1.0 μg/ml (Käyhty et al 1983b).

Natural acquisition of anticapsular antibodies starts after 2 years of age when the child's immune system becomes mature enough to respond to Hib polysaccharide and corresponds to a rapidly decreasing incidence of Hib

disease. Alaskan children who present with the disease at younger ages develop higher levels of antibody at earlier ages, a finding that suggests earlier and greater exposure to antigen stimulation (Ward et al 1981). The age dependent pattern of infection could thus be explained by the pattern of acquisition of specific anti-Hib antibodies. It is hypothesized that genetic factors contributing to impaired or delayed synthesis of anti-Hib antibodies could increase susceptibility to the disease and that the higher incidence in some populations might be due to a high prevalence of particular antibody genotypes.

8.1.2 Hib osteomyelitis/septic arthritis (OM/SA)

There is experimental evidence suggesting that osteomyelitis and septic arthritis are manifestations of the same disease process in infants and children (Alderson et al 1986). Acute haematogenous OM/SA is a consequence of a bacteraemia or septicaemia which leads to gross destruction of the metaphysis, growth plate and epiphysis (Alderson et al 1986). Although the pathogenesis of the two illnesses is similar, the site of initial infection and local anatomical and cellular defence mechanisms may affect the clinical presentation. In this study acute bone and joint infections have been considered as a spectrum of the same disease.

8.1.2.1 *Epidemiology/incidence*

Septic arthritis accounts for 5-8% of all Hib systemic disease in children (Dajani et al 1979) and is the most common cause of septic arthritis in infants under 2 years of age (Nelson 1972; Lebel and Nelson 1988). In reviews of childhood osteomyelitis the incidence of Hib infections varied from 0 to 6.7% with a mean of 1.9% (Lebel and Nelson 1988). The majority of patients were less than 30 months of age which is similar to the distribution of Hib meningitis infection, but contrasts to the age distribution

of *S.aureus* OM/SA where 50% of children are older than 2 years (Fink and Nelson 1986). Males are more commonly affected (2.3:1) (Dich et al 1975; Rotbart and Glode 1985; Hoffman et al 1990). Acute osteomyelitis of children frequently affects the femur and tibia (Nade 1983a) and acute septic arthritis usually involves the hip and knee joints (Nade 1983b).

8.1.2.2 *Immune response*

The immune response to Hib OM/SA arthritis is presumably similar to that seen in Hib meningitis but this had not been extensively studied.

8.1.3 *S.aureus* osteomyelitis/septic arthritis (OM/SA)

8.1.3.1 *Epidemiology/incidence*

Acute haematogenous OM/SA is predominantly a disease of children. *S.aureus* is the major aetiological agent accounting for about 70% of cases.

In the Western Cape the incidence of acute osteomyelitis (all *S.aureus*) and septic arthritis (predominantly *S.aureus* and *H.influenzae*) is five times higher than that reported from developed communities (Hoffman et al 1990). The reason for this high incidence in the Western Cape is not known.

Little is known of specific risk factors predisposing to oesteomyelitis. A review assessing the importance of possible risk factors (age, sex, genetic, socioeconomic, distant foci of infection, minor trauma) based on pathogenic mechanisms of osteomyelitis detected no such factors (Wald 1985). In some diseases such as sickle cell anaemia, the haemoglobinopathies and chronic granulomatous disease a predisposition to osteomyelitis is recognised. Males are more often affected 2-3:1 (Dich et al 1975; Hoffman et al 1990).

8.1.3.2 *Organism*

S.aureus is a gram positive coccus occurring in pairs, short chains and clusters. *S.aureus* is one of the earliest recognized and most studied human pathogens. This organism is a common cause of invasive infections in both normal and abnormal hosts, and causes a number of toxin mediated diseases. Staphylococcal infections in children range from mild furuncles to severe disseminated septicaemia and the toxic shock syndrome (Cohen 1986). *S.aureus* commonly colonizes the skin and mucous membranes and can cause a variety of severe infections such as septicaemia, pneumonia, endocarditis, osteomyelitis and arthritis (Sheagren 1984).

8.1.3.3 *Immunity*

The major components of the cell wall of *S.aureus* are peptidoglycan (50%), ribitol teichoic acid (TA) (40%) and protein A (5%). Peptidoglycan consists of alternating links of N-acetyl glucosamine and N-acetylmuramic acid. Attached to the carboxyl group of each N-acetylmuramic acid is a tetrapeptide cross-linked by a pentaglycine bridge to a neighbouring unit. *S.aureus* teichoic acid has either α or ß linked N-acetyl-glucosamine residues in contrast to teichoic acid of other gram positive organisms which is usually composed of glycerol residues. Protein A binds non-specifically to IgG heavy chains.

The interactions of staphylococci and host defences are not completely understood. The occurence of antibodies to *S.aureus* cell wall determinants including peptidoglycan, teichoic acid and capsule (Verburgh et al 1981; Granström et al 1983; Wilkinson 1983) are widespread in humans.

Antibodies against many staphylococcal antigens occur in sera from healthy subjects as well as from patients with infections other than those caused by *S.aureus* (Rydén et al 1990). This can be explained by the fact that some antigens are commonly shared in gram positive bacteria.

Serology to several antigens such as: extracellular toxins (e.g. alpha-toxin, beta-toxin, gamma-toxin and leucocidin) extracellular enzymes (e.g. nuclease, coagulase, staphylokinase and lipase); as well as cell surface components (such as teichoic acid, lipoteichoic acid and peptidoglycan) have been proposed for the diagnosis of staphylococcal infections (Rydén et al 1990). *S.aureus* has a unique teichoic acid (a polymer of ribitol) as part of its cell wall. Teichoic acid antigens are in themselves poor immunogens, but when bound to peptidoglycan incite specific antibody formation. Antibody production to teichoic acid has been used as an aid to the diagnosis of staphylococcal infections. In adults with staphylococcal infection, sera containing teichoic acid antibodies (Granström et al 1983; Wheat et al 1984; Hammarström et al 1985) suggest that these antibodies are important in the defence against microorganisms.

Teichoic acid antibody may rise significantly during staphylococcal bacteraemia. Following invasive *S.aureus* infection, antibody to teichoic acid can be detected within 11-12 days. Titres begin to decline within 2-4 weeks after appropriate treatment and antibody is undetectable 2-5 months post infection (Tuazon and Sheagren 1976).

Uncomplicated bacteraemia or non-bacteraemic *S.aureus* infections either do not stimulate an antibody response or produce a low titre.

Antibodies against teichoic acid have been demonstrated in patients with various staphylococcal infections especially endocarditis and osteomyelitis (Crowder and White 1972; Tuazon and Sheagren 1976; Mackowiak and Smith 1978; Wheat et al 1978; Tenebaum and Archer 1980; Gramström et al 1983; Julander et al 1983; White et al 1983; Wheat et al 1985; Jacob et al 1987).

Tests which have been used to measure teichoic acid antibody include counterimmunoelectrophoresis (CIE), ELISA, solid phase RIA and agar gel diffusion (Tuazon and Sheagren 1976; Wheat et al 1978; Mackowiak and Smith 1978; Granström et al 1983). The latter test has been the most widely used as it is commercially available and easy to use. Thisyakorn et al (1984) compared the sensitivity of gel diffusion and ELISA for the detection of teichoic acid antibodies in the serum of children with staphylococcal infections and found the latter to be superior.

Sera from healthy children and adults contain teichoic acid antibodies (Granström et al 1983; Wheat et al 1986). Thus for optimal results the antibody levels of patients should be compared to age matched controls. Granström et al (1983) found that using age-correlated normal values their ELISA was a sensitive and specific diagnostic method for various staphyloccal infections.

8.2 IgG SUBCLASSES (ISOTYPES)

IgG isotype restriction and functional differences between the IgG subclasses have important implications in host defences and disease. Deficiencies can be manifested either as a deficiency of the IgG isotype or as an inability to make antibodies of a particular subclass to antigens despite normal concentrations of that isotype.

The inability to produce antibodies of the appropriate IgG subclass or to produce enough to protect against infection may lead to increased susceptibility to infection.

Although the exact role of the IgG subclasses is still unknown it is of interest that a predominance of one or other of the IgG subclasses is specifically stimulated in the IgG responses to defined antigens. Anti-carbohydrate antibodies are relatively restricted to the IgG2 subclass while antibodies to protein antigens are mainly of the IgG1 subclass.

8.2.1 Introduction

Human IgG subclasses were first mentioned in 1960 by Dray (1960). Using antisera against normal human IgG that were prepared in rheseus monkeys, and adsorbed with selected IgG myeloma proteins he was able to discriminate between three antigenically different components of IgG. A few years later Terry and Fahey (1964) and Grey and Kunkel (1964) showed that there were four subclasses of IgG. These four subclasses were officially recognised in a World Health Organization publication (1966) and termed IgG1, IgG2, IgG3 and IgG4.

The basic immunoglobulin molecule is a four chain structure composed of two identical heavy chains and two identical light chains. Each chain is made up of a genetically distinct variable region and a constant region. Antibodies are categorised into their various isotypes depending upon which heavy chain constant region gene is utilized. In humans these isotypes are IgM, IgD, IgA1, IgA2, IgE and four IgG subclasses IgG1, IgG2, IgG3 and IgG4. Each represents the product of a specific gene that encodes for the constant region of human immunoglobulin heavy chains. The four IgG subclasss are antigenically distinct and have different physiochemical

properties. Subclasses of human IgG differ in their biologic activities. IgG1, IgG2 and IgG3 fix complement whereas IgG4 does not. Although IgG2 is less able to activate the classical and alternative complement pathway, it is capable of doing so when the antibody and antigen ratio is high (Valim and Lachmann 1991). Three types of Fc gamma receptors (Fc γ R) with different tissue distribution and physiological roles have been described on the basis of immunochemical and physiochemical differences: Fc γ R1 (CD 64), Fc γ R11 (CD 32) and Fc γ R111 (CD 16). Fc γ R11a occurs in two allelic forms defined as low responders which interact with IgG2 and high responders which do not (Warmerdam et al 1991; Jefferis et al 1994) (Figure 8.3). This may prove to be of clinical significance for protection against encapsulated bacterial sepsis as IgG2 is the predominant subclass produced in response to bacterial polysaccharide antigens. The relative functional activities of each of the different IgG subclasses is not fully established.

TABLE 8.3: PROPERTIES OF IgG SUBCLASSES

ISOTYPE	IgG1	IgG2	IgG3	IgG4
Proportion of total IgG	60-70%	15-25%	5-10%	3-6%
Complement binding	+++	+	+++	0
Placental transfer	++	+	++	++
t ½ (days)	21-23	20-23	7-8	21-23
Specific antibody responses				
Tetanus toxoid	+++	+	+	++
Polysaccharides	++	+++	+	(+)
Blocking activity in allergy	0	0	0	+
Binding to Staph protein A	+	+	0	+
Fc receptor binding				
Fc γ R1	+++	0	+++	++
Fc γ R11a				
Low responder	+	+	+	0
High responder	+	0	+	0
Fc γ R111	+	0	+	0

8.2.1.1 *Factors affecting normal development and expression of IgG subclasses*

- Antigen restriction

  In view of the biological differences among the IgG subclasses, it is not surprising that the IgG subclasses may be selectively produced. Although antigenic stimulation may potentially give rise to antibody of any IgG subclass it is usually restricted to one or two subclasses depending on the type of antigen. IgG1 and IgG3 generally respond to the protein antigens of bacteria, viruses, vaccines and foods. IgG2 antibodies are predominantly stimulated by carbohydrate antigens and are important in protection against polysaccharide encapsulated organisms such as *Streptococcus pneumoniae, H.influenzae* and *Neisseria meningitidis*.

- Maturational delay

  Each IgG subclass has an individual pattern of development with IgG1 and IgG3 attaining adult levels at an early age whereas adult levels of IgG2 and IgG4 are not reached until adolescence. It is speculated that the delayed ability to produce an immune response to carbohydrate antigens is linked to this late maturation of IgG2. In children IgG2 deficiency is predominant whereas in adults IgG3 deficiency is more common (Hanson et al 1988).

- Race

  Several studies have reported racial differences of IgG subclass values. Shackelford et al (1985b) found Blacks had higher IgG, IgG1 and IgG2 concentrations than Whites. In contrast, Ambrosino et al (1991) found the IgG1, IgG2 and IgG4 subclass concentrations of Black children were lower than those of White children.

- Sex

  IgG subclass deficiencies are reported to be more common amongst boys than girls but are more frequent in woman than men (Hanson et al 1988). This shift occurs around puberty suggesting that additional factors e.g. hormones, may affect IgG subclass levels.

- Linkage

  The genes encoding IgG2, IgG4, IgA, IgE and IgD are closely linked. It follows that structural or regulatory defects on a genomic transcriptional or translational level may affect one or more of these closely related immunoglobulins. It is interesting that the physical relationship of the IgG subclasses on the genomic level is compatible with the combined IgG subclass deficiencies most frequently found in patients. In humans, the 5' → 3' C_H region gene order is C_μ, C_δ, $C_{\gamma 3}$, $C_{\gamma 1}$, $C_{\alpha 1}$, $C_{\gamma 2}$, $C_{\gamma 4}$, C_ϵ, $C_{\alpha 2}$. IgG2 deficiency is often associated with low IgG4 levels and selective IgA deficiency and may reflect this close linkage of the heavy chain genes (Oxelius et al 1981).

- Genetic factors

  Gm allotypes are genetic markers resulting from inherited amino acid differences in the IgG subclasses. The frequency of these immunoglobulin alleles varies between population groups, and Gm allotypes may influence the serum IgG subclass levels (Oxelius 1990b) thereby affecting normal ranges for IgG subclasses in different population groups.

IgG subclass deficiency has been reported in two patients with defective expression of MHC class II antigens (Bremard-Oury et al 1986).

- Environmental factors

  Environmental factors are also likely to influence the IgG subclass values. Repeated exposure to antigens is likely to stimulate the production of antibodies of a particular subclass more than would otherwise be expected. Egyptian patients with schistosomiasis have high concentrations of IgG4 (Iskander et al 1981).

8.2.1.2 *IgG subclass deficiency*

The field has been extensively reviewed - Heiner 1987; Ochs and Wedgwood 1987; Schur 1987; Morgan and Levinsky 1988; Hanson et al 1988; Jefferis and Kumararatne 1990; Morell 1990; Scott et al 1990; Smith 1992.

Deficiencies of individual subclasses of IgG were first described over 20 years ago. IgG subclass deficiencies are increasingly being recognized as significant entities within the spectrum of antibody-deficiency syndromes. The exact prevalence and clinical significance of IgG subclass deficiencies is not accurately known but they do appear to be relatively common (Schur 1987; Jefferis and Kumararatne 1990). Persons with immunoglobulin subclass deficiency range from healthy individuals to those with severe recurrent infections.

8.2.1.2.1 Disease associations

Many associations between IgG subclass deficiencies and disease conditions have been suggested (Table 8.4).

TABLE 8.4: ASSOCIATION OF ABNORMAL SERUM IgG SUBCLASS LEVELS AND DISEASE

	Change in IgG subclass
Infections	
Recurrent infections with capsulated bacteria	↓ IgG2/IgG4
Otitis media in children	↓ IgG2, ↓ IgG2 specific antibodies to pneumococcus
AIDS	Variable subclass deficiencies
Allergic disease	
Bronchial asthma	↓ IgG2/IgG3/IgG4; ↑ IgG4
Atopic eczema dermatitis	↑ IgG4
Allergy	↑ IgG4
Autoimmune diseases	↓ IgG2
Neurologic disorders	
Ataxia telangiectasia	↓ IgG2/IgG4
Frederich's ataxia	↓ IgG3
Miscellaneous immunodeficiencies	
C3 deficiency	↓ IgG2
IgA deficiency	↓ IgG2/IgG4
Adenosine deaminase deficiency	↓ IgG2

Patients with an IgG subclass deficiency commonly show an increased frequency of infections especially in the respiratory tract, including sinusitis, otitis media and bronchopneumonia, but also osteomyelitis, meningitis, septicaemia and various skin infections (Hanson et al 1988).

The importance of IgG subclass immunity to bacteria with polysaccharide capsular antigen is suggested as patients with decreased concentrations of IgG subclasses in the blood, especially IgG2 and/or IgG4 are more likely to

have recurrent infections than healthy persons with normal levels (Schur et al 1970; Oxelius 1984; Stanley et al 1984; Freijd et al 1984; Shackelford et al 1986, 1990; Hanson et al 1988; Moss et al 1992).

Lack of IgG2 has been shown to be related to low concentrations of antibodies against polysaccharides (Siber et al 1980; Insel and Anderson 1986b) and this might explain the relationship between IgG2 deficiency and infection with polysaccharide encapsulated bacteria. However, a number of observations show that decreased concentrations of IgG2 are not strictly related to recurrent infections (Hammarström and Smith 1983; Out et al 1986; Hammarström et al 1987; Nahm et al 1990).

Beard et al (1990a) found that impaired antibody production may be a predisposing factor in osteomyelitis/septic arthritis in children. Osteomyelitis has been reported in association with IgG2 subclass deficiency (Gottsegen 1987) and in an IgG4 deficient patient (Heiner et al 1988).

8.2.1.2.2 Carbohydrate specific antibody responses

It is known that the four IgG subclasses may have different affinities for an antigen (Persson et al 1988; Devey et al 1989).

Ideally it is desirable to measure antibodies against a range of bacterial antigens in order to obtain an insight into the immune competence of patients. This also permits a distinction to be made between specific antibody defects found in subjects with normal IgG subclass concentrations (Ambrosino et al 1987, 1988; Herrod et al 1989) and IgG subclass deficiencies of a more general type (Hammarström et al 1987).

The interaction of carbohydrate antigens with the immune system has a number of unusual characteristics. Responses to protein antigens are polyclonal, probably reflecting the many unique epitopes of these molecules. Polysaccharide antigens in contrast are large molecules with repeating determinants and relatively few epitopes. Antibodies to polysaccharides have been found to be oligoclonal rather than polyclonal. The influence of T-lymphocytes is limited and multiple exposure to antigen does not necessarily lead to a secondary immune response. The age intervals of natural immunity to carbohydrate antigens differs from most protein antigens (Briles et al 1987). IgG anti-polysaccharide antibodies are mainly of the IgG2 subclass. This subclass matures later than the other subclasses, resulting in a physiologic immunodeficiency up to 18-24 months of age or beyond.

Antibody against the polysaccharide capsule of Hib has been shown to play a major role in protection against invasive Hib disease. This protection correlates with the presence of serotype-specific antibodies against bacterial capsular PRP (Schreiber et al 1986).

Whether induced by infection or by immunization with PRP or PRP conjugate vaccines, the antibody response to PRP is clonally restricted (Insel et al 1985). Most people express a predominant idiotype (Hib idiotype -1 [Hib Id-I]) in the antigen-binding site of PRP-specific antibody (Lucas 1988; Lucas and Granoff 1990) reflecting the response of the VII A2Z light chains to PRP.

Several early studies have indicated that human polysaccharide antibodies (IgG) are predominantly IgG2 (Yount et al 1968; Barret and Ayoub 1986). The restriction of anti-carbohydrate antibodies to IgG2 is not absolute.

Recent reports indicate that after natural infections the IgG antibody response to some polysaccharide antigens is composed of IgG1 and IgG2 antibodies (Lane and MacLennan 1986; Claesson et al 1988). Most adults vaccinated with Hib or meningococcal group A polysaccharide develop substantial amounts of IgG1 as well as IgG2 antibody (Rautonen et al 1986; Mäkelä et al 1987; Shakelford et al 1987; Granoff et al 1988a) whereas pneumococcal IgG antibodies (types 2, 14 and 18c) are predominantly IgG2 (Sarvas et al 1989).

Immunization with Hib PRP covalently bound to a protein carrier shifted the polysaccharide specific IgG subclass from predominantly IgG2 to a mixture of IgG1 and IgG2. This suggests that a switch from one activating pathway to another may have occurred.

Children show predominantly IgG1 responses to Hib polysaccharide (Granoff et al 1988b; Käyhty et al 1988) whereas adults produce both IgG1 and IgG2 responses (Mäkelä et al 1987; Shackelford et al 1987; Granoff et al 1988a; Seppälä et al 1988).

8.2.1.2.3 T-cell Influences

One of the most fundamental concepts regarding an antibody response is that the amount of antibody produced is regulated by T-lymphocytes. Although it is clear that T-helper and T-suppressor cells control the antibody response to some bacterial polysaccharides, (meningococcal type A, type III *Streptococcus pneumonia*) it is not known whether this regulation is common to other clinically relevant bacterial polysaccharide antigens. The mechanisms by which T-suppressor and T-helper cells are activated and their exact mode of functioning remains unclear.

T-helper cells and/or their secreted cytokines regulate (i) the magnitude of the immune response; (ii) the isotype switch in immunoglobulin classes (IgM to IgG); (iii) the affinity maturation of antibody and (iv) the memory capacity (Abbas et al 1991).

8.2.1.2.4 T-dependent and independent responses

Based on T-cell involvement in antibody synthesis, antigens can be classified as thymus dependent (Td) or thymus independent (Ti) immunogens. Ti antigens are capable of activitating B-cells without T-cell help to produce mainly IgM antibody with few memory B-cells. Td antigens elicit stronger, earlier antibody responses, produce IgG antibody and induce memory cells (Stein 1992) (Table 8.5)

Antibodies to protein antigens tend to be T-cell dependent and are usually IgG1 and/or IgG3. Antibodies to carbohydrate antigens and polysaccharides including bacterial capsules tend to be T-cell independent and are usually IgG2 (Yount et al 1968; Freijd et al 1984; Barrett 1985). There is evidence that although T-cells are not required for the initiation of the antibody response they can influence the magnitude of the anti-polysaccharide response (Baker et al 1981).

Capsular polysaccharide antigens cannot be processed by antigen presenting cells and alpha beta T-cell receptor bearing T-cells cannot recognise carbohydrate epitopes. There is some evidence that gamma/delta T-cell receptor bearing T-cells may respond to carbohydrate antigens.

TABLE 8.5: PROPERTIES OF T-CELL DEPENDENT AND T-CELL INDEPENDENT ANTIGENS

	T-DEPENDENT	T-DEPENDENT	
		TYPE 1	TYPE II
Example	**Proteins**	**Lipo-Saccharide**	**Poly-Saccharide**
T-cell required for ab production	Yes	No	No
Ontogeny of response	Present at birth	Early	3-18 months
Response in athymic mice	No	Yes	Yes
Isotype switching	Yes	No	No
Secondary (memory) response	Yes	No	No
Antibody response	Heterogeneous	Restricted	Restricted
Affinity maturation	Yes	No	No
Polyclonal B cell activation	No	Yes	No

In humans the predominant subclass in response to T-dependent antigens is IgG1 although IgG2 is also produced in adequate amounts. In contrast, responses to T-independent antigens show significant but varying degrees of restriction to IgG2 (Stein 1992). When a T-independent antipolysaccharide response is converted into a T-dependent response the IgG2 isotype restriction seen with the polysaccharide antigens is lost.

Purified capsular polysaccharides are classified as type II T-cell-independent antigens, and the immature immune system of young infants appears

when they are conjugated to carrier proteins, capsular polysaccharides become more T-cell dependent and are more immunogenic in young infants. The involvement of helper T-lymphocytes is associated with a greater initial antibody response as well as with a true anamestic response with repeat immunization.

The exact reason for the absence of an immune response to Ti antigens such as polysaccharides is not clear and various hypotheses have been proposed to explain it (Bixler and Pillai 1989).

8.2.1.2.5 Lymphokine influences

The differences in magnitude of antibody response may result from inherent differences in the ability of specific carrier proteins to induce T-cell proliferation and lymphokine release.

Lymphokines regulate B-cell terminal differentiation and immunoglobulin production (Muraguchi et al 1988). In addition in humans and murine studies lymphokines appear to regulate IgG subclass selection (Snapper and Paul 1987; Lundgren et al 1989; Gascan et al 1991). Polysaccharide specific B-cells have also been shown to respond to interleukin-6 (IL-6) (Ambrosino et al 1990;).

T-cells activated by protein antigen such as tetanus toxoid might preferentially secrete gamma-interferon and thus induce antigen specific B-cells to produce IgG1 antibody whereas T-cells activated by polysaccharide antigen secrete IL-4 and IL-5 and possibly IL-6 to provide help for the differentiation of antigen specific B-cells into IgG2 producing plasma cells.

Ambrosino et al (1990) underlined the need for IL-6 in the in-vitro antibody response to polysaccharides. In addition it has been shown that gamma interferon and IL-6 can augment in-vitro antibody responses to both protein and polysaccharide antigens provided B-cells are primed in-vivo with T-cell dependent conjugate vaccine (Peeters et al 1992).

8.3 SUSCEPTIBILITY

Young age is the most important risk factor for Hib disease although genetic and environmental factors can affect responses to individual antigens. Socioeconomic risk factors for invasive Hib disease have been reviewed recently by Takala and Clements (1992). These risk factors can be broadly divided into two groups: i) factors that increase exposure to Hib (e.g. day care outside the home, overcrowding, presence of young siblings); and ii) factors that increase the child's susceptibility to Hib (parental smoking, short duration of or no breast feeding).

There is no explanation why only a small proportion of children develop invasive disease prior to the ability to synthesize protective levels of anti-Hib PRP or anti-teichoic acid antibody. However, the following evidence suggests that antibody responses to carbohydrate antigens and susceptibility to these bacterial infections may be under genetic control:

- Children who have recovered from Hib meningitis have lower antibodies to the polysaccharide capsule than siblings (Whisnant et al 1976) and do not respond to immunisation as well as controls (Norden et al 1975).
- Certain ethnic populations such as Alaskan Eskimos have very high incidences of infection with Hib (Ward et al 1981).
- Hib disease expression also differs in various races e.g. epiglottitis is common in Whites and rare in Blacks and Eskimos.

- Siblings of patients with meningitis have lower antibody responses to Hib-pertussis complex vaccine than control children (Granoff et al 1983).
- Children who develop bacterial meningitis or acute haematogenous osteomyelitis/septic arthritis are normally well nourished. Separate studies at Baragwanath Hospital and in Natal have shown that the incidence of malnourished children with bacterial meningitis is no greater than that of the general population (Rosen and Davis 1980; Mulla et al 1984). Most other infectious diseases are more common in undernourished children.

It appears that nutritional factors, unlike in other common infections, have only a minor role to play while genetic factors affecting antibody formation to carbohydrate antigens could be important in determining susceptibility to Hib meningitis, Hib OM/SA, and *S.aureus* OM/SA.

8.3.1 Allotypes

The risk of disease in relation to the frequency of different allotypes is of interest as allotypes are hereditary antigenic determinants present on immunoglobulin molecules and are polymorphic. All allotypes are inherited in a Mendelian fashion and are co-dominant. Human allotypes are genetic markers expressed as antigens on the constant regions of heavy or light chains of immunoglobulions.

Since 1956, 24 human immunoglobulin (Ig) allotypes have been described. Four of these allotypes are localized on IgG1 molecules (G1m), 1 on IgG2 (G2m), 13 on IgG3 (G3m), 2 on IgA2 (A2m), 1 on IgE (Em) and 3 on the Kappa-light chains (Km). Alphameric nomenclature for the allotyopes has

been used in this study. The corresponding numeric nomenclature is found in Appendix F.

Population studies have shown that some Gm haplotypes are characteristic for a particular race (de Lange 1991).

Ig allotypes have been studied in relation to the humoral immune response. Gm allotypes have been shown to influence IgG subclass levels (Morell et al 1972; Steinberg et al 1973; Oxelius 1990a, 1993; Sarvas et al 1991). Studies have demonstrated the interaction between the Gm and Km genes in antibody responses particularly to Epstein-Barr virus infection and to immunization with type III group B streptococcal antigen (Whittingham and Propert 1986). Schanfield et al (1979) showed an association between Gm (1,3,5,11,13) and the response to tetanus toxoid in Melanesians. Recent studies indicate that genes associated with certain Gm and/or Km immunoglobulin allotypes may be associated with altered responsiveness to vaccines containing bacterial polysaccharides (Pandey et al 1979, 1981; Granoff et al 1984, 1988a; Ambrosino et al 1985; 1988a).

The determination of allotypic markers is useful in the investigation of immune responses and disease susceptibility.

8.3.1.1 *Km light chain allotype*

The first allotype of immunoglobulin light chains was described by Robartz et al (1961) and termed InV (*Inhibiteuir virmontois*, named after the donor of the original antiserum), now designated Km (Kappa marker). The Km allotypes are inherited independently from the Gm allotypes. Human k light chains are encoded for by a single gene on chromosome 2 and are thus unlinked to the HLA loci. Three allotypes Km(1,2) and Km(3) controlled

by the Km(1), Km(1,2) and Km(3) alleles have been described. These allotypes are found in the constant portion of k light chains in all immunoglobulin classes. Usually Km(1) and Km(3) behave as antithetical alleles.

Most allotypes have originated from gene mutation of only one of a few nucleotides resulting in a difference of 1 or 2 amino acids. Studies of the aminoacid sequences of k chains have shown that substitutions of amino acids at position 153 and position 191 result in homozygous and heterozygous phenotypes (Table 8.6).

TABLE 8.6: RELATION BETWEEN THE Km ALLOTYPES AND THE AMINO ACID SEQUENCE OF THE CONSTANT REGION OF THE KAPPA LIGHT CHAIN

ALLOTYPE	ALLELE	AMINO ACID RESIDUE	
		POSITION 153	POSITION 191
Km(1,2)	Km(1,2)	ala	leu
Km(1)	Km(1)	val	leu
Km(3)	Km(3)	ala	val

In almost all Caucasian subjects the Km(2) factor is found when the Km(1) factor is present since these factors are transmitted together by the Km(1,2) allele. Subjects with the phenotype Km(-1,2,3) or Km(-1,2,-3) have not been reported (Lima and Newall 1990). Km(1) seems to be least frequent in Caucasians (10-18%) and most frequent in South American Indians (94%)

and varies from 80% to 50% for Mongoloids and Negroids (Lefranc and Lefranc 1990). The Km(3) allotype occurs frequently and is found in approximately 95% of Caucasians.

The accessibility of the Km(1) allotype to the anti-Km antibody depends on the isotype of the associated heavy chain and on the antibody quaternery structure. The gamma chain subclass influences the accessibility: the γ 1 and γ 3 chains strengthen it, whereas the γ 4 and the γ 2 chains suppress it by epitope masking (Terry et al 1965; Steinberg and Rostenberg 1969).

Allotypes have been associated with increased and decreased antibody responses to a variety of antigens. Various studies have shown substantial racial differences in associations between immunoglobulin allotypes and antibody responsiveness to the polysaccharide antigens of encapsulated bacteria. For example highly significant associations have been demonstrated between the Km(1) allotype and greater post-immunization antibody levels to *H.influenzae* and meningococcal C polysaccharides in white children but not in black children (Pandey et al 1979; 1981). Granoff et al (1984) found that the presence of the Km(1) allotype in both Black and White children was associated with a higher antibody response to Hib PRP than in children who lacked this allotype. The relative risk of meningitis was also lower among black children with the Km(1) allotype than in those who lacked this allotype although this effect was not observed in White children.

8.3.1.2 *G1m allotypes*

The four allotypes that may be present on the γ 1 chain of IgG1 molecules are G1m(z), G1m(a), G1m(x), and G1m(f) (Appendix F). G1m(f) was initially reported by Steinberg and Wilson (1963) but was independently

discovered and characterized by Gold et al (1965). G1m(f) is located on the Fd fragment of the IgG1 chain. Only a few allotypes are mutually exclusive, and therefore cannot be present on the same chain and G1m(f)/G1m(z) is one of them. It is known that the G1m(z) and G1m(f) allotypes correlate with lysine and arginine respectively at position 214 of the γ 1 chain.

There have been very few studies looking at the interactive effect of G1m(f), antibody responses and disease susceptibility (Whittingham and Propert 1986). Considering the increasing recognition of the importance of IgG1 responses to carbohydrate antigens in children the influence of the G1m(f) allotype in Hib meningitis, Hib OM/SA and *S.aureus* OM/SA was examined.

8.3.1.3 *G2m allotypes*

G2m(n) (G2m[23] in American literature) is the only allotype known on IgG2 molecules. It is found in 60-70% of Caucasians (Ambrosino et al 1985). It occurs as two alleles G2m(n) +ve or G2m(n) -ve (Kunkel et al 1966). The G2m(n) allele occurs at a frequency of 0.4 to 0.6 in Europe and 0.1 to 0.8 in Asia. It has been shown that this allele is present in Australian Aborigines at a frequency of 0.7 and that it is extremely rare in Blacks (Shackelford et al 1985b; de Lange 1991).

The exact location of the G2m(n) allotype on the CH2 domain of the γ2 chain is not yet known (Lefranc and Lefranc 1990).

It is well known that Gm allotypes influence IgG subclass levels (Sarvas et al 1991). Healthy adults expressing the G2m(n) marker have been reported to have higher concentrations of IgG2 in serum than those who were

negative (Morell et al 1972b; Steinberg et al 1973; van der Giessen et al 1973; Ambrosino et al 1985; Rautonen et al 1989; Oxelius 1993). There were no differences with respect to G2m(n) on IgG1 concentrations (Morell et al 1972b).

There is considerable controversy about the effect of the G2m(n) allotype on the specific antibody response to polysaccharide antigens (Table 8.76). Ambrosino et al (1985) found that G2m(n) positive individuals had better IgG responses to several polysaccharide vaccines than G2m(n) negative individuals. However, other studies have failed to confirm these findings (Granoff et al 1984, 1986a, 1988a). Although Granoff et al (1988a) found no significant differences in the magnitude of total IgG or IgG1 antibody responses between n-positive and n-negative individuals the n-positive individuals had higher IgG2 anticapsular antibody than n-negative individuals. More recently it was shown that the IgG2 specific response to several polysaccharide antigens was higher in n-positive than n-negative individuals (Morell et al 1989; Sarvas et al 1989, 1990). Interestingly Sarvas et al (1990) only found the effect of G2m(n) on the antibody response when the immunogen was the Hib PRP polysaccharide and not the Hib PRP conjugated to diphtheria toxoid. All these studies have been done in Caucasians.

TABLE: 8.7: EFFECT OF POSITIVE OR NEGATIVE G2m(n) ON ANTIBODY RESPONSE TO POLYSACCHARIDE VACCINES

REFERENCE	AGE	POLY-SACCHARIDE	SPECIFIC ab	G2m(n)+:G2m(n)-
Ambrosino et al 1985	Adults	Hib PRP	IgG	Increase
Granoff et al 1984	Children	Hib PRP - pertussis	IgG	No difference
Granoff et al 1986a	Children	Hib PRP - pertussis	IgG	No difference
Granoff et al 1988b	Adults	Hib PRP vaccine	IgG, IgG1 IgG2	No difference Increase
Sarvas et al 1989	Adults Children	Pneumococcal 3, 14, 18	IgG2 IgG1	Increase No difference
Sarvas et al 1990	Adults/ children Adults	Hib PRP Hib conjugate Hib PRP/ PRP conjugate	IgG2 IgG2 IgG1	Increase No difference No difference

Ambrosino et al (1985) have indicated an increased risk of Hib infections other than epiglottitis in G2m(n) negative individuals. However, other studies with larger numbers of patients have failed to confirm these findings (Granoff et al 1984; Granoff et al 1989; Takala et al 1991). The presence of G2m(n) may also be associated with a lower relative risk of vaccine failure (Granoff et al 1986b).

8.3.1.3.1 Zygosity of G2m(n)

It has been suggested that one of the paired G2m(n) alleles may be more productive than the other (Morell et al 1972b). In view of the functional differences between alleles it is useful to divide IgG2 into positive and negative forms and also to determine intermediate heterozygotes.

Heterozygous status cannot be easily determined as there are no antibodies available to the negative alleles of the markers. Rautonen et al (1989) have developed a triple well precipitation method to determine G2m(n) homo- and heterozygosity. Using this method Sarvas et al (1989) and Granoff and Holmes (1992) found adults homozygous positive for G2m(n) had higher IgG2 antibody responses to pneumococcal types 14 and 18c polysaccharides or Hib PRP than did those who were G2m(n) negative. Heterozygotes had intermediate levels.

8.3.2 C4

Other important defence mechanisms against invasive Hib disease are the complement system and phagocytosis. The complement system enhances clearance of *H.influenzae* from the bloodstream through its action as an opsonin in both non-immune and immune hosts. Complement deficiency is very rare and could not explain the incidence of Hib disease seen.

IgG1 anti-capsular antibody seems to be more efficient than IgG2 anti-capsular antibody at promoting complement-dependent opsonization of Hib and at initiating complement dependent bactericidal activity (Amir et al 1990). IgG1 subclass specific antibodies to Hib PRP are able to kill significantly more Hib than are comparable amounts of IgG2 Hib PRP antibodies in the presence of excess complement. In addition IgG1 requires lower concentrations of complement than IgG2 to generate comparable amounts of bacteriolytic activity.

The fourth component of complement (C4) is necessary for host defense against infection since one of its cleavage products (C4b) forms part of the enzyme (C4b, 2a) responsible for activating C3 and C5-C9 via the classical pathway. C4 is encoded by two closely linked loci within the major histocompatability complex. The gene products C4A and C4B share major structural characteristics but differ with respect to electrophoretic mobility, haemolytic activity and binding specificities. C4B preferentially binds to hydroxy groups and has four times the functional haemolytic activity than C4A on a mol-per-mol basis (Isenmen and Young 1984). C4A preferentially binds to amino groups and is more efficient at inhibiting immune precipitation.

Most individuals express four gene products although various combinations of deletions and duplications exist. The null alleles (QO = quantity zero, $C4A^{*}QO$, $C4B^{*}QO$) are defined by the hetero-or homozygous lack of C4A or C4B in the serum. Partial deficiencies of C4 are relatively common. It has been estimated that only 50-65% of people have four functional C4 genes, 30-38% have three, and that 5-10% have only two functional C4 genes. However, complete C4 deficiency is uncommon as $C4A^{*}QO$ and

C4B*QO genes rarely present on the same chromosome (Hauptmann et al 1988).

The prevalence in the general population of homozygous C4A deficiency is 1% and homozygous C4B deficiency is 3% (Awdeh and Alper 1980). The prevalence of homozygous C4B deficiency has been shown by some to be independent of ethnicity whereas others have shown population group differences in the frequency of the complement genes (Rowe et al 1989; Bishof et al 1990; Imanishi et al 1991).

No consistant immunological abnormalities have been detected in healthy individuals with C4A*QO or C4B*QO alleles. Recently a relatively common deficiency of one isotype of C4 (C4B) has been shown to be associated with invasive Hib disease (Bishof et al 1990) suggesting that defects in complement-mediated host defence may be more common in systemic Hib infections than previously thought.

Two independent studies have found an increase in C4B deficiency in children with systemic infections with encapsulated bacteria (Rowe et al 1989; Bishof et al 1990). However more recent studies have not confirmed these findings (Cates et al 1992).

Susceptibility to disease may be influenced by other genes within the MHC. C4A/21-OH deletion has been reported to be increased in patients with IgA deficiency/common variable immunodeficiency (French and Dawkins 1990).

The reason that a C4B deficiency may predispose an individual to bacterial meningitis is unknown. Possibilities include:

- As C4B preferentially binds to hydroxyl groups and since bacterial cell walls and capsules are rich in carbohydrates it is possible that C4B is more efficient in binding to bacterial surfaces, in assembling the C3-cleaving enzyme (C4b, 2a) and thus generating opsonically active C3b.
- C4B is encoded by genes located within the MHC class III complex in humans and may be in linkage disequilibrium with other genes in the MHC which are themselves in some way responsible for an abnormal immune response.

8.4. MANNOSE BINDING PROTEIN (MBP)

MBP is an acute phase protein synthesized in the liver (Esekowitz et al 1988; Thiel et al 1992). It is a C-type lectin with a binding specificity towards carbohydrate structures containing mannose or N-acetyl glucosamine. It is able to activate complement by the classical pathway upon binding to a mannose-rich surface (Ikeda et al 1987). These carbohydrates are found on a number of bacteria and yeasts and can activate the complement system without the involvement of specific antibodies.

Failure to opsonize baker's yeast is a defect found in 5-7% of the general population of healthy Caucasians. The presence of the defect has been linked to low levels of MBP (Super et al 1989). The frequency of MBP deficiency is similar in both Caucasians and Chinese but is unknown in Black and Coloured populations. The deficiency has been reported to be significantly more common in infants with recurrent respiratory tract infection, otitis media and chronic diarrhoea (Turner et al 1991).

MBP activation of the classical pathway of complement may be significant when other partial defects of the immune system exist leaving individuals compromised and susceptible to disease.

MBP may be especially important in early life. It has been speculated that infants between 6 and 24 months of age are most at risk when antibody independent mechanisms of complement activation such as MBP may play a critical role in protection against infections.

8.5 CONCLUSION

This review of the literature indicates that IgG subclass levels, specific IgG subclass antibody production, allotype regulation of IgG subclasses, C4B deficiency and MBP deficiency may be factors associated with an increased susceptibility to invasive *H.influenzae* and *Staphlococcus aureus* infections.

CHAPTER 9

HEALTHY CHILDREN AND ADULTS - RESULTS AND DISCUSSION

This chapter describes:

9.1 **CONTROL STUDY POPULATION** - this describes the population used to establish the normal ranges for the IgG subclass levels.

9.2 **IgG AND IgG SUBCLASSES** - this describes the frequency distribution, the transformation and statistical analysis of the IgG subclasses which resulted in the establishment of age, race and sex related percentile charts of normal ranges of IgG and IgG subclasses.

9.3 **ALLOTYPES** - describes the frequency of G1m(f), G2m(n) and Km(3) allotypes in the control groups. The effect these allotypes have on the IgG and IgG subclass levels is assessed.

9.4 **C4 PROTEIN TYPING** - describes the measurement and determination of the frequency of C4B*QO in the Black and Coloured population of the Western Cape.

9.5 **MANNOSE BINDING PROTEIN (MBP)** - describes the measurement of and determination of MBP levels in Black adults of the Western Cape.

9.6 **SUMMARY OF MAIN FINDINGS**

9.1. STUDY POPULATION AND COLLECTION OF SAMPLES

Permission for collection of samples was obtained from the Ethics and Research Committee, University of Cape Town, from the Divisional Council which run the well baby clinics and from the children's parents.

In this chapter children and adults of different ethnic backgrounds are referred to as Black, Coloured (mixed population inheritance) and White. Although population classification and apartheid have been abolished these racial groups have been retained because of important heritable and social differences that continue to exist.

Blood was collected under sterile conditions. Test serum was stored at -70°C in small aliquots to avoid repeated freezing and thawing of the samples.

9.1.1 Estimation of possibility of HIV infection in controls

The majority of specimens for this study were collected prior to 1990. At that time the incidence of human immunodeficiency virus (HIV) in the Western Cape was estimated to be very low (0.76%) (Epidemiological Comments 1993). Random samples of control specimens were sent for anonymous HIV testing. None of the 200 control specimens tested were positive. It was therefore concluded that the presence of HIV infection in the populations studied was most unlikely.

9.1.2 Children

Blood specimens were collected from 730 healthy children attending schools, community health clinics or from preoperative patients admitted for elective surgery. Four hundred and sixty seven of the samples were from Coloured children, 253 from Black children and 10 from White children.

The race and sex distributions of the controls are shown in Table 9.1. The racial distribution is representative of the ethnic groups attending this hospital. Because of the small number of both white patients and controls these have not been included in further analysis. One hundred specimens from Black children came from rural school children in the Transkei. All other specimens were collected from children resident in the Western Cape. Children with a history of previous hospitalization for an infective illness, or with an acute infectious illness, or who gave a history of frequent infections or immunodeficiency were excluded.

TABLE 9.1: RACE AND SEX DISTRIBUTIONS OF THE CONTROLS

CHILDREN			
Race	**Female**	**Male**	**Total**
Black	119	134	253
	(47.0%)	(53.0%)	(34.7%)
Coloured	154	313	467
	(33.0%)	(67.0%)	(64.0%)
TOTAL	**273**	**447**	**720**

ADULTS			
Race	**Female**	**Male**	**Total**
Black	50	25	75
	(66.7%)	(33.3%)	(44.1%)
Coloured	19	29	48
	(39.6%)	(60.4%)	(28.2%)
White	25	22	47
	(53.2%)	(46.8%)	(27.6%)
TOTAL	**94**	**76**	**170**

9.1.3 Adults

Samples from 170 healthy adults were obtained from the Western Province Blood Transfusion Service (Table 9.1). All blood donors were screened for HIV infection.

9.2 IgG AND IgG SUBCLASSES

IgG was measured by nephelometry using a Behring Nephelometer analyser (Software version 2.2.EXT). IgG subclasses were measured by ELISAs as described in Chapter 2.

9.2.1 Distribution

The IgG and IgG subclass distributions in 720 randomly selected healthy control children were studied. Frequency histograms of IgG and IgG1 in Black children and Coloured male children were Normally distributed. Figure 9.1 shows the distribution histograms of the IgG2, IgG3 and IgG4 levels of the Black children. The IgG1 results of Coloured female children and the IgG2, IgG3 and IgG4 data of all groups of children showed non-Normal negatively skewed frequencies (Table 9.2). The data were thus log transformed.

Log_{10} transformation of the data 'normalized' the frequency distribution except in the case of the IgG of the Coloured male children, the IgG2 of the Black children and the IgG4 of children of both races (Table 9.2). As the IgG, IgG1, IgG2, IgG3 and IgG4 data were neither constantly Normally nor log_{10} Normally distributed, the Box Cox transformation regression model was evaluated.

TABLE 9.2: TEST OF GOODNESS OF FIT OF OBSERVED FREQUENCY DISTRIBUTIONS OF CONTROL DATA WITH NORMAL DISTRIBUTIONS

	Untransformed	Log_{10} Transformed	Box Cox Transformed
IgG B	Normal	Normal	Normal
IgG CM	Normal	non-Normal	Normal
IgG CF	Normal	Normal	Normal
IgG1 B	Normal	Normal	Normal
IgG1 CM	Normal	Normal	Normal
IgG1 CF	non-Normal	Normal	Normal
IgG2 B	non-Normal	non-Normal	Normal
IgG2 C	non-Normal	Normal	Normal
IgG3 B	non-Normal	Normal	Normal
IgG3 CM	non-Normal	Normal	Normal
IgG3 CF	non-Normal	Normal	Normal
IgG4 B	non-Normal	non-Normal	non-Normal
IgG4 CM	non-Normal	non-Normal	non-Normal
IgG4 CF	non-Normal	non-Normal	non-Normal

B = Black children **CM = Coloured male children**
CF = Coloured female children

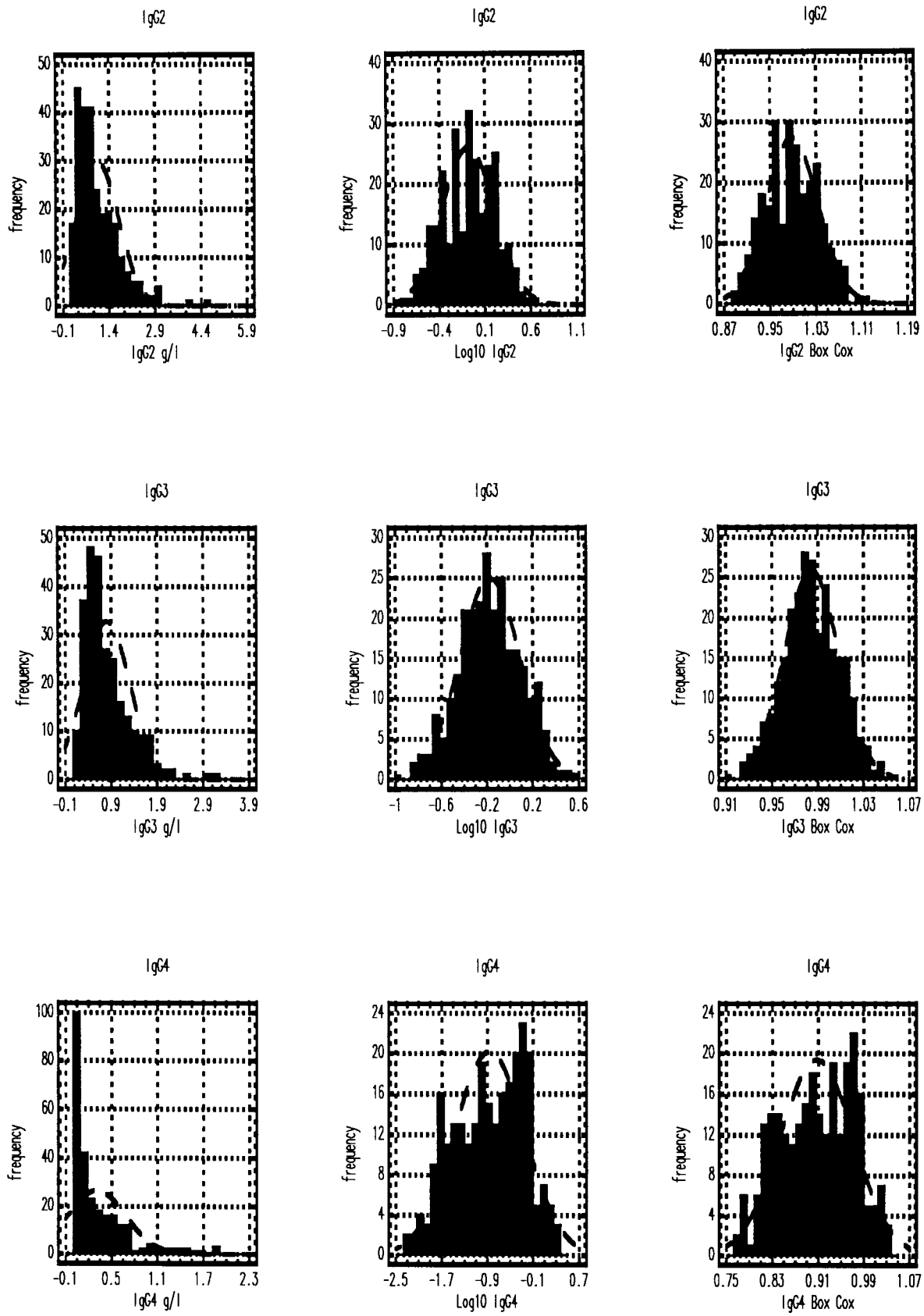


Figure 9.1: Distribution histogram of the untransformed, the $\log_{10}$ transformed and Box Cox transformed data of the IgG2, IgG3 and IgG4 subclass levels of the Black children. The expected Normal distribution is shown by the broken line (-)

The Box Cox transformation regression model was used for IgG, IgG1, IgG2, IgG3 and IgG4 (Box and Cox 1964). Age and sex and their interaction were included in the model in finding the appropriate Box-Cox transform (i.e. the λ parameter). The resulting transformed data were then checked for Normal distribution. The models fitted are in Table 9.3.

TABLE 9.3: FITTED MODELS OF BOX COX TRANSFORMATION

IgG	B	$(IgG)^{0.34}$	$= 2.24075 + 0.007927\ age - 0.000028681\ age^2$
IgG	CM	$(IgG)^{0.47}$	$= 2.8988 + 0.007212\ age$
IgG	CF	$(IgG)^{0.60}$	$= 3.7241 + 0.024909\ age - 0.000070779\ age^2$
IgG1	B	$(IgG)^{0.24}$	$= 1.7945 + 0.001406\ age$
IgG1	CM	$(IgG1)^{0.14}$	$= 1.3657 + 0.000421\ age$
IgG1	CF	$(IgG1)^{0.23}$	$= 1.6784 + 0.001405\ age$
IgG2	B	$(IgG2)^{0.07}$	$= 0.9565 + 0.000483\ age$
IgG2	C	$(IgG2)^{0.21}$	$= 0.89903 + 0.001274\ age$
IgG3	B	$(IgG3)^{0.04}$	$= 0.9694 + 0.000209\ age$
IgG3	CM	$(IgG3)^{-0.01}$	$= 1.008901 + 0.000008041\ age - 0.000000501\ age^2$
IgG3	CF	$(IgG3)^{0.08}$	$= 0.9311 + 0.000408\ age$
IgG4	B	$(IgG4)^{0.05}$	$= 0.8176 + 0.002836\ age - 0.000014193\ age^2$
IgG4	CM	$(IgG4)^{0.26}$	$= 0.4257 + 0.005653\ age - 0.000024659\ age^2$
IgG4	CF	$(IgG4)^{0.22}$	$= 0.4927 + 0.003837\ age - 0.000013110\ age^2$

B = Black children **CM = Coloured male children**
CF = Coloured female children

The Box Cox transformations of the IgG, IgG1, IgG2 and IgG3 were symmetrical and not statistically different from the Normal distribution (Table 9.2). IgG4 Box Cox transformation was symmetrical although this distribution differed from the Normal distribution (Figure 9.1).

The statistical significance of the discrepancy between the observed frequency distributions of the untransformed, log transformed and Box Cox

transformed data with the Normal distributions was evaluated with the Chi-square goodness of fit test. The $\log_{10}$ and Box Cox transformation significantly Normalised the data distribution in most groups (Table 9.2). The frequency histograms of the $\log_{10}$ transformed data showed Normal distribution in all groups except for IgG of Coloured male children, IgG2 of Black children and IgG4 levels of both groups. Box Cox transformation of IgG2 values of the Coloured male children produced a Normal distribution although log transformation of the same data did not (Table 9.2).

Although the frequency distribution of Box Cox transformated IgG4 differed significantly ($p = \leq 0.05$) from the Normal distribution, this transformation gave the data a symmetrical distribution not seen in the untransformed or $\log_{10}$ transformed data (Figure 9.1). For this reason in the statistical analyses of IgG4, Box Cox transformed data has been treated as having a Normal distribution.

9.2.1.2 *Discussion*

The frequency distributions of the four human subclasses have not been extensively investigated although the distribution of data is of importance when choosing a method of analysis. These data show that generally the IgG and IgG1 untransformed values of Black and Coloured children, were Normally distributed and that the Box Cox transformed data of the IgG, IgG1, IgG2 and IgG3 values, but not the IgG4 values were Normally distributed in Black children and Coloured children (Table 9.3). With the exception of IgG values of Coloured males, IgG2 values of Coloured children and IgG4 of both races the $\log_{10}$ transformed data was Normally distributed. In some cases inclusions resulting from visual assessment of the histograms were different from those based on statistical tests of goodness of fit. This highlights the importance of using a statistical test of goodness of

fit although most of the previous reports of frequency distribution of subclasses have relied on visual inspection of the frequency histograms (Shakib et al 1975; French and Harrison 1984; Bird et al 1985). The IgG4 data did not have a Normal distribution and neither $\log_{10}$ nor Box Cox transformation was able to Normalize the data.

As concentrations of the four subclasses are neither consistently Normal or log Normal distributed (Djurup et al 1988) calculations of normal ranges should not be based on the assumption of such frequency distributions.

In normal adults there is general agreement about the frequency distribution of each subclass (Shakib et al 1975, French and Harrison 1984). IgG1 is Normally distributed about the mean whereas the frequency distributions of the other subclasses particularly IgG4 are skewed. In children IgG1 has been found to be Normally distributed whereas IgG2 and IgG3 were approximately log Normally distributed and no transformation normalized the IgG4 data (Bird et al 1985).

A recent study by Djurup et al (1988) compared IgG subclass concentrations in sera from 200 normal adults measured in 2 different laboratories using the same technique (radial immunodiffusion) and reagents. In one laboratory the distributions of IgG1 and IgG2 were normal whereas those of IgG3 and IgG4 were skewed, whereas in the other laboratory all four subclasses were significantly different from a Normal distribution. The observed frequency distribution of a subclass may be influenced by the technical performance of the assay. Thus each laboratory evaluating IgG subclasses should determine the frequency distribution of the subclasses before undertaking statistical analysis and determination of normal ranges, and should not interpret results from a reference range that has been established in another laboratory.

9.2.2 Age

A regression analysis of the IgG and IgG subclasses against age demonstrated a correlation with age (Figure 9.2). The respective r and p values were: IgG r = 0.61 ($p = \leq 0.0001$), IgG1 r = 0.38 ($p = \leq 0.0001$), IgG2 r = 0.45 ($p = \leq 0.0001$), IgG3 r = 0.42 ($p = \leq 0.0001$) and IgG4 r = 0.41 ($p = \leq 0.0001$).

Age had to be taken into account in Box Cox transformation of IgG and the four IgG subclasses (Table 9.3). The concentration of IgG and IgG subclasses increase with age and the ages at which the IgG and IgG subclass concentrations of the children in this study reached adult and peak levels are variable and are listed in Table 9.4

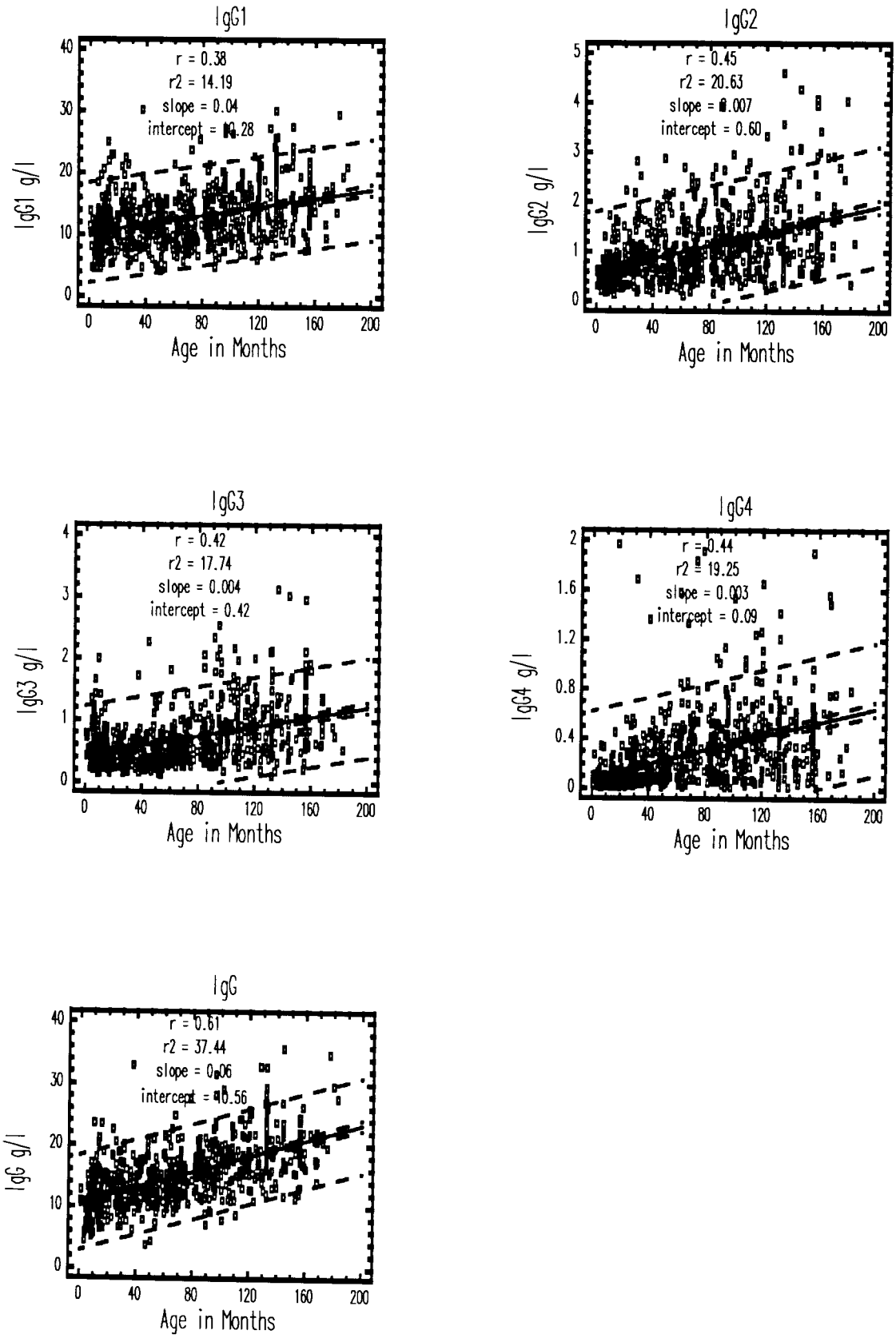


Figure 9.2: Regression analysis of IgG and IgG subclass levels against age in healthy children in the Western Cape.

TABLE 9.4: IgG and IgG SUBCLASS LEVELS - 50TH PERCENTILE

	AGE AT WHICH LEVEL REACHED	
	PEAK LEVEL	ADULT LEVEL
IgG B	12 y	6 y
IgG CM	12 y	7 y
IgG CF	12 y	6 y
IgG1 B	12 y	1 y
IgG1 CM	> 12 y	4 y
IgG1 CF	> 12 y	0.5 y
IgG2 B	> 12 y	> 12 y
IgG2 C	> 12 y	> 12 y
IgG3 B	> 12 y	10 y
IgG3 CM	> 12 y	10 y
IgG3 CF	> 12 y	9 y
IgG4 B	8 y	6.5 y
IgG4 CM	> 12 y	> 12 y
IgG4 CF	> 12 y	8 y

B = Black children **CM = Coloured male children**
CF = Coloured female children

The attainment of mean adult levels was not synonymous with the peak level. The peak level, with the exception of IgG2, was greater than the mean adult level and generally had not been reached by 12 years of age. The IgG, IgG1 and IgG3 of the Blacks reached adult levels at an earlier age than the Coloured patients.

IgG declines over the first 6 months of life; thereafter a slow increase in serum IgG levels is observed with 70% of adult levels being reached by the end of the second year of life; adult levels are reached between 7-13 years

of age (Buckley et al 1968; Schur et al 1979; Jolliff et al 1982; Shackelford et al 1985b).

In published studies of normal ranges there is no consensus about the exact age at which the IgG subclasses reach adult levels. IgG subclasses attain adult serum concentrations at variable ages with IgG1 and IgG3 reaching adult levels at an earlier age than IgG2 and IgG4 (Lee et al 1986). IgG2 has been reported to be the most delayed and probably does not do so until after 12 years of age (Aucouturier et al 1988). The findings in this study are in agreement with these reports. IgG2 was the most delayed and had not reached adult levels by 12 years of age. Children of 2 years had only 27% of adult levels of IgG2 whereas those of 12 years had 58% (Figure 9.3).

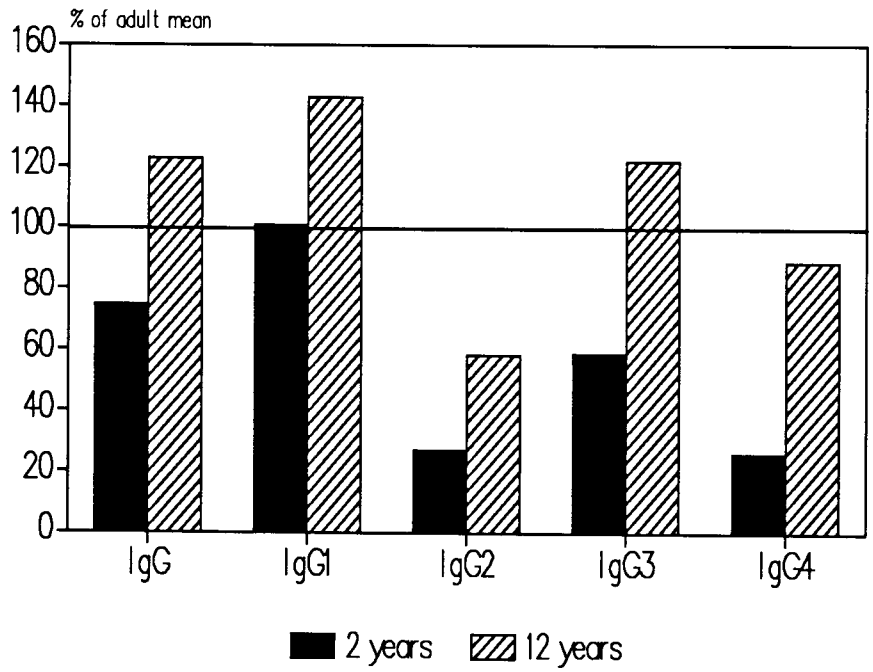


Figure 9.3: Concentrations of IgG, IgG1, IgG2, IgG3 and IgG4 levels of children 2 and 12 years of age expressed as a % of the adult mean.

9.2.3 Proportion of IgG subclasses of the total IgG

Although the total serum IgG levels may vary considerably between healthy adults, the proportions of the 4 subclasses are maintained within a relatively narrow range (Table 9.5). Adult controls tested in this study exhibited similar proportions (Table 9.5). In children of 2 and 12 years of age IgG1 levels were proportionately higher, IgG2 and IgG4 proportionately lower and IgG3 proportions were similar to adult concentrations (Table 9.5). These results show that the proportion of each subclass present in adults does not exist in children due to variable ontogeny of subclasses. There are no previously published reports on the proportion of the IgG subclasses in young children.

TABLE 9.5: IgG SUBCLASSES AS A % OF MEASURED TOTAL IgG ACCORDING TO AGE AND RACE

		Children		Adults	ADULTS (Jefferis & Kumararatne 1990)
		2 yrs	12 yrs		
IgG1	B	94.4%	89.2%	71.6%	
	CM	90.8%	70.7%	74.2%	60 - 65%
	CF	92.8%	83.1%	64.1%	
IgG2	B	4.9%	7.4%	14.8%	
	CM	6.6%	5.5%	17.4%	20 - 25%
	CF	6.2%	5.2%	16.4%	
IgG3	B	4.1%	5.1%	5.3%	
	CM	3.9%	5.8%	5.4%	5 - 10%
	CF	4.2%	4.7%	4.9%	
IgG4	B	0.6%	1.2%	2.2%	
	CM	0.9%	1.7%	2.9%	3 - 6%
	CF	0.5%	1.6%	1.6%	

B = Black **CM = Coloured male** **CF = Coloured female**

9.2.4 Percentile Ranges

Predictive percentiles were obtained from the Box Cox scale of IgG or IgG subclass and transformed back to the original scale.

Smooth age-specific reference ranges for IgG and IgG subclasses have been produced. The data points and the percentile ranges (2.5%, 5%, 25%, 50%, 75%, 95% and 97.5%) for IgG and IgG subclasses are shown in Figures 9.4a, 9.4b and 9.4c (enlarged figures of these percentile charts can be found in Appendix I). The percentile values for racially matched adults are also shown.

As the IgG subclass levels of the Black and Coloured children were significantly different and as a sex difference was noticed in the Coloured children for the IgG, IgG1, IgG3 and IgG4 values, normal ranges have been independently determined for the Black children, Coloured male and Coloured female children. Tables of age, race and sex related normal ranges for IgG subclasses can be found in Appendix J.

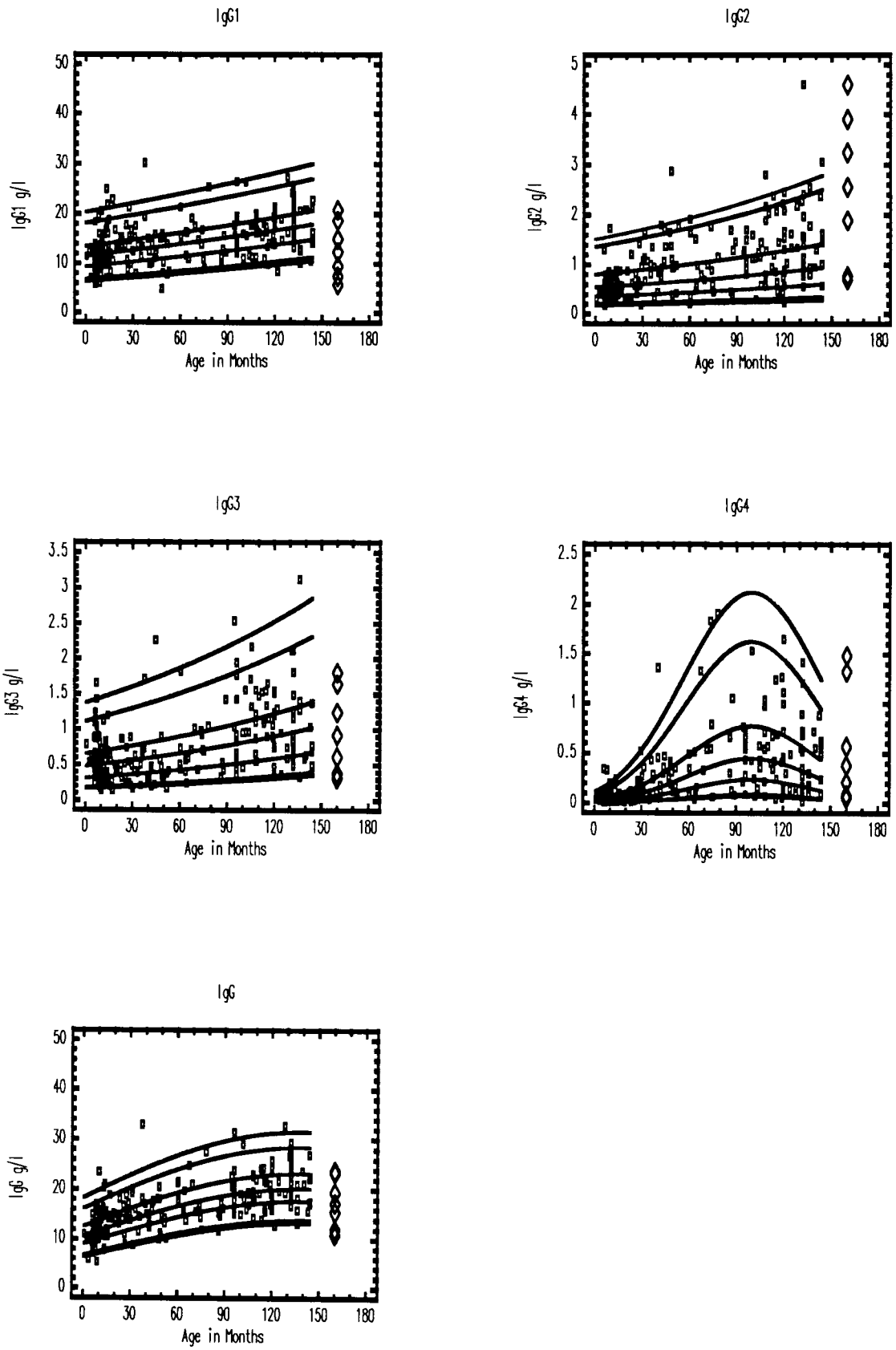


Figure 9.4a: IgG1, IgG2, IgG3, IgG4 and IgG concentration percentiles for Black children. Percentile lines are 2.5%, 5%, 25%, 50%, 75%, 95% and 97.5%. The equivalent adult centile points are shown (◇)

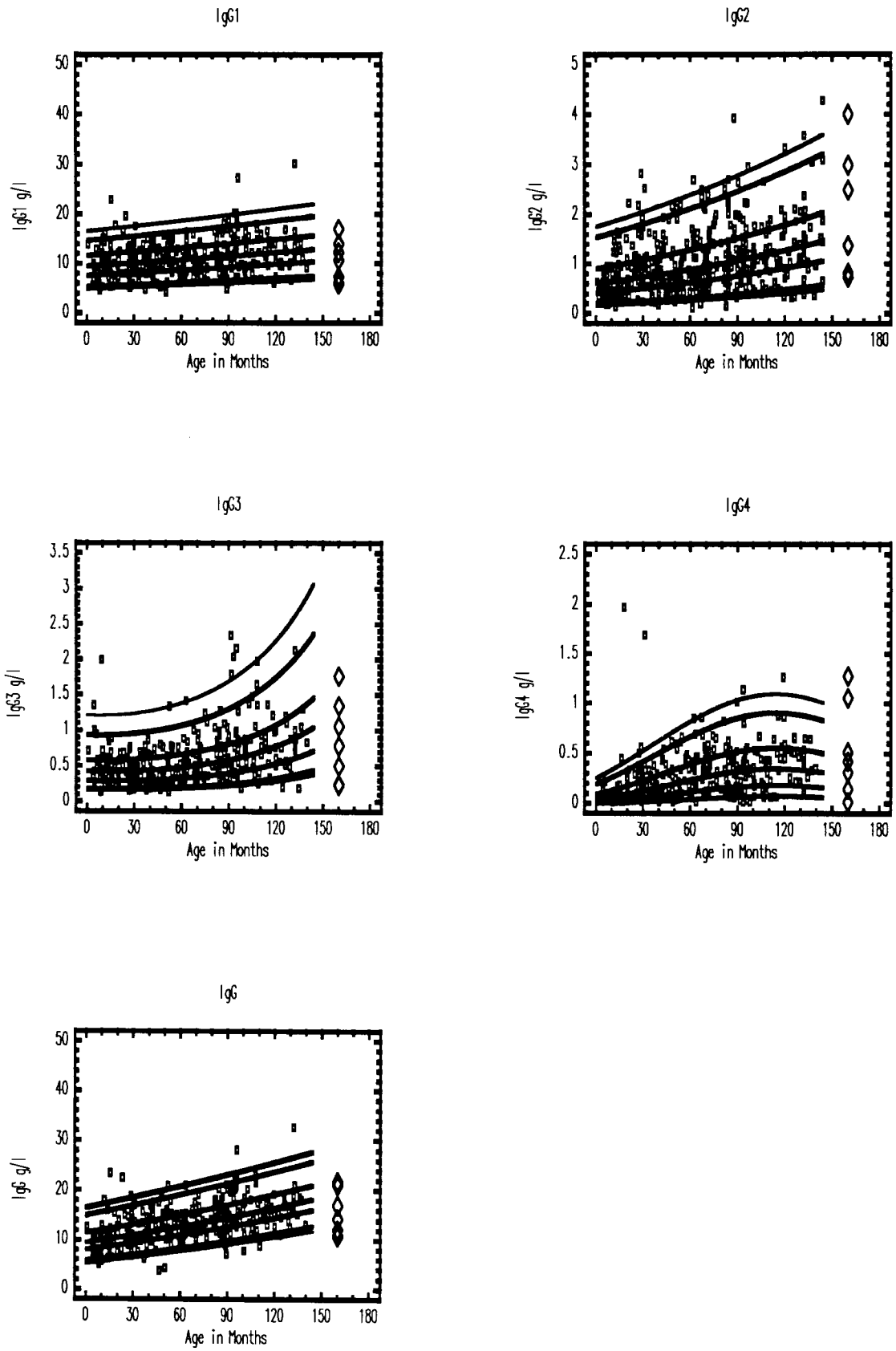


Figure 9.4b: IgG1, IgG2, IgG3, IgG4 and IgG concentration percentiles for Coloured male children. Percentile lines are 2.5%, 5%, 25%, 50%, 75%, 95% and 97.5%. The equivalent adult centile points are shown (m)

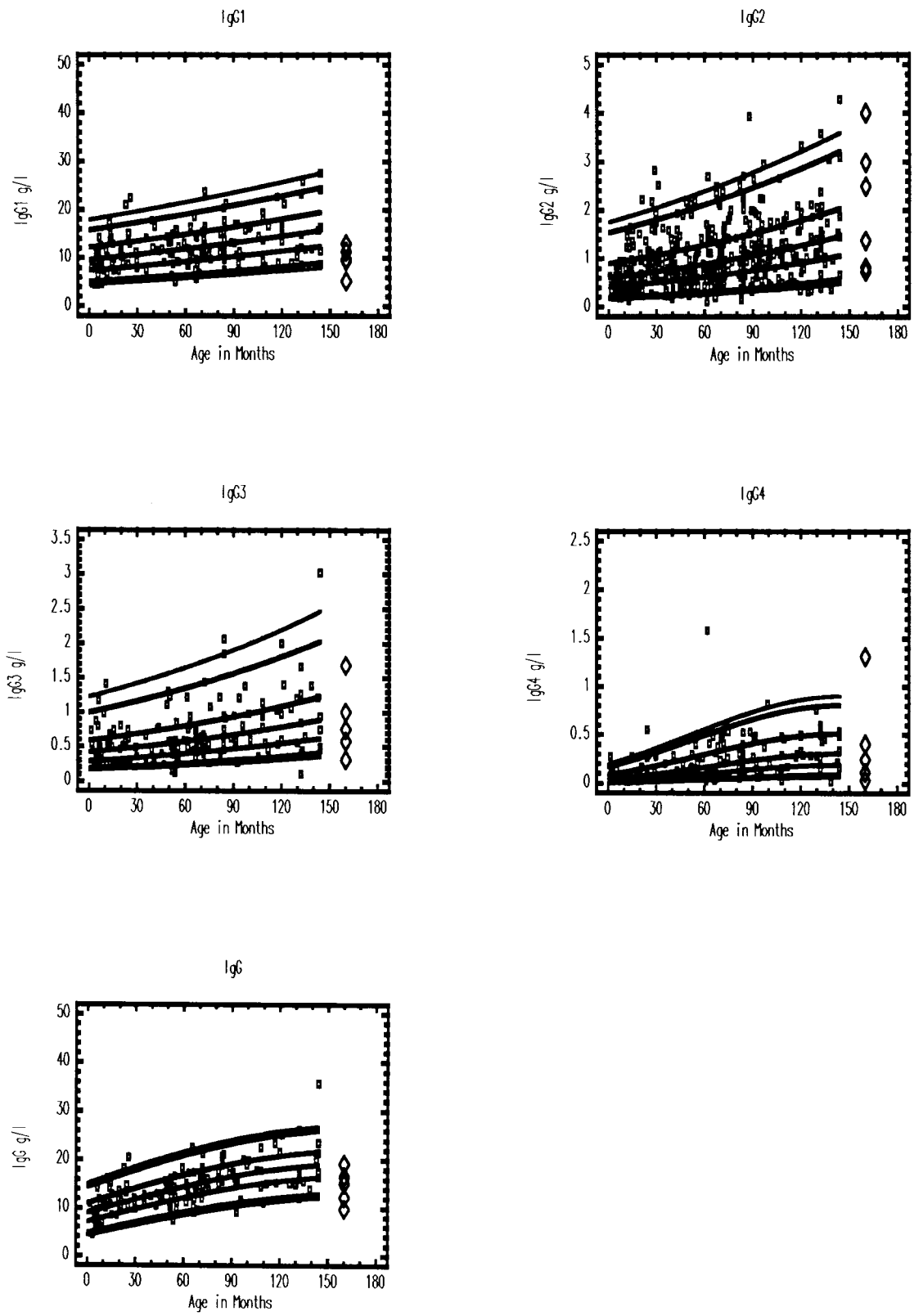


Figure 9.4c: IgG1, IgG2, IgG3, IgG4 and IgG concentration percentiles for Coloured female children. Percentile lines are 2.5%, 5%, 25%, 50%, 75%, 95% and 97.5%. The equivalent adult centile points are shown (◇)

9.2.4.1 *Discussion*

Normal values are needed to identify individuals with IgG subclass deficiencies. The choice of statistical method can greatly influence the calculated normal range. A comparison of normal reference ranges from various studies is complicated because different statistical analyses have been applied and the frequency distributions have not always been taken into consideration. Details of published IgG subclasses normal ranges and the methods used to obtain them are shown in Table 9.6. The most common practice of establishing reference ranges for IgG subclasses has been to use the mean of the data plus or minus two standard deviations. This method assumes the data to have a Normal distribution. Most studies have shown that the IgG subclasses (with the possible exception of IgG1) are not Normally distributed (Table 9.6). If the data is either Normal or log-Normal distributed, normal range estimates obtained by non-parametric methods requires more samples to obtain the same precision of estimation as those obtained using parametric methods. When data are not Normally distributed as assumed, non-parametric estimates are more accurate (Reed et al 1971). Reference ranges derived by parametric methods are more affected by outliers than when non-parametric methods are used. IgG subclasses in children are age dependent and it is difficult to analyse data with non-parametric statistical methods and to acount for the effect of age. Reed et al (1971) and Hapke and Patil (1981) recommend that non-parametric percentile estimates of the normal range are superior to parametric estimates when applied to biological data. The percentile method avoids the need to assess the frequency distribution of data. The use of non-parametric reference intervals does not necessitate the use of non-parametric statistical tests. Unless the distribution of data being compared is markedly skewed parametric tests are 'robust' enough to analyse data that do not have a perfectly Normal distribution (Hapke and Patil 1981).

TABLE 9.6: SERUM IgG SUBCLASS LEVELS - NORMAL RANGES

REFERENCES	AGES IN YEARS	NO OF SERA	METHOD	ANTISERA	REFERENCE STANDARD	EXPRESSION OF NORMAL RANGE	FREQUENCY DISTRIBUTION
Morell et al 1972a	0-2	95	RIS	PC	WHO 67/97	a. Mean ± SD b. Range	
Shakib et al 1975	Adults	111	RID	PC	Myeloma	Mean ± SD	IgG1 normal IgG2,3,4 skewed
van der Giessen et al 1975	4-12	141	RID	PC	Myeloma	a. Mean ± SD b. Range	
	Adults	107	RID	PC	Myeloma	a. Mean ± SD b. Range	Adults IgG1,2,3,4 skewed
Oxelius 1979	0.12-15	162	EIA	PC	WHO 67/97	a. Mean ± SD b. Range	
Schur et al 1979	0.5-16	281	RID	PC	Myeloma WHO 67/95	a. Log data b. Mean ± SD c. Range	
Zegers et al 1980	0.25-1.2	160	RID	PC	HDO-01 (CLB)	a. Mean ± SD	b. Range
French & Harrison 1984	Adults	172	RID	MC	WHO 67/97	95% confidence limits	IgG1 normal IgG2,3,4 skewed
Bird et al 1985	0.5-6	215	RID	MC	WHO 67/97	95% confidence limits	IgG1 normal IgG2,3 log normal IgG4 skewed

Table 9.6 continued

REFERENCES	AGES	NO OF SERA	METHOD	ANTISERA	REFERENCE STANDARD	EXPRESSION OF NORMAL RANGE	FREQUENCY DISTRIBUTION
Papadea et al 1985	Adults	63	ELISA	MC	USNRP	95% confidence limits	
Shackelford et al 1985b	0.6-16	114	RIA	MC IgG1	Myeloma proteins	a. Log data b. Mean ± SD c. Range	
Aucouturier et al 1987	1-16	128	ELISA	MC		Mean ± SD	
Aucouturier et al 1988	1-17	225	ELISA	MC		a. mean b. range	All subclasses skewed
Singh 1988	0-15	56	RID	PC	WHO 67/97		
Plebani et al 1989	0.5-18 Adults	448 141	RID	MC	WHO 67/97	Age related percentile ranges	All subclasses skewed
Beard et al 1990b	0.33-15	292	ELISA	MC	WHO 67/97	Age related percentile curves	
Ambrosino et al 1991	0.5-5 y	664	ELISA	MC	WHO 67/97	Age related percentile curves	
Lau et al 1993	16-20	350	RID	PC	WHO 67/97	Age related percentile ranges	
Goddard et al 1994	0.5-15	720	ELISA	MC	WHO 67/97	Age, race and sex related percentile ranges	All subclasses skewed

RIS = Radioimmunosorbent **EIA = electroimmunoassay**

Variations in normal ranges found in studies using different methods, different antisera, different reference standards and different cut-off criteria indicate the need for the standardization of IgG subclass measurement methods and statistical analyses if meaningful comparison of results obtained in different laboratories is to be possible. Very few methods have demonstrated the correctness of their statistical methods.

The determination of reference ranges for serum immunoglobulins has often been hampered by the ethical and practical difficulties of obtaining blood from healthy children. Many previous studies have used sera from hospital in-patients (Oxelius 1979; Aucouturier et al 1987). In other studies too few subjects of different ages have been tested leading to unreliable reference ranges (Oxelius 1979; Singh 1988; Plebani et al 1989).

In this study normal references ranges were established from randomly selected healthy children rather than attempting to obtain matched controls for the following reasons:

- The patient groups with Hib infections were much younger than the group with *S.aureus* infections which would have necessitated two separate control groups.
- As it is ethically and practically difficult to obtain blood from healthy children, it is easier to take blood from randomly selected controls rather than to select controls to match patients.
- Selection of a matched group of controls from the randomly selected group is likely to show some bias.

It is preferable to calculate age-related percentiles that vary smoothly with age rather than considering subclass concentrations at each age independently of those at other ages. The different age groups used in reference ranges vary between studies and are usually determined by the number of specimens obtained in each group rather than by any biologically significant factor. Using mean $\pm$ 2 SD to estimate normal values for IgG and IgG subclasses, 2.5% of normal donors will be considered deficient in one or other subclass.

In this study the Box Cox (Box and Cox 1964) transformation was used to construct age, race and sex related IgG and IgG subclass percentiles. Dr Derek Chalton, Division of Biostatistics, Medical Research Council, calcuated the approximate Box Cox transform (i.e. the λ parameter) including the effects of age, race and sex for each subclass. Once the λ parameter had been calculated this could be used in the Box Cox of Statgraphics 6 programme to draw age related percentile charts and for analysis of various groups compared to the controls.

As it was known that the IgG and IgG subclass values change with age and that Box Cox transformation 'Normalized' the data, the analysis of covariance (ANCOVA) with age as a covariant was used to determine the effect of race, sex or allotype on the IgG subclass levels using the Box Cox transformed data.

Although the distribution of the IgG4 data could not be 'Normalized' using either $\log_{10}$ or Box Cox transformation in this study the latter transformation gave a symmetrical frequency distribution. Percentile ranges have been used to establish reference ranges and parametric statistical methods have been used to analyse data.

IgG and IgG subclasses were found to be much higher than those reported from infants in developed countries (Bird et al 1985; Shackelford et al 1985b; Singh 1988; Beard et al 1990b). The differences are large enough to influence the interpretation of laboratory results if the correct age, race and sex matched reference ranges are not used.

Although great care was taken to achieve valid and plausible reference ranges, the end result is largely dependent on the representativeness of the children in the study. One hundred of the Black school children lived in a rural area of the Transkei and the environmental effect on the immunoglobulins of these children could be different to those of Black children from poor urban areas of Cape Town.

It will be important to collect further specimens from Black school children in Cape Town to ascertain if their levels differ from the Black children in the rural areas of the Transkei.

9.2.5 Race

There were statistically significant differences in the IgG, IgG1, IgG2 and IgG3 subclasses of the Black and Coloured racial groups (Figures 9.4a, 9.4b and 9.4c). The Blacks had higher levels than the Coloureds (IgG $p = < 0.0001$; IgG1 $p = < 0.0001$; IgG2 $p = 0.0136$; IgG3 $p = < .0001$). There was no significant difference in the IgG4 levels of the Black and Coloured groups.

In South Africa higher levels of IgG have been reported in Blacks than in Whites in both adults and children (Milner and Calitz 1971; Shulman et al 1975; Shulman and Gilich 1976) and newborns (Van Rijswyk et al 1985).

The IgG values of the Cape Coloured population have also been found to be significantly higher than the Whites (Fraser et al 1981).

There is only one study directly comparing Black and Cape Coloured immunoglobulin levels (which was done on cord blood). The Black and Coloured IgG levels were significantly higher than the Whites but there was not a significant difference in the IgG values of the Black and Coloured subjects (Van Rijswyk et al 1985).

In North-America Buckley et al (1968) reported signicantly higher concentrations of IgG in Black subjects betweeen 6 and 14 years of age compared to those in Whites of comparable age. No significant differences in IgG concentrations were found in Black and White children less than 6 years of age, however, no correction was made for age. Correcting for age Shackelford et al (1985b) found higher concentrations of IgG in Black children. They also reported that although Black adults had higher IgG1 and IgG2 concentrations than White adults, the IgG1 and IgG2 levels in Black and White children were not significantly different. In contrast, in a more recent study with larger numbers of children, the IgG1, IgG2 and IgG4 subclass concentrations of Black childen were lower than White children (Ambrosino et al 1991).

In this study the Black children had significantly higher IgG, IgG1, IgG2 and IgG3 levels than the Coloured children. It would have been interesting to have compared these levels to those of White children but unfortunately insufficient controls were collected for statistical analysis. There are no previous reports of IgG subclass values in South African children of any racial group.

9.2.6 Sex

In Black children and adults the IgG and IgG subclass levels were not significantly different in males and females. In Coloured children the IgG, IgG1, IgG3 and IgG4 levels were significantly higher in males than in females but no sex difference was noted for IgG2. This sex effect was not seen in the IgG and IgG subclass levels of Coloured adults.

No significant sex differences for IgG values have been reported in Black subjects (Roode 1980) but Cape Coloured males had significantly higher values than females (Fraser et al 1981). Sufficient information concerning the effect of sex on serum IgG and IgG subclass concentrations has only been reported in a small number of studies. Several studies have shown that IgG4 concentrations are higher in males than females (van der Giessen et al 1975, Merrett et al 1983, Aucouturier et al 1984; French and Harrison 1984; Plebani et al 1989; Beard et al 1990b) although this has not been found in all studies. In contrast studies reporting sex differences for IgG1 (Shackelford et al 1985b), IgG2 (van der Giessen et al 1975; Beard et al 1990b) and IgG3 (French and Harrison 1984) have found females to have higher concentrations than males. However, these sex differences for the IgG subclass values are not constant findings. Shakib et al (1975) and Plebani et al (1989) found no differences in IgG1, IgG2 or IgG3 levels in adults or children. Beard et al (1990b) did not find a sex difference between males and females for IgG1 values. Differences in the relative proportions of males and females in different studies could be a factor accounting for discrepancies between studies. This may also explain the differences between races noted in this study as the male:female ratio was 2:1 in the Coloured children where a sex difference was noted and 1:1 in the Blacks where no sex difference was found.

9.3. ALLOTYPES

9.3.1 Statistical Analysis

The frequency of the allotypes amongst the patients was compared to normal controls. The potential risk associated with an allotype was estimated with the odds ratio (OR) with 95% confidence intervals (CI).

Due to the known association of IgG subclass levels with age, analysis of variance (ANCOVA) (using Box Cox tranformed data) with age as a covariant was used in evaluating the effect that an allotype may have on IgG subclass or subclass specific antibody concentration. The Kruskal Wallis test (using untransformed data) gave statistically similar significance values to the analysis of variance (ANCOVA) and in no case were discordant conclusions reached by the two methods.

9.3.2 Determination of zygosity of G1m(f) and G2m(n) allotypes

To determine G1m(f) zygosity, the amount of IgG1 as measured by the G1m(f) ELISA was expressed as a percentage of the amount of IgG1 determined by the IgG1 subclass ELISA. The G1m(f) negative samples had undetetable levels in the assay. All the G1m(f) positive samples had a value of $\geq$ 28% of the IgG1 value. A frequency histogram of these ratios was plotted for the controls (Figure 9.5). From this plot there appeared to be two populations. A cut-off of 75% was taken to distinguish G1m(f) positive homo- and heterozygosity i.e. those with a level below 75% were defined as being heterozygous positive for the marker and those with a percentage value greater than 75% were considered homozygous positive. Similar measurements and calculations were undertaken using the G2m(n) and the IgG2 ELISA to determine the zygosity of G2m(n).

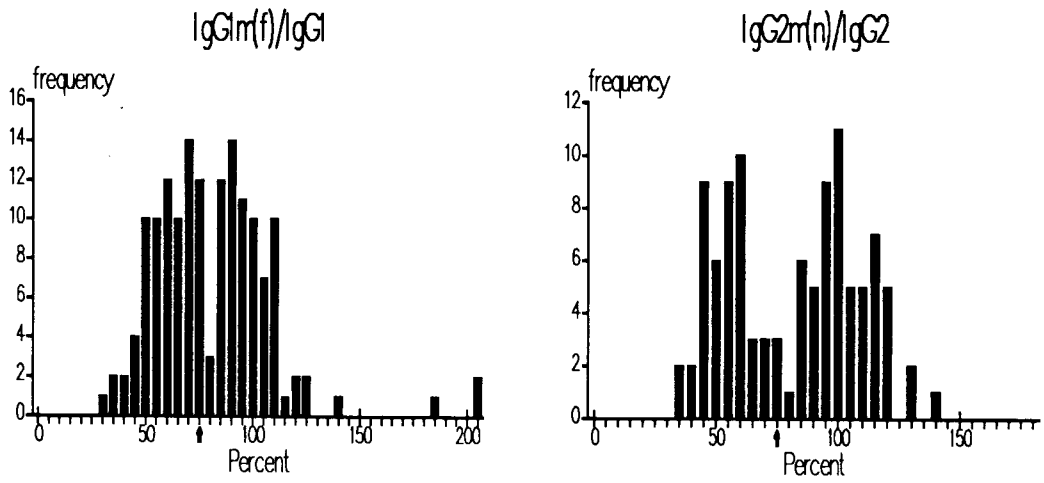


Figure 9.5: Frequency histograms of the G1m(f) positive IgG1 and G2m(n) positive IgG2 expressed as a percentage of IgG1 and IgG2. The cut-off of 75% used to distinguish between positive homo- and heterozygosity is indicated with an arrow

The G2m(n) negative samples had undetectable levels in the assay. All the G2m(n) positive samples had a value of at least 29%. A frequency histogram of these ratios was plotted (Figure 9.5). The division between positive homo- and heterozygosity was not as obvious as for the G1m(f). However, a cut-off point of 75% was selected and all values of greater than

75% were considered homogygous positive and below which they were considered heterozygous.

9.3.3 Prevalence of G1m(f), G2m(n) and Km(3) allotypes in controls

The prevalence of these allotypic markers is shown in Table 9.7. The G1m(f) allotype is uncommon in Black children but occurred in 52% of Coloured children. Amongst the G1m(f) positive Coloured children 54% were homozygous and 46% heterozygous using the criteria described above. Two of the three G1m(f) positive Black children were homozygous and the other heterozygous for the marker. The presence of G1m(f) allotype in these two population groups is statistically different (OR = 49.47 (15.82 < OR <247.02); p = 0.0001).

TABLE 9.7: FREQUENCY OF THE G1m(f), G2m(n) AND Km(3) ALLOTYPES IN BLACK AND COLOURED CHILDREN

	G1m(f)+/+	**G1m(f)+/-**	**G1m(f)-/-**
Black n = 138	2 (1.5%)	1 (0.7%)	135 (97.8%)
Coloured n = 271	76 (28.0%)	65 (24.0%)	130 (48%)
	G2m(n)+/+	**G2m(n)+/-**	**G2m(n)-/-**
Black n = 138	0 (0%)	4 (2.9%)	134 (97.1%)
Coloured n = 271	53 (19.6%)	40 (14.8%)	178 (65.7%)
	Km(3) +ve	**Km(3) -ve**	**NT***
Black n = 138	124 (88.9%)	7 (5.1%)	7 (5.1%)
Coloured n = 271	260 (95.9%)	6 (2.2%)	5 (1.8%)

* = Not typable

The G2m(n) marker, like the G1m(f) allotype, is uncommon in Black children (2.9%) whereas the G2m(n) allotype was found in approximately a third of the Coloured children. This difference was statistically significant (OR = 18.55 (6.72 < OR < 70.82) p = 0.0001). The four G2m(n) positive Black children were all heterozygous for this marker, and 57% of Coloured children were homozygous and 43% were heterozygous.

The Km(3) allotype is common in both the Coloured children (95.9%) and Black children (88.6%) (Table 9.7).

Sex had no effect on the frequency of either the G1m(f), G2m(n) or Km(3) allotypes.

9.3.3.1 *Discussion*

The frequency of the G1m(f), G2(m)n and Km(3) allotypes in South African Black and Coloured populations is not known. The G1m(f) and G2m(n) allotypes are typical Caucasian alleles, the frequency of which varies in Europe from 0.6 to 0.8 and 0.4 to 0.6 respectively. The frequency of the Km(3) allotype is about 0.9 in Caucasoids and varies from 0.5 to 0.8 for Negroids (Lefranc and Lefranc 1990).

The G1m(f) and G2m(n) frequencies differ between the Black and Coloured children. The frequencies of the G1m(f), G2m(n) and Km(3) allotypes were respectively 52.4%, 35.6% and 93.97% in Coloured children and 2.2%, 2.9% and 93.4% in Black children in the Western Cape. G1m(f) and G2m(n) have been reported to be completely absent in Black populations (de Lange 1991). However, Granoff et al (1984) found the G2m(n) allotype infrequently (13%) in North American Blacks. When present in Black

populations this allotype may reflect racial admixture. In this study the G2m(n) was present in 2.9% of Black children and the fact that these were all heterozygous supports this hypothesis. In studies of immunoglobulin allotypes appropriate local race matched control groups are therefore important in assessing whether a specific allotype is significantly over- or under-represented in the study groups.

9.3.4 Influence of allotype on IgG and IgG subclass concentrations

9.3.4.1 *Influence of G1m(f) on IgG, IgG1, IgG2, IgG3, IgG4*

In male Coloured children the G1m(f) positive allotype was associated with lower IgG and IgG1 levels and higher IgG2 and IgG3 levels when compared to children with absence of this allotype. In female Coloured children the G1m(f) allotype was only statistically associated with higher IgG2 levels although the other trends seen in male children were detectable (Table 9.8). This effect of the G1m(f) allotypes on the IgG, IgG1 and IgG3 subclass levels in Coloured male children was unaffected by the presence or absence of the G2m(n) allotype.

There were insufficient G1m(f) positive Black children (3/138) on which to do meaningful statistical analysis.

TABLE 9.8: RELATIONSHIP BETWEEN THE G1m(f) AND G2m(n) ALLOTYPE AND IgG SUBCLASS LEVELS IN COLOURED CHILDREN

	Geometric Mean Antibody Concentration (g/l)			
	Coloured male G1m(f)		Coloured female G1m(f)	
	n = 107	n = 90	n = 34	n = 40
	+	-	+	-
IgG	12.57*	13.61	13.47	13.56
IgG1	10.12*	11.03	11.70	11.15
IgG2	1.10**	0.83	1.00*	0.73
IgG3	0.53*	0.46	0.60	0.48
IgG4	0.23	0.22	0.13	0.19
	Coloured male G2m(n)		Coloured female G2m(n)	
	n = 74	n = 123	n = 19	n = 55
	+	-	+	-
IgG	12.65	13.28	13.14	13.65
IgG1	10.40	10.83	11.19	11.48
IgG2	1.25**	0.83	1.27**	0.73
IgG3	0.50	0.48	0.57	0.52
IgG4	0.23	0.22	0.13	0.18

* p = < 0.05 ** p = < 0.01

The effects of homo- and heterozygosity of G1m(f) on Coloured male and female children's IgG subclass levels are shown in Table 9.9. In most cases where differences were noted between G1m(f) homozygote positives and negatives (IgG, IgG1, IgG2, IgG3), intermediate levels were seen in heterozygotes.

TABLE 9.9: RELATIONSHIP OF HOMO- AND HETEROZYGOUS G1m(f) ALLOTYPES AND IgG SUBCLASS LEVELS IN COLOURED CHILDREN

G1m(f) Males	**+/+ n = 61 (31.0%) g/l**	**+/- n = 46 (23.3%) g/l**	**-/- n = 90 (45.7%) g/l**
IgG	12.17	12.93	13.61
IgG1	9.51*	10.94	11.03
IgG2	1.16	1.02	0.83
IgG3	0.52	0.54	0.46
IgG4	0.25	0.19	0.22
G1m(f) Females	**+/+ n = 15 (20.3%) g/l**	**+/- n = 19 (25.7%) g/l**	**-/- n = 40 (54.0%) g/l**
IgG	13.24	13.19	13.56
IgG1	10.49	12.60	11.15
IgG2	0.88	1.06	0.73
IgG3	0.55	0.63	0.48
IgG4	0.12	0.13	0.19

*** p = < 0.05 denotes a significant difference of the subclass levels between homozygous and heterozygous G1m(f) allotype individuals**

9.3.4.2 *Influence of G2m(n) on IgG, IgG1, IgG2, IgG3 and IgG4*

The effect of G2m(n) allotypes on subclass levels is shown in Table 9.8. Both male and female Coloured children had significantly higher IgG2 levels associated with the G2m(n) positive allotype.

Heterozygotes for G2m(n) had higher mean levels than either G2m(n) homozygous positive or negative children (Table 9.10).

As in the G1m(f) analysis, there were too few G2m(n) positive Blacks (4/134) for accurate statistical analysis on the effect of the marker on the IgG subclass levels.

TABLE 9.10: RELATIONSHIP OF HOMO- AND HETEROZYGOUS G2m(n) ALLOTYPES AND IgG SUBCLASS LEVELS IN COLOURED CHILDREN

G2m(n) Males	**+/+ n = 42 (21.3%) g/l**	**+/- n = 32 (16.2%) g/l**	**-/- n = 123 (62.4%) g/l**
IgG	12.45	12.82	13.28
IgG1	9.86	10.26	10.83
IgG2	1.05	1.56*	0.83
IgG3	0.53	0.50	0.48
IgG4	0.21	0.25	0.22
G2m(n) Females	**+/+ n = 11 (14.9%) g/l**	**+/- n = 8(10.8%) g/l**	**-/- n = 55 (74.3%) g/l**
IgG	13.45	13.47	13.65
IgG1	11.90	11.72	11.48
IgG2	1.22	1.47*	0.73
IgG3	0.62	0.53	0.52
IgG4	0.13	0.14	0.18

*** p = < 0.05 denotes a significant difference of the subclass levels between heterozygous and homozygous positive and negative G2m(n) individuals**

9.3.4.3 *The combined effect of G1m(f) and G2m(n) on IgG subclass levels*

The combined presence of the G1m(f) and G2m(n) allotypes resulted in a statistically significant increase in IgG2 levels (Tables 9.11). This is probably largely due to the influence of the G2m(n) allotype on IgG2 levels

as the IgG2 level was significantly higher in G2m(n) positive Coloured male and female children (Table 9.8). The presence or absence of the G1m(f) marker did not influence the IgG2 levels of the G2m(n) positive male controls (p= 0.42) (Table 9.11).

TABLE 9.11: THE COMBINED EFFECT OF G1m(f) AND G2m(n) ON IgG SUBCLASS LEVELS IN COLOURED MALE CHILDREN

	IgG1m(f)+ve COLOURED MALE CONTROLS		
	IgG2m(n)+ve	IgG2m(n)-ve	p
	n = 69	n = 38	
	g/l	g/l	
IgG	12.53	12.42	0.853
IgG1	10.04	10.20	0.784
IgG2	1.25	0.86	0.0001
IgG3	0.55	0.50	0.340
IgG4	0.23	0.22	0.913

These IgG2 levels were unaffected by the accompanying G1m(f) +ve or -ve haplotype (Table 9.11). The G2m(n) negative control children had a low mean concentration of IgG2 whether they were G1m(f) positive or negative. Heterozygotes (n/-) and positive homozygotes had high IgG2 levels regardless of the accompanying haplotype.

The G2m(n) allotypic marker did not affect the IgG, IgG1, IgG3 or IgG4 subclass levels of Coloured control children.

9.3.4.4 *Influence of Km(3) on IgG, IgG1, IgG2, IgG3, IgG4*

The IgG and IgG subclass levels were unaffected by the accompanying Km(3) allotype. Although there were very few Km(3) negative samples the subclass levels of these specimens all fell within the mean +/- 2 SD range of the Km(3) positive controls.

9.3.4.5 *Summary*

Allotype-associated differences have been reported for IgG1, IgG2 and IgG3 (van der Giessen et al 1975; Grubb et al 1986; Sarvas et al 1991; Oxelius 1993). The molecular mechanism which explains why allotypes affect IgG subclass concentrations is unknown.

Analysis of the effect of the G1m(f) and G2m(n) allotypic markers on the IgG, IgG1, IgG2, IgG3 and IgG4 subclass levels was not possible in Black children due to the small numbers which were positive for these markers.

In Coloured children the G1m(f) allotype was associated with decreased IgG and IgG1 levels and increased IgG2 and IgG3 levels. The G1m(f) positive heterozygotes were mostly associated with intermediate levels. Although the male and female Coloured children were different when analysing the effect of the G1m(f) allotype, these differences were only noted in the degree of significance and not in the trends. The fact that a greater statistical significance was found in male children may be due to the greater numbers in this group. It may be valid to draw similar conclusions on the effects of allotypes on IgG subclass levels in both male and female Coloured children.

Litwin and Balaban (1972) found G1m(f) individuals to have higher concentrations of IgG1 than those lacking the marker. G1m(f) negative

Finnish individuals have significantly higher IgG1 levels than G1m(f) positive individuals (Sarvas et al 1991).

IgG2 levels were higher in the Coloured children who have the G2m(n) allotype when compared to those negative for the marker. Average concentrations of IgG2 were 50.6% and 74.9% higher respectively in G2m(n) positive children than in G2m(n) negative Coloured male and female children. There was no effect of this marker on the other IgG subclass concentrations. A high concentration of IgG2 has been found to be associated with the G2m(n) allotype in several studies (Morell et al 1972b; van der Giessen et al 1975; Sarvas et al 1990; Sarvas et al 1991). Steinberg et al 1973 did not find a correlation between the G2m(n) allotype and IgG2 levels.

The male and female Coloured children who were G2m(n) positive heterozygotes, had IgG2 concentrations 88% and 101% higher respectively, than homozygous negative individuals. The homozygous positive male and female Coloured children had IgG2 levels 26% and 67% higher respectively than the homozygous negative individuals.

There is no obvious explanation why the G2m(n) heterozygotes had higher IgG2 levels than G2m(n) homogygotes. Steinberg et al (1973) previously reported that IgG2 concentrations of G2m(n) heterozygotes were higher than G2m(n) positive homogyzotes. Other studies have found the heterozygotes to have intermediate levels between positive and negative homozygotes (Sarvas et al 1991).

The positive effect of G1m(f) on IgG2 was only seen in G2m(n) positive children. This increase in IgG2 is mainly due to the effect of the G2m(n) allotype.

Because of the infrequency of both the G1m(f) and G2m(n) allotype in Blacks it was not possible to analyse the effects of these allotypes on IgG subclass levels. However, it is worth noting that Black children had higher levels of IgG1 and IgG2 even though the majority were G1m(f) and G2m(n) negative whereas in Coloured children this resulted in lower IgG1 and IgG2 levels.

The Km(3) negative allotype was infrequent in both the Black and Coloured children and had no effect on the IgG subclass levels.

9.4 C4 PROTEIN TYPING

9.4.1 Measurement of C4A and C4B

Assays for the determination of C4A and C4B were performed by Dr P Creemers, Department of Tissue Immunology, Medical School, University of Cape Town using the following methods.

Sera were pretreated overnight at room temperature with carboxypeptidase B and neuraminidase. Electrophoresis was performed in 0.45% (w/v) agarose gel at 500 V for 3 hours using a discontinuous Tris-glycine-barbital buffer system. The C4 migration patterns were detected by immunofixation with a sheep anti-human C4 serum and stained with 0.1% Coomassie blue. C4A and C4B proteins were distinguished by haemolytic overlay gel (Mauff et al 1983; Sim and Cross 1986; Zhang et al 1988). Alleles were assigned by visual comparison with known alleles, as well as by calculation of the relative migration. These assignment methods were performed

independently by two different technologists. In the case of disagreement the samples were re-run.

9.4.2 Samples

The control groups for the C4 typing were 260 Black adults, 100 Coloured adults and 69 White adults. These normal control sera were supplied by the Department of Tissue Immunology from their sera bank.

9.4.3 C4 isotype frequencies

Black adults had a statistically significant increased frequency of C4B deficiency (C4B*QO) ($p = < 0.001$) and a decreased frequency of C4A deficiency (C4A*QO) ($p = < 0.001$) as compared to White adults (Table 9.12). The difference in these antigen frequencies between Black and Coloured adults and between Coloured and White adults was not statistically significant.

TABLE 9.12: C4A*QO AND C4B*QO ANTIGEN FREQUENCY

	**ANTIGEN FREQUENCY	
	C4A*QO	**C4B*QO**
Controls		
White (n = 69)	0.241	0.060
Coloureds (n = 100)	0.093	0.130
Blacks (n = 260)	0.069	0.250

** **Distribution for the null alleles conformed to the Hardy Weinberg equilibrium for homozygosity**

Population group differences in the frequency of the complement genes have been shown (Imanishi et al 1991) but no significant differences in the frequency of homozygous C4B deficiency have been found between adults

and children or between healthy Black and Caucasian Americans (Rowe et al 1989; Bishof et al 1990). However, in these reports the groups were small and may not have been suitable for accurate statistical analysis. Greater admixture of Caucasian genes in the American Blacks, as compared to South African Blacks may also explain these differences.

9.5 MANNOSE BINDING PROTEIN (MBP)

9.5.1 MBP Assays

Measurement of human MBP levels was performed by Professor M W Turner, The Molecular Immunology Unit, Institute of Child Health, University of London. The method used was a sandwich ELISA (Lipscombe et al 1992). Briefly, a mouse monoclonal antibody to human MBP (MAB 37) diluted to 1 μg/ml in carbonate-bicarbonate buffer was coated onto Dynatech microtitre plates overnight at 4°C. After washing with PBS/T serum samples were incubated in the coated plates for 100 minutes at 37°C. After washing, horseradish peroxidase conjugated streptavidin was added and incubated for 60 minutes at 37°C before the addition of the substrate OPD.

9.5.2 Control samples

The normal controls for the MBP measurement were 55 healthy Black adults supplied from the Western Province Blood Transfusion Service, Cape Town.

9.5.3 MBP levels

The median levels and 95% confidence intervals of serum MBP in the South African Blacks were 2386 (1256-3706) ng/ml with a range (mean $\pm$ 2 SD) of $\leq$ 10-5779 ng/ml.

In this study MBP deficiency was defined as a MBP concentration ≤ 10 ng/ml. Using this definition 3.6% (2/55) of the Black adult controls had a MBP deficiency. The frequency of MBP deficiency in healthy Caucasian and Chinese populations has been estimated to be between 5% and 10% (Turner 1991).

Racial differences of MBP have been reported (Lipscombe et al 1992). They found the Chinese had significantly higher levels than the Caucasians and Gambians. The levels of the Black South African adult controls were not significantly different to those of the Caucasians and Gambians (Figure 9.6).

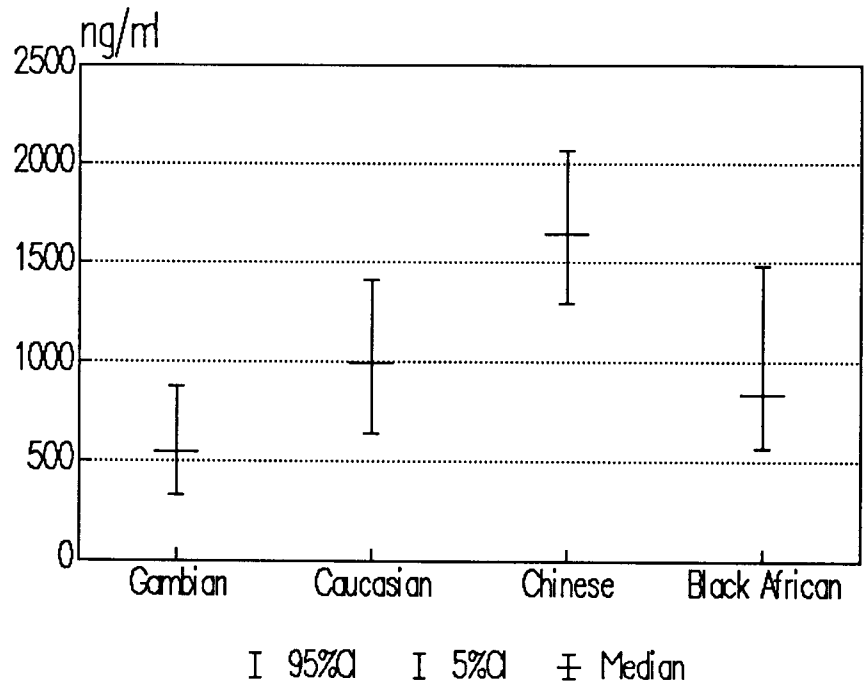


Figure 9.6: The median levels, 95% confidence intervals (95% CI) and 5% confidence intervals (5% CI) of serum mannose binding protein levels in Gambian, Caucasian, Chinese (Lipscombe et al 1992) and South African Black adults

9.6 SUMMARY OF MAIN FINDINGS

- IgG and IgG subclass age related percentile ranges were established for children in the Western Cape from 4 months to 12 years of age.
- Due to statistically significant racial differences separate normal ranges were established for Black and Coloured children.
- A sex difference was found in Coloured children for IgG, IgG1, IgG3 and IgG4 but not IgG2. Thus separate charts were created for Coloured male and Coloured female children.
- The serum IgG subclasses do not increase in parallel
- The proportions of the IgG subclasses in children differ from those of adults
- The G1m(f) allotype was present in 52% of the Coloured children but in only 2.2% of the Black children. The G2m(n) marker occurred in 34.4% of the Coloured children and in 2.9% of the Black children. The Km(3) allotypes was very common in both Coloured and Black children.
- As the G1m(f) and G2m(n) allotypes are uncommon in Black children it was not possible to assess the effect of these markers on the IgG subclasses in these children.
- The G1m(f) marker had a negative influence on IgG and IgG1 levels and a positive effect on IgG2 and IgG3 levels of Coloured children.
- G2m(n) negative Coloured children had lower IgG2 levels than G2m(n) positive children.
- Km(3) allotype did not affect the IgG subclass levels.
- The Black and Coloured adult controls had a higher frequency of $C4B^{*}QO$ deficiency than the White adult controls.
- Mannose Binding protein deficiency was found in 3.6% of Black South Africans which is similar to that reported for Caucasians, Chinese and Gambians.

CHAPTER 10

PATIENTS - RESULTS AND DISCUSSION

This chapter reports the results obtained in children with *Haemophilus influenzae* meningitis or osteomyelitis/septic arthritis and children with *S.aureus* osteomyelitis/septic arthritis.

10.1 **PATIENTS** - a description of the patient groups including details of infection and the race, sex, age and nutritional status of the patients.

10.2 **IgG and IgG SUBCLASSES** - an analysis of immunoglobulin levels in the patient groups compared to the normal age matched controls and analysis of changes during the course of the disease.

10.3 **ANTI-Hib PRP ANTIBODIES** - Results of specific antibody assays for anti-Hib PRP during the course of infection

10.4 **ANTI-*S.AUREUS* TEICHOIC ACID ANTIBODY** - Results of specific teichoic acid antibody assays in sera from patients with *S.aureus* osteomyelitis/septic arthritis

10.5 **ANTI-TETANUS TOXOID ANTIBODIES** - used as an assessment in patients of the capacity of their immune system to respond to antigenic stimulation with tetanus toxoid immunization

10.6 **G1m(f), G2m(n) AND Km(3) ALLOTYPES** - the frequency of these allotypes in the patient groups and comparison to those obtained in normal control groups

10.7 **C4 PROTEIN TYPING** - a description of the frequency of C4A*QO and C4B*QO in the Hib meningitis patients and comparison with the frequency of these haplotypes in normal control subjects

10.8 **MANNOSE BINDING PROTEIN** - a descriptiion of levels of MBP in the patient groups and comparison with local and reported levels of MBP in normal groups

10.9 **SUMMARY**

10.1 PATIENTS

Children admitted to the Red Cross War Memorial Children's Hospital between February 1988 and December 1991 with bacteriologically proven *H.influenzae* type b (Hib) meningitis, Hib osteomyelitis/septic arthritis (OM/SA) and *S.aureus* OM/SA were enrolled into the study. Permission for collection of samples was obtained from the Ethics and Research Committee, University of Cape Town and from the children's parents.

Blood was collected under sterile conditions. Test serum was stored at -70°C in small aliquots in order to avoid repeated freezing and thawing of the samples.

None of the 100 randomly selected samples from these patients were HIV positive.

10.1.1 Meningitis

Sera from 89 children with bacteriologically proven Hib meningitis were collected. All patients had either a positive cerebrospinal fluid (CSF) or blood culture for *H.influenzae*. *H.influenzae* capsular type b was confirmed by slide agglutination (Murex, Dartford, England, Catalogue No ZM21).

In addition all the patients had a CSF compatible with bacterial meningitis including: pleocytosis with predominance of polymorphs, and reduced CSF glucose ($< 60\%$ of serum glucose) and/or a raised CSF protein (≥ 0.5 g/l).

Blood specimens were taken on admission, 7-10 days later (2nd specimen) and 6 weeks later at a follow-up appointment (3rd specimen).

10.1.2 Osteomyelitis/septic Arthritis (OM/SA)

- 207 children admitted to the Red Cross War Memorial Children's Hospital during the period 1987-1991 with clinically diagnosed OM/SA were studied. In all patients *S.aureus* or Hib were isolated from pus aspirates or blood culture.
- The diagnosis of acute osteomyelitis was made when pus was found during surgical drainage, or if not operated, on when subsequent bony changes developed on X-ray.
- Only patients with primary acute bone and joint infections were included in this study. Patients with subacute osteomyelitis and with bone and joint infection secondary to compound fractures or penetrating joint injuries were excluded.
- Patients were immunized with 0.5 ml adsorbed tetanus toxoid vaccine on admission. Blood specimens were taken on admission prior to the tetanus toxoid immunization and again 3-4 weeks later at a follow-up visit.

10.1.3. Race

The ethnic distribution of the patients is shown in Table 10.1. There were two white children with Hib meningitis and 2 with *S.aureus* OM/SA. Because of the small number of white patients and controls these have not been included in further analysis.

TABLE 10.1: ETHNIC AND SEX DISTRIBUTION OF PATIENTS

	Hib Meningitis		
	Female	**Male**	**Total**
Black	18	14	32
Coloured	28	27	55
TOTAL	**46**	**41**	**87**

	Hib OM/SA		
	Female	**Male**	**Total**
Black	8	8	16
Coloured	5	12	17
TOTAL	**13**	**20**	**33**

	S.aureus OM/SA		
	Female	**Male**	**Total**
Black	17	29	46
Coloured	51	75	126
TOTAL	**68**	**104**	**172**

In the Hib meningitis patients and *S.aureus* OM/SA patients the majority of patients were Coloured children. This ethnic distribution is comparable to hospital in-patient admission ratios and to the control group. However, in the Hib OM/SA patients there was an equal distribution of Black and Coloured patients (Table 10.1).

10.1.4 Sex

The sex distribution of the patients is shown in Table 10.1. In the patients with OM/SA the overall sex ratio of 1.53:1 of male to female patients is in keeping with the male preponderance in most previous studies (Dich et al 1975; Waldvogel and Vasey 1980; Hoffman et al 1990). In contrast there was an equal distribution of male to female patients in the Hib meningitis group.

10.1.5. Age

The patients with Hib meningitis and Hib OM/SA were of similar ages and in both groups the patients were significantly younger than those with *S.aureus* OM/SA (Table 10.2). There was no significant difference between the ages of the Coloured and Black patients in any of the groups.

TABLE 10.2: AGE DISTRIBUTION IN MONTHS OF PATIENTS

	Mean	Median	25 centile	75 centile
Hib meningitis	12.2	8.4	6.1	11.9
Hib OM/SA	12.6	11.2	8.7	16.6
S.aureus OM/SA	82.5	85.8	53.5	118.8

10.1.6 Nutritional status

A child was considered undernourished if the weight for age percentiles was lower than the third percentile.

There was no significant difference in the weight for age percentiles between the Hib OM/SA and *S.aureus* OM/SA patient groups (Table 10.3). There were statistically significantly fewer Hib meningitis children with weight for age percentiles below the third percentile when compared to Hib OM/SA group ($p = 0.03$) or the *S.aureus* OM/SA group ($p = \leq 0.01$). Previous studies (Rosen and Davis 1980; Mulla et al 1984) have shown no association between meningitis and malnutrition and this is confirmed in this study. Although the *S.aureus* OM/SA group of children are older than the Hib OM/SA group the above findings did not alter whether the comparison was made to the *S.aureus* group as a whole or just to those children less than 48 months.

TABLE 10.3: WEIGHT FOR AGE PERCENTILE OF PATIENTS

	≤ 3rd Percentile	≥ 3rd Percentile
Hib meningitis	15.2%	84.8%
Hib OM/SA	27.2%	72.7%
S.aureus OM/SA	20.9%	79.1%

It was not possible to determine the weight for age percentiles in the control group of children but a study from Khayelitsha (a black housing area of Cape Town) found 10-20% of preschool (< 6 years) children fell below the third percentile for weight for age (Hugo-Hamman et al 1987). An earlier

study (Jacobs et al 1988) found similar percentages of Coloured preschool children ≤ 3rd percentile weight for age.

Malnutrition has been linked to many abnormalities which may leave the patient more vulnerable to bacterial infection (Gross and Newberne 1980). This does not seem to be a major contributing factor in the predisposition of patients to Hib meningitis, Hib OM/SA and *S.aureus* OM/SA in this study.

10.2 IgG AND IgG SUBCLASSES

The IgG and IgG subclasses of Hib meningitis, Hib OM/SA and *S.aureus* OM/SA patients (taken on admission) were compared to the age, sex and race matched normal range of healthy children comparing frequencies based on quartile categories. The four categories compared were ≥ 75th percentile; 50-75th percentile; 25-50th percentile and ≤ 25th percentile. The Chi-square test was used to determine whether there were equal numbers of patients in each category.

As IgG subclass levels are age related, statistical analysis was done using ANCOVA with age as a covariant, to counteract this, so the comparisons were age related.

10.2.1 Patients vs controls

Results in patients are displayed graphically using multiple notched Box-and-Whisker plots. These plots display the median value and the range and distribution of data (Figure 10.1). The central box represents the range of data values from the 25th to the 75th percentile. The 'whiskers' extend out to the minimum and maximum values that are within 1.5 times the interquantile range. The other values (beyond 1.5 times the box length (interquantile range)) are plotted as separate points. The central line is the

median (50th percentile). The notch of each box corresponds to the width of the confidence interval for the median. The confidence level on the notches allows comparisons to be made at the 95% level by examining whether the two notches overlap. The width of the box is proportional to the square root of the number of observations in the data set.

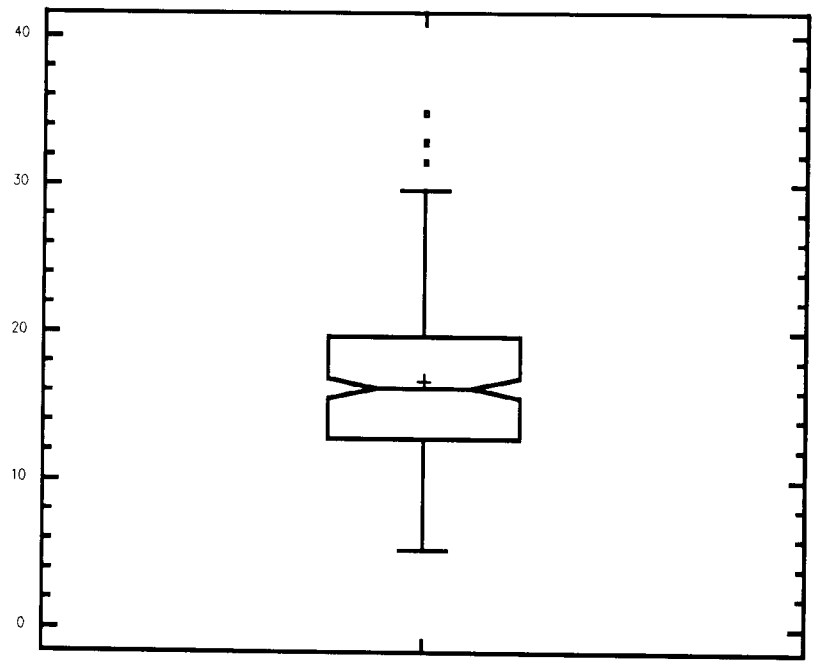


Figure 10.1: Example of a notched Box-and-Whisker plot

10.2.1.1 *Hib meningitis*

The IgG1, IgG2 and IgG4 levels of the Black children with Hib meningitis were significantly decreased compared to control values (Figure 10.2a). Both male and female Coloured children showed significantly decreased IgG4 levels (Figures 10.2b and 10.2c).

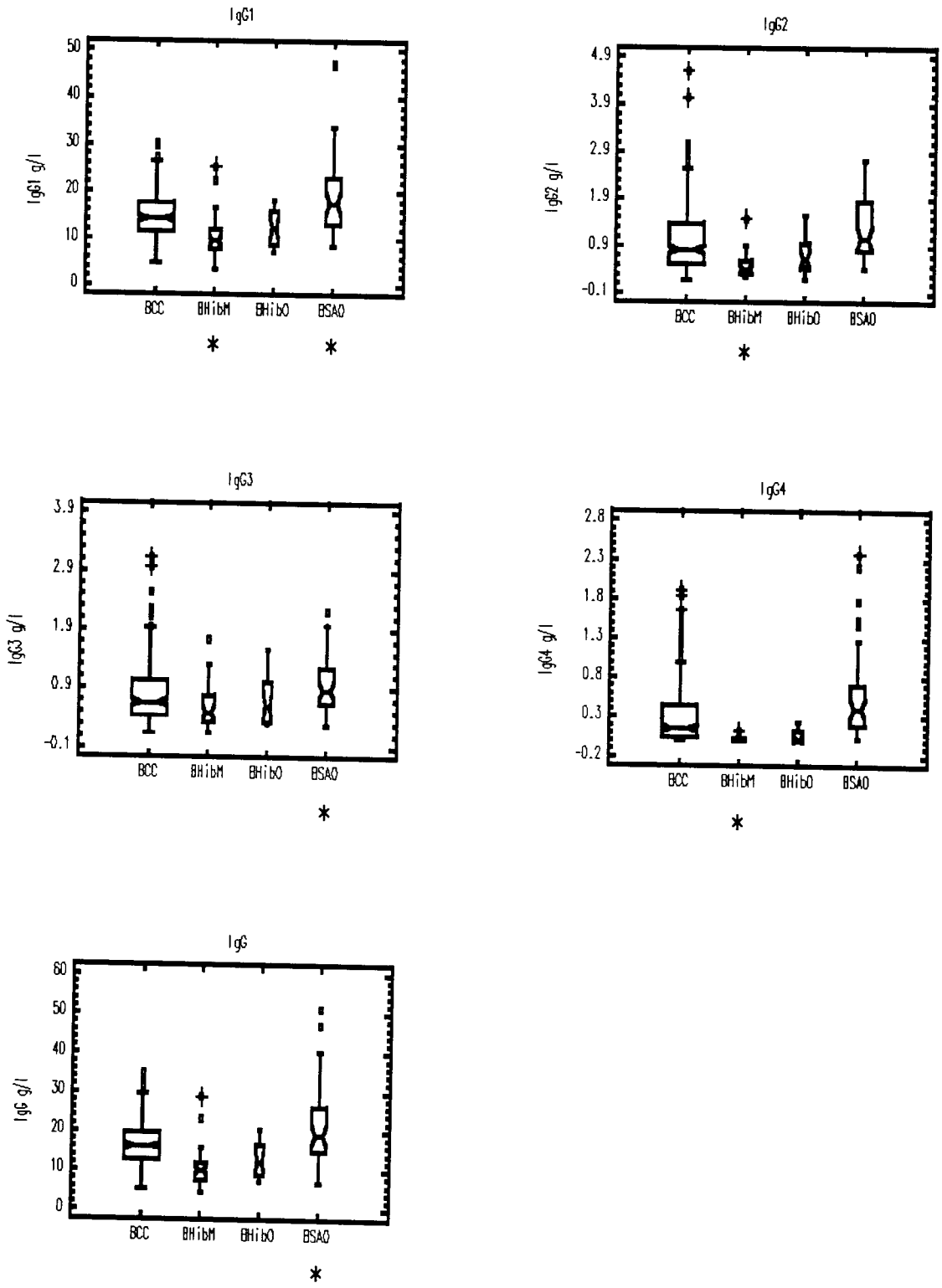


Figure 10.2a: Comparison of the distribution of IgG and IgG subclass values in Black children: child controls (BCC); children with Hib meningitis (HibM); children with Hib osteomyelitis/septic arthritis (BHibO) and children with *S.aureus* osteomyelitis/septic arthritis (BSAO). A * denotes a statistically significant difference ($p \leq 0.05$) between the patient groups and controls.

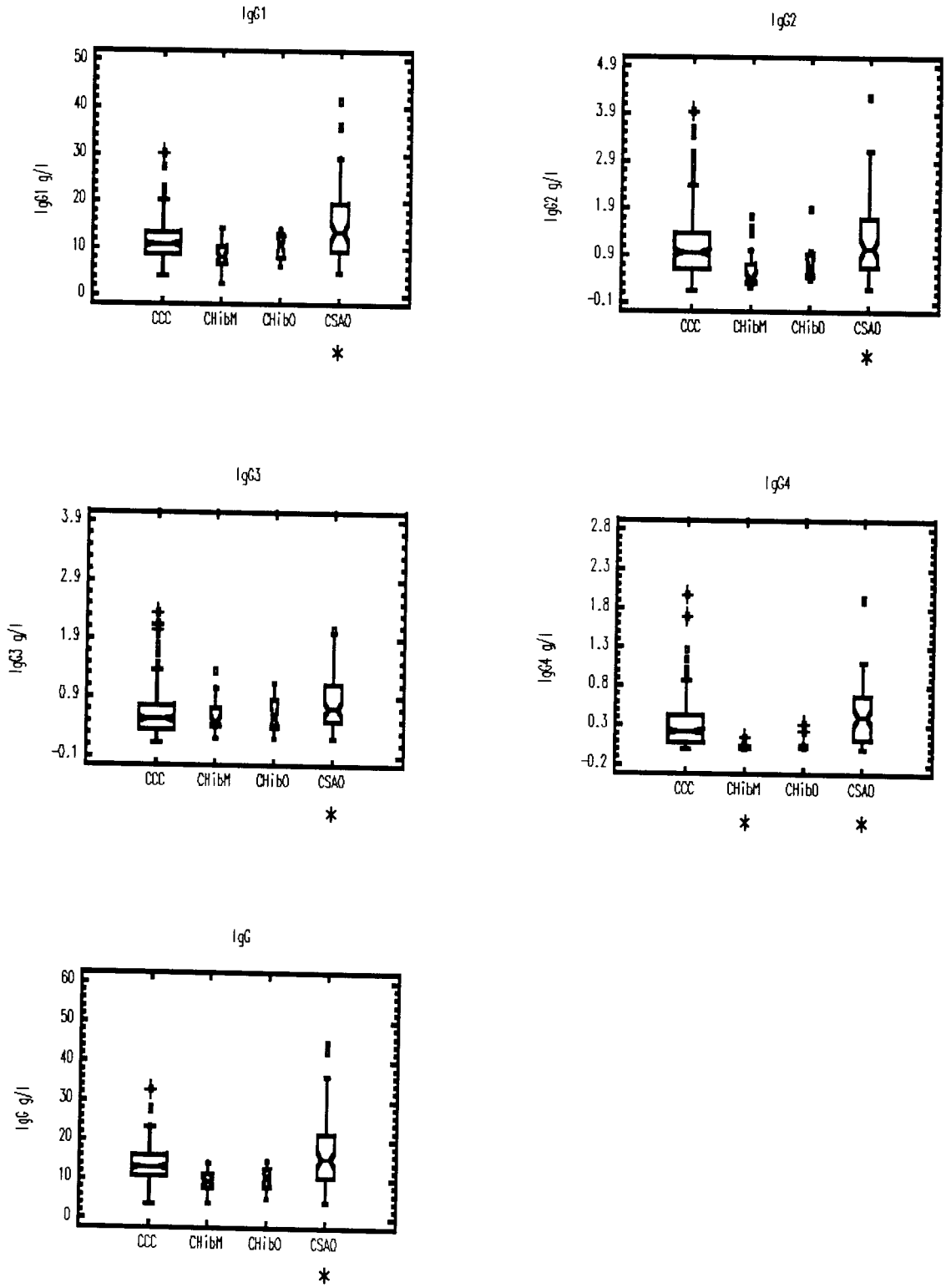


Figure 10.2b: Comparison of the distribution of IgG and IgG subclass values in Coloured male children: controls (CCC); children with Hib meningitis (CHibM); children with Hib osteomyelitis/septic arthritis (CHibO) and children with *S.aureus* osteomyelitis/septic arthritis (CSAO). A * denotes a statistically significant difference ($p \leq 0.05$) between the patient groups and controls.

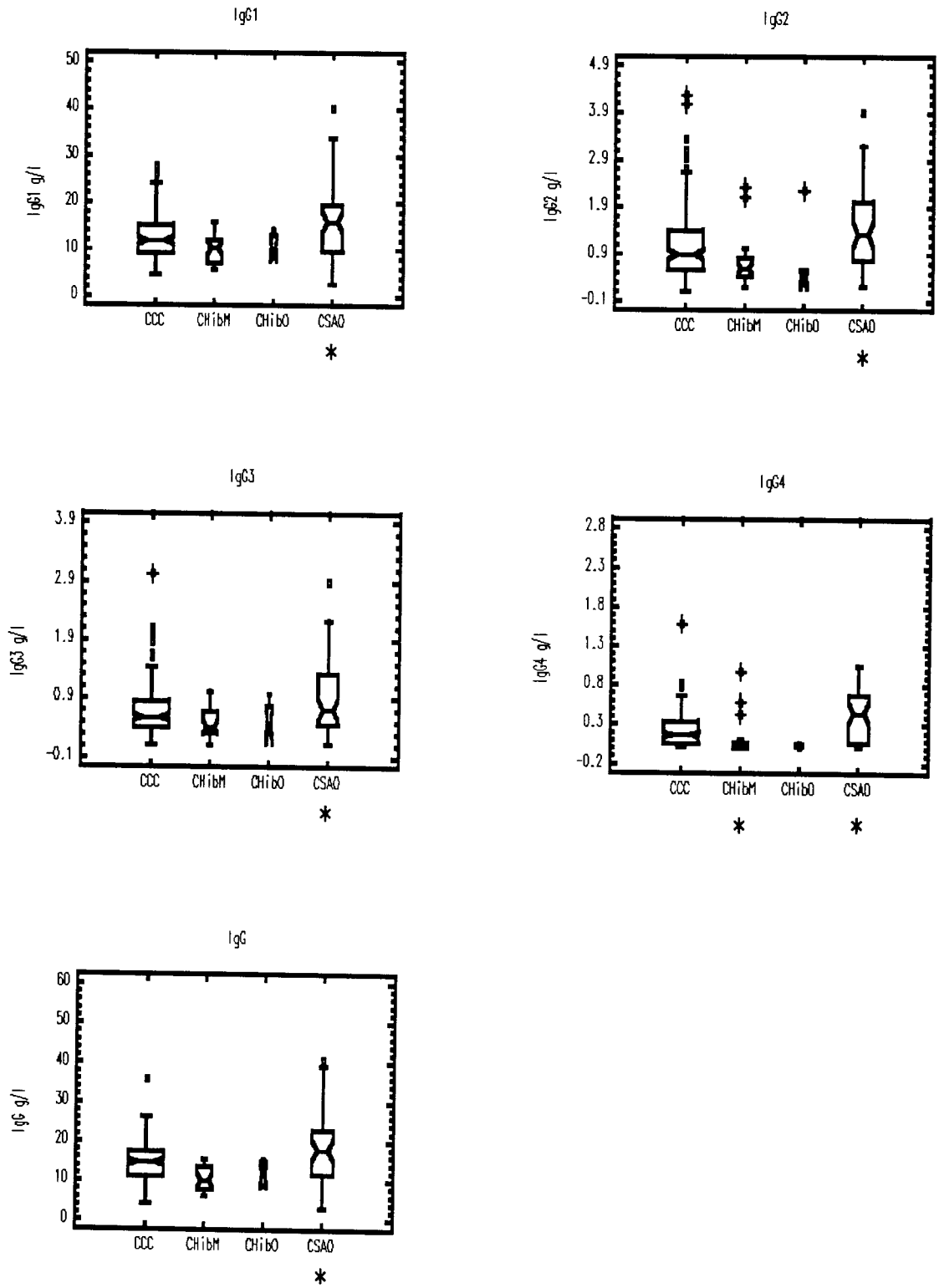


Figure 10.2c: Comparison of the distribution of IgG and IgG subclass values in Coloured female children: child controls (CCC); children with Hib meningitis (CHibM); children with Hib osteomyelitis/septic arthritis (CHibO) and children with *S.aureus* osteomyelitis/septic arthritis (CSA0). A * denotes a statistically significant difference ($p \leq 0.05$) between the patient groups and controls.

10.2.1.2 *Hib OM/SA*

No significant differences in the IgG and IgG subclasses of the Hib OM/SA patients in either racial group were found when compared to the controls (Figures 10.2a, 10.2b and 10.2c).

10.2.1.3 *S.aureus OM/SA*

The IgG, IgG1 and IgG3 of Black patients with *S.aureus* OM/SA were significantly increased. The IgG and all the IgG subclasses were significantly increased in male and female Coloured children.

10.2.1.4 *Discussion*

The relationship between selective IgG subclass deficiencies and susceptibility to infection is controversial and has been reviewed recently by Smith (1992). The first report of a selective IgG deficiency and susceptibility to recurrent pyogenic infection was from Schur et al (1970). Subnormal levels of one or more of the IgG subclasses have been shown to be associated with an increased susceptibility to bacterial infections especially of the respiratory tract (Schur et al 1970; Oxelius 1974; Stanley et al 1984) and otitis media (Friejd et al 1985).

Decreased IgG subclass concentrations have been reported in children with osteomyelitis and septic arthritis (Gottsegen 1987; Beard et al 1990). The incidence of subclass aberrations in patients with Hib meningitis, Hib OM/SA and *S.aureus* OM/SA has not been well documented.

In this study Black children with Hib meningitis had statistically lower levels of IgG1, IgG2 and IgG4 whereas Coloured children had only lower IgG4 levels. The IgG and IgG subclasses of the Hib OM/SA group were no different to the controls. In contrast the *S.aureus* OM/SA patients generally

had increased IgG and IgG subclass levels which may be interpreted as an appropriate response to a bacterial infection.

The age difference in patients with infections due to *S.aureus* or Hib (Table 10.2) may be one explanation as to why the *S.aureus* OM/SA patients but not the Hib OM/SA group of patients responded to the infection with an increase of IgG and IgG subclass levels. As the majority of people have detectable teichoic acid antibody levels (from previous exposure to cross-reacting antigens) a superimposed *S.aureus* OM/SA infection could give rise to a secondary antibody response in older patients.

No evidence was found for a deficiency of IgG or IgG subclass levels in acute Hib OM/SA or *S.aureus* OM/SA.

This finding is in disagreement of those of Eid et al (1980) who found that the majority of patients with primary *S.aureus* osteomyelitis had a depression of their IgG, IgM and IgA immune response to an acute infection.

The results in this study point to a possible association between susceptibility to Hib meningitis and low IgG1, IgG2 and IgG4 subclasses in Black patients and low IgG4 levels in Coloured patients. Although not statistically significant both the Coloured male and female children with Hib meningitis also showed a trend of low IgG1 and IgG2 levels.

10.2.2 IgG and IgG subclass levels during disease

10.2.2.1 *Serum IgG and IgG subclass levels*

The IgG or IgG subclass levels did not increase during the course of the disease in patients with Hib meningitis or Hib OM/SA. A significant increase of IgG, IgG1 and IgG2 levels occurred during the course of the disease in patients with *S.aureus* OM/SA. There was no increase in IgG3 levels. The IgG4 levels of the of *S.aureus* OM/SA patients tended to increase during the course of the disease but this difference was only statistically significant in the Coloured female patients (Table 10.4).

TABLE 10.4: COMPARISONS OF IgG AND IgG SUBCLASS VALUES IN INITIAL AND FOLLOW-UP SPECIMENS DURING THE COURSE OF THE DISEASE IN *S.AUREUS* OM/SA PATIENTS

	Black Patients		Coloured Male Patients		Coloured Female Patients	
	1	2	1	2	1	2
IgG	20.23	26.32*	16.21	22.56*	17.14	22.17*
IgG1	17.96	23.81*	13.87	18.90*	14.37	18.98*
IgG2	1.15	1.88*	1.03	2.15*	1.03	2.15*
IgG3	0.88	1.03	0.74	0.82	0.73	0.77
IgG4	0.36	0.50	0.33	0.49	0.27	0.40*

*** p = < 0.05**
1 = Initial specimen taken at the time of diagnosis of disease
2 = Specimen taken at time of follow-up (3-4 weeks later)

10.2.2.2 *Discussion*

Earlier studies have shown an impaired IgG antibody response in young children with Hib meningitis (Norden et al 1972; O'Reilly et al 1975).

Kouvalainen et al (1977) found that although IgG levels increased over 2 weeks during bacterial meningitis due to other organisms no rise in IgG levels were seen in children with Hib meningitis. This is in agreement with the findings in these studies. Children with *S.aureus* OM/SA showed an increase of IgG, IgG1 and IgG2 antibodies during the course of disease although children with Hib OM/SA did not. This suggests that impairment of IgG and IgG subclass responses may be involved in the pathogenesis of Hib meningitis and Hib OM/SA but not *S.aureus* OM/SA. It may also, however, be a reflection of the different mean age of the groups and the capacity for older children to increase their IgG levels in response to infection.

10.3 ANTI-Hib PRP ANTIBODIES

An impaired antibody response has been described in children with IgG subclass deficiencies (Umetsu et al 1985; Insel and Anderson 1986). Selective antibody deficiency to polysaccharide antigens can also be present in patients with normal IgG subclass concentrations (Ambrosino et al 1988).

10.3.1 Analysis of PRP antibodies

The assays used to measure the IgG, IgG1 and IgG2 subclass specific anti-Hib PRP antibodies have been detailed in Section A, Chapter 4.3. The assays are specific and sensitive with lower limits of detection of 0.011 μg/ml for IgG, 0.012 μg/ml for IgG1 and 0.006 μg/ml for IgG2.

The minimum concentration of PRP antibody necessary for protection is not precisely known. Robbins et al (1973) estimated the protective serum level of anti-Hib antibody to be 0.06-0.1 μg/ml, whilst Käyhty et al (1983) estimated it to be 0.15 μg/ml in non-vaccinated serum. A level of 1 μg/ml

of anti-Hib PRP antibody was associated with protection after immunization with a polysaccharide vaccine (Käyhty et al 1983b; Anderson 1984).

These levels cannot be equated with the values obtained in this study as all the methods measured total PRP antibody with a RABA assay. In this study IgG, IgG1 and IgG2 subclass specific antibodies were measured using an ELISA.

The antibody concentrations in the majority of Hib meningitis and Hib OM/SA patients were less than 0.15 μg/ml. To aid in the analysis cut-off values were selected based on the lower limits of detection of the assays and on estimated protective values from the literature as discussed above. The data were analysed in 4 groups: undetectable; less than 0.06 μg/ml; between 0.06 and 0.15 μg/ml; and greater than 0.15 μg/ml (Table 10.5). The lower limit of detection for IgG was 0.012 μg/ml, for IgG1 was 0.012 μg/ml and for IgG2 was 0.006 μg/ml.

There was only sufficient serum to measure Hib PRP antibodies from 69 of the 87 patients with Hib meningitis. Of these only 58 had a second sample (on average 10 days later) and 27 a third sample (on average 40 days later). There was only sufficient serum on 31 of the 33 patients with Hib OM/SA for Hib PRP antibody measurement and only 19 patients had a second specimen.

10.3.1.1 *IgG Hib PRP antibody levels*

The Hib meningitis and Hib OM/SA patients had very low levels of Hib PRP antibody levels (Table 10.5)

TABLE 10.5: CONCENTRATIONS OF IgG, IgG1 AND IgG2 Hib PRP ANTIBODIES IN CHILDREN WITH Hib MENINGITIS AND Hib OM/SA ON ADMISSION

	IgG PRP	IgG1 PRP	IgG2 PRP
	Hib Meningitis		
Undetectable	11.6%	33.3%	96.7%
< 0.06 μg/ml	55.1%	52.2%	1.6%
0.06-0.15 μg/ml	27.5%	11.6%	1.6%
> 0.15 μg/ml	5.8%	2.9%	0%
	Hib OM/SA		
Undetectable	12.9%	19.4%	83.9%
< 0.06 μg/ml	61.3%	74.2%	16.1%
0.06-0.15 μg/ml	22.6%	10.5%	0%
> 0.15 μg/ml	3.2%	0%	0%

27.5% of the Hib meningitis and 25.8% of the Hib OM/SA patients had an IgG Hib PRP antibody level greater than the minimum level considered to be protective (0.06 μg/ml) and only 5.8% and 3.2% respectively had a level greater than the more generally accepted protective level (0.15 μg/ml).

14.5% of the Hib meningitis patients and 10.5% of the Hib OM/SA had a IgG1 Hib PRP level ≥ 0.06 μg/ml and only 2.9% of the Hib meningitis and none of the Hib OM/SA patients had levels ≥ 0.15 μg/ml. Only 3.2% of Hib meningitis and 16.1% of Hib OM/SA patients had detectable IgG2 Hib PRP antibodies present.

10.3.1.2 *Hib PRP antibody levels during disease*

The IgG or IgG1 specific Hib PRP antibody levels did not increase significantly during the course of the disease in Black or Coloured patients with Hib meningitis or with Hib OM/SA. However, 5% and 10% of follow-up specimens of the Hib meningitis and Hib OM/SA patients respectively had an IgG PRP level $\geq$ 1.0 μg/ml whereas none of these patients had baseline levels $\geq$ 1.0 μg/ml (data not shown).

The IgG2 Hib PRP data was not analysed as the majority of the patients had undetectable antibody levels.

10.3.1.3 *Race*

The Chi-square test was used to analyse any differences of Hib PRP antibody levels between Black and Coloured patients. Race had no effect on the IgG, IgG1 or IgG2 Hib PRP antibody levels in either disease group.

10.3.1.4 *Discussion*

Using the assays developed in this study, which are sensitive and specific, the majority of infants had low ($\leq$ 0.15 μg/ml) baseline levels of Hib PRP antibodies. These levels are too low for statistical comparison with regard to the age of patients, to serum IgG and IgG subclass levels and to the influence of the allotypic markers G1m(f), G2m(n) and Km(3).

There was a lack of specific Hib PRP antibody response in the children with Hib meningitis or Hib OM/SA during the course of the disease. This finding is in contrast to Rijkers et al (1988) who found that children below 2 years of age do develop anti-PRP antibodies during the course of Hib meningitis.

Levels of Hib PRP antibodies were measured in five healthy children (12-15 months old) pre- and two weeks post-Hib Titer vaccination. All of these children had detectable ($\geq$ 0.012 μg/ml) pre-immunization IgG and IgG1 Hib PRP levels but none had protective levels ($\geq$ 0.06 μg/ml).

Four responded to immunization with post immunization antibody levels increasing to protective levels ($\geq$ 0.15 μg/ml) although none had levels $\geq$ 1.0 μg/ml (Table 10.6). The baseline levels of IgG and IgG1 antibodies to Hib PRP in the patients with Hib meningitis and Hib OM/SA were similar to those measured pre-immunization in these 5 healthy control children and to published data from similarly age matched children (Greenberg et al 1987) and were generally less than 0.06 μg/ml. The majority of patients had low levels (not protective) of IgG and IgG1 Hib PRP antibodies and only 5% had detectable IgG2 Hib PRP antibody. In the immunized control children none of them had detectable IgG2 Hib PRP antibody before or after immunization (Table 10.6).

TABLE 10.6: Hib PRP ANTIBODY LEVELS (μg/ml) IN 5 HEALTHY CHILDREN IMMUNIZED WITH Hib TITER VACCINE

	IgG		IgG1		IgG2	
	Pre	Post	Pre	Post	Pre	Post
1	0.031	0.315	0.024	0.303	0.0	0.0
2	0.057	0.140	0.036	0.210	0.0	0.0
3	0.057	0.035	0.056	0.037	0.0	0.0
4	0.032	0.715	0.027	0.638	0.0	0.0
5	0.018	0.416	0.017	0.434	0.0	0.0

A strong correlation between IgG2 specific Hib PRP antibody and total PRP antibody in young children with invasive Hib disease has been shown (Ramadas et al 1986). Few of the patients in this study had IgG2 specific Hib PRP antibody. There are increasing reports that in young children most PRP antibody is of the IgG1 subclass and a gradual shift to IgG2 is seen as the immune system matures (Hammarström and Smith 1986b). Although this assay is sensitive, specific and accurate and allows IgG, IgG1 and IgG2 specific Hib PRP antibodies to be quantitated it is not possible to determine the proportion of each subclass making up the total PRP antibody. Using these assays it has been demonstrated that young children with invasive Hib meningitis or OM/SA do not have or develop protective anti-Hib PRP antibodies which confirms previous findings (Ramadas et al 1986).

10.4 ANTI-*S.AUREUS* TEICHOIC ACID ANTIBODIES

The ELISA assays used to measure the IgG, IgG1 and IgG2 antibodies have been detailed in Section A, Chapter 5.3. These assays are specific and sensitive with lower limits of detection of 0.001 units for IgG, and 0.005 units for IgG1 teichoic acid and IgG2 teichoic acid antibodies.

The IgG, IgG1 and IgG2 teichoic acid specific antibodies were measured in patients at the time of diagnosis of *S.aureus* OM/SA and again 3-4 weeks later.

Levels of teichoic acid antibodies were also measured in 4 children with pneumococcal OM/SA, 11 children with Hib OM/SA and 16 children with group A ß-haemolytic streptococcal OM/SA. The mean age of these children was 47.4 months with a range of 2-145 months.

10.4.1 Anti-teichoic acid antibody levels

S.aureus OM/SA patients all have IgG antibody to *S.aureus* ribitol teichoic acid. The majority of the antibody is IgG1 and very few of the patients have significant levels of IgG2 (Figure 10.3).

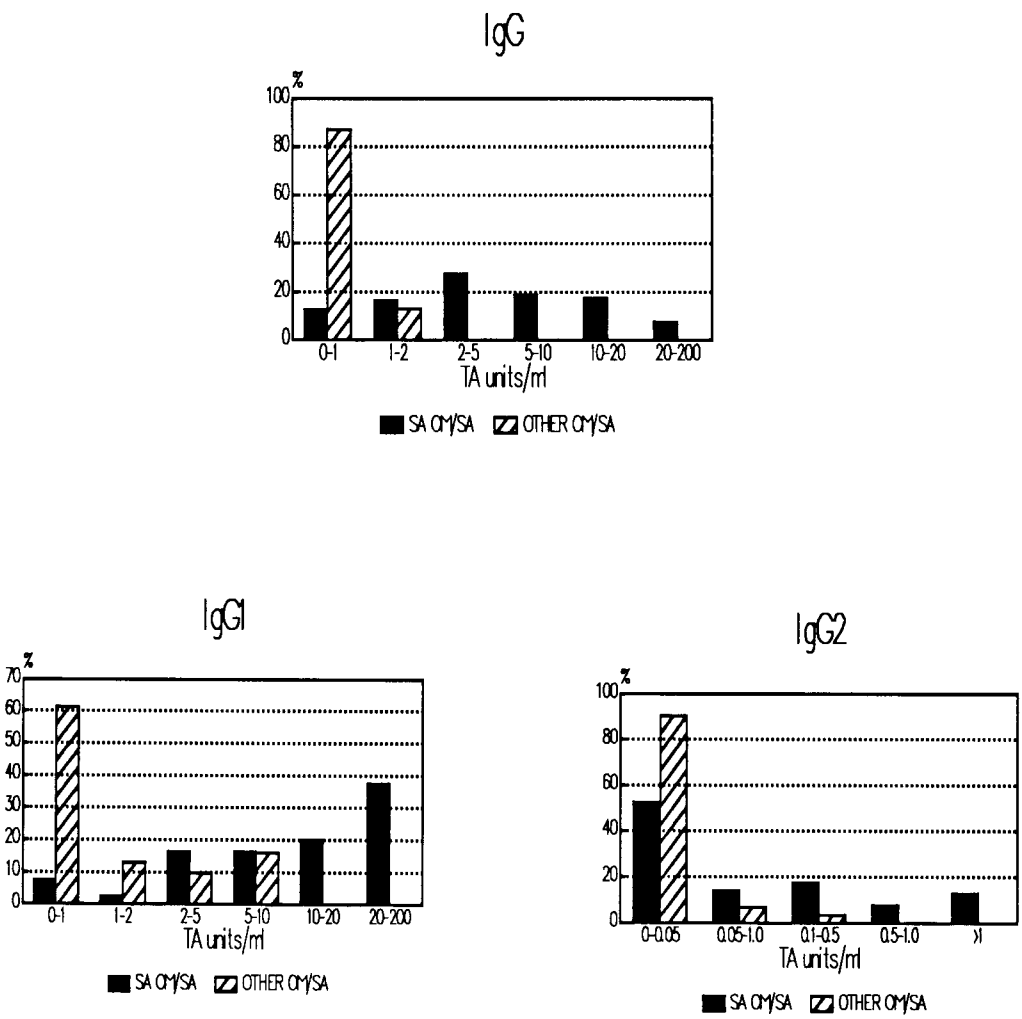


Figure 10.3: Serum IgG, IgG1 and IgG2 teichoic acid (TA) antibody concentrations (units/ml) in children with *S.aureus* OM/SA (SA OM/SA) and in children with OM/SA due to organisms other than *S.aureus* (other OM/SA)

Overall, there were no significant differences in the teichoic acid antibody levels measured in the specimens taken at the time of diagnosis and the

specimen taken at follow-up a few weeks later. Because the antibody measurements were performed on sera obtained at various intervals after illness the effect of the length of time after illness on antibody level was also analysed. There was no significant correlation with time after illness and the IgG, IgG1 and IgG2 teichoic acid specific antibody concentrations.

These results were compared to the levels of teichoic acid antibody levels in patients with acute OM/SA due to organisms other than *S.aureus* i.e. *S.pneumoniae* (4 patients), Hib (11 patients) and group A ß-haemolytic streptococcus (16 patients). Arbitrary cut-off values for IgG, IgG1 and IgG2 teichoic acid antibody levels were selected as shown in Figure 10.3.

None of the 31 patients with OM/SA caused by organisms other than *S.aureus* had IgG teichoic acid levels greater than 2 units/ml whereas 71.2% of *S.aureus* OM/SA patients had levels above this.

Similarly for IgG1, only 25.7% of patients with OM/SA other than *S.aureus* had levels greater than 2 units/ml whereas 90.1% of the *S.aureus* OM/SA patients had levels in this range.

16.25% of the *S.aureus* OM/SA patients had IgG2 teichoic acid levels greater than 0.5 units/ml and none of the other patients with OM/SA had levels above this.

10.4.2 Race and age

There was no difference in the teichoic acid antibody levels between the Black and Coloured patients. Nor was there any correlation between the IgG, IgG1 and IgG2 teichoic acid antibody levels and age ($r = 0.2$; $r = 0.24$ and $r = 0.03$ respectively).

10.4.3 Discussion

Rising or elevated serum levels of antibodies to staphylococcal cell wall teichoic acid correlates with the development of serious infections due to *S.aureus* (Crowder and White 1972; Nagel et al 1975; Tuazon and Sheagren 1976; Wheat et al 1978).

Levels of teichoic acid antibodies vary substantially in normal control populations and in patients with various types of staphylococcal infections (Granström et al 1983; Wise and Tosolini 1992).

The ELISA has been used to determine antibodies to teichoic acid (MacKowiak and Smith 1978; Verburgh et al 1981; Granström et al 1983; Hammarström et al 1984). Granström et al (1983) found increased IgG titres in 87% of patients with endocarditis, 79% with complicated septicaemia and 50% with uncomplicated septicamia in serum samples drawn between 7-30 days of disease onset.

In the *S.aureus* OM/SA patients only 12.5% had IgG and 7.5% had IgG1 teichoic acid antibody levels less than 1 unit/ml. In patients with OM/SA due to organisms other than *S.aureus* 87.1% had IgG and 61.3% had IgG1 teichoic acid antibody levels less than 1 unit/ml. In contrast 97.5% of *S.aureus* OM/SA patients and 100% of patients with OM/SA due to organisms other than *S.aureus* had IgG2 teichoic acid antibody levels less than 1 unit/ml.

Of the patients with *S.aureus* OM/SA 43.8% and 72.8% respectively had IgG and IgG1 teichoic acid antibody levels greater than 5 units/ml. Of the patients with OM/SA due to organisms other than *S.aureus* 0% and 16.0%

respectively had IgG and IgG1 teichoic acid antibody levels greater than 5 units/ml (Figure 10.3).

These data show that in most children IgG1 antibodies form the majority of the IgG teichoic acid antibodies and that very few children produce quantitative IgG2 teichoic acid antibody levels. In contrast previous studies have shown that anti-teichoic acid antibodies and antibodies against whole staphylococcus were primarily of the IgG2 subclass (Hammarström et al 1984; Monteil et al 1990).

No rise of anti-teichoic acid antibodies was observed in children during the course of *S.aureus* OM/SA. This latter finding makes the value of measuring IgG teichoic acid antibodies in acute *S.aureus* infection doubtful.

10.5 ANTI-TETANUS TOXOID ANTIBODIES

The response to booster tetanus toxoid immunization as measured by levels of anti-tetanus toxoid antibodies in serum was used as an indicator of immunological competence. Anti-tetanus antibody levels were determined in pre- and post-tetanus toxoid immunization samples in the children with Hib OM/SA and *S.aureus* OM/SA. In most of the Hib meningitis patients tetanus toxoid immunization was not given as they had recently had a routine diphtheria, pertussis, and tetanus immunization in the previous two months (documented on the clinic card). The tetanus toxoid antibody levels measured on the admission specimen were regarded in these patients as the post immunization specimens.

The assays used to measure the IgG, IgG1 and IgG4 tetanus toxoid antibodies are detailed in Section A, Chapter 3.3. The lower limit of detection for IgG was 0.003 U/ml and for IgG1 and IgG4 0.005 U/ml.

An antitoxin level of 0.01 IU/ml is widely employed as an indicator of immunity (Sedgwick et al 1983, Virella et al 1985; Porter et al 1992), although others feel that levels of ≥ 0.1 IU/ml are safer (Simonsen et al 1987). A level ≥ 2 IU/ml is considered suitable for production of human anti-tetanus immunoglobulin (Barr et al 1975).

The units of the IgG tetanus toxoid assays are calibrated against a commercially available reference standard. The units for the IgG1 and IgG4 tetanus toxoid ELISAs are arbitrary. To aid in the analysis, cut-off values of tetanus toxoid antibody levels were selected based on protective values quoted in the literature. They were used only to aid in analysis and not to comment on whether the levels are protective or not.

10.5.2 Tetanus toxoid antibody levels

All the patient groups had post IgG tetanus toxoid or IgG1 tetanus toxoid antibody levels ≥ 0.01 U/ml which is considered a protective level. Most of the Hib meningitis patients had post-IgG4 tetanus toxoid levels < 0.1 U/ml whereas 23.8% of Hib OM/SA and 64.2% of *S.aureus* OM/SA patients had levels ≥ 0.1 U/ml (Table 10.7).

The percentage of patients in each group with pre- and post-IgG and IgG1 tetanus toxoid levels of greater than 0.01 U/ml were not significantly different. In contrast the Hib meningitis patients produced less IgG4 tetanus toxoid than the *S.aureus* OM/SA or Hib OM/SA patients.

The IgG1 tetanus toxoid levels correlated strongly with the IgG tetanus toxoid levels (r = 0.93) whereas there was little correlation between the

IgG4 tetanus toxoid and IgG tetanus toxoid levels (r = 0.1). There was no correlation between the IgG, IgG1 and IgG4 tetanus toxoid levels and age.

TABLE 10.7: IgG, IgG1 and IgG4 TETANUS TOXOID ANTIBODY LEVELS PRE- AND POST-TETANUS TOXOID IMMUNIZATION IN HEALTHY INFANTS AND PATIENTS WITH Hib MENINGITIS, Hib AND *S.AUREUS* OSTEOMYELITIS/SEPTIC ARTHRITIS

	U/ml	Hib Men	Hib OM/SA	*S.aureus* OM/SA
IgG Pre	≥ 0.1		90.5%	92.6%
	≥2.0		23.8%	31.5%
	≥10.0		4.8%	6.5%
IgG Post	≥0.1	100%	100%	98.2%
	≥2.0	36.7%	85.7%	89.8%
	≥10.0	3.3%	52.4%	69.9%
IgG1 Pre	≥0.1		95.3%	94.3%
	≥2.0		19.1%	32.1%
	≥10.0		4.8%	6.6%
IgG1 Post	≥0.1	100%	100%	98.1%
	≥2.0	35.7%	85.7%	92.5%
	≥10.0	0%	42.9%	58.5%
IgG4 Pre	≥0.1		0%	39.6%
	≥2.0		0%	8.5%
	≥10.0		0%	0.9%
IgG4 Post	≥0.1	3.6%	23.8%	64.2%
	≥2.0	0%	4.8%	44.3%
	≥10.0	0%	0%	20.8%

Hib Men = *H.influenzae* type b meningitis
Hib OM/SA = *H.influenzae* osteomyelitis/septic arthritis
***S.aureus* OM/SA = S.aureus osteomyelitis/septic arthritis**

10.5.3 Discussion

The interpretation of results of randomly collected individual samples is difficult since there are no established normal limits for different age groups. This is complicated by factors such as the age of the patient and the number and timing of previous tetanus toxoid immunizations. The use of

paired samples pre- and post-immunization avoids establishing normal values of anti-tetanus toxoid antibody levels. However, previous studies have shown great variability of individual responses to tetanus toxoid immunization in healthy controls (French and Harrison 1985; Virella et al 1985).

The limit of detection of IgG tetanus toxoid antibody (0.0022 U/ml) is more than adequate for detecting levels greater than 0.01 U/ml which was taken as the lower protective limit in this analysis.

The main objective of establishing these assays was to determine the immunocompetency of the patients when confronted with a protein antigen. The patients all showed an appropriate antibody response to tetanus toxoid antigen and can be regarded as immunocompetent with respect to tetanus toxoid. All but one patient had post-immunization IgG tetanus toxoid antibody levels $\geq$ 0.1 U/ml and that patient had a level between 0.01 and 0.1 U/ml. Earlier authors have also observed a predominance of IgG1 tetanus toxoid antibodies (French and Harrison 1985; Rubin et al 1986; Moss et al 1987). The distribution of IgG1 and IgG4 tetanus toxoid antibodies is interesting. The post-immunization IgG4 tetanus toxoid antibody levels of the Hib meningitis patients were much lower than the Hib OM/SA and *S.aureus* OM/SA patients. The following factors may have influenced this:

- The time interval between the tetanus toxoid immunization and the 'post' specimen was greatest for the Hib meningitis patients
- The majority of Hib meningitis patients were immunized prior to getting the disease whereas the other patient groups were immunized at the time of diagnosis of the disease.

A healthy group of young Black children tested as part of another study showed similar results to the patient groups. IgG, IgG1 and IgG4 tetanus toxoid antibody levels of greater than 0.1 U/ml pre- and post-tetanus toxoid immunization were: pre-IgG tetanus toxoid = 90.7%; post-IgG tetanus toxoid = 97.3%; pre-IgG1 tetanus toxoid = 93.3%; post-IgG1 tetanus toxoid = 98.7%; pre-IgG4 tetanus toxoid = 0%; post-IgG4 tetanus toxoid = 5.3%. The low IgG4 tetanus toxoid levels in this healthy group of children may have been due to their young age (range 6-9 months; mean 6.9 months).

10.6 G1m(f), G2m(n) AND Km(3) ALLOTYPES

10.6.1 Frequency of G1m(f), G2m(n) and Km(3) in patients

Data were analysed by Chi-square and the odds ratios and 95% confidence intervals were calculated. The significance levels of the Fisher two-tailed test were used due to the small numbers in some groups.

10.6.1.1 *Hib meningitis*

G1m(f) was present in 53.2% of Coloured Hib meningitis patients which was not significantly different to the frequency of the marker in the control population (Chi-square value = 0.01; p = 0.92; OR = 1.03 (0.53 < OR < 2.01)) (Table 10.8). However, the frequency of the G2m(n) marker (8.5%) was significantly lower in the Coloured Hib meningitis patients than in the controls (35.6%) (Chi-square value = 13.65; p = 0.0002; OR = 0.17 (0.04 < OR < 0.48)) (Table 10.9).

TABLE 10.8: FREQUENCY OF G1m(f) IN CONTROLS AND PATIENTS

	G1m(f)+	G1m(f)-	p value*
Coloured			
Controls	144 (52.4%)	131 (47.6%)	
Hib meningitis	25 (53.2%)	22 (46.8%)	0.92
Hib OM/SA	6 (35.3%)	11 (64.7%)	0.18
S.aureus OM/SA	61 (48.4%)	65 (51.7%)	0.51
Black			
Controls	3 (2.2%)	135 (97.8%)	
Hib meningitis	0 (0%)	30 (100%)	
Hib OM/SA	0 (0%)	15 (100%)	
S.aureus OM/SA	3 (6.5%)	43 (93.5%)	

* **Statistical analysis of patients groups compared to controls**

TABLE 10.9: FREQUENCY OF G2m(n) IN CONTROLS AND PATIENTS

	G2m(n)+	G2m(n)-	p value
Coloured			
Controls	98 (35.6%)	177 (64.4%)	
Hib meningitis	4 (8.5%)	43 (91.5%)	0.0002
Hib OM/SA	0 (0%)	17 (100%)	0.002
S.aureus OM/SA	41 (32.5%)	85 (67.5%)	0.50
Black			
Controls	4 (2.9%)	134 (97.1%)	
Hib meningitis	0 (0%)	30 (100%)	
Hib OM/SA	0 (0%)	15 (100%)	
S.aureus OM/SA	1 (2.2%)	45 (97.8%)	

* **Statistical analysis of patient groups compared to controls**

No Black patients were either G1m(f) or G2m(n) positive but the frequency of these markers is so low in the control group (G1m(f) = 2.2% and G2m(n) = 2.9%) that this was not statistically significant (Tables 10.8 and 10.9).

The frequency of the Km(3) positive allotype in both the Black and Coloured Hib meningitis patients was significantly decreased compared to the control population. Chi-square value = 4.82; p = 0.02; OR = 0.21 (0.05 < OR < 0.96) for the Black patients and Chi-square value 4.37; p = 0.03; OR = 0.21 (0.05 < OR < 1.06) for the Coloured patients (Table 10.10).

TABLE 10.10: FREQUENCY OF Km(3) IN CONTROLS AND PATIENTS

	Km(3)+ve	Km(3)-ve	NT*	p value**
Coloureds				
Controls	260 (95.9%)	6 (2.2%)	5 (1.9%)	
Hib meningitis	36 (83.7%)	4 (9.3%)	3 (7.0%)	0.03
Hib OM/SA	16 (94.1%)	1 (5.9%)	0 (0%)	0.35
S.aureus OM/SA	117 (92.9%)	5 (4.0%)	4 (3.1%)	0.49
Blacks				
Controls	124 (88.6%)	7 (5.0%)	9 (6.4%)	
Hib meningitis	19 (73.1%)	5 (19.2%)	2 (7.7%)	0.02
Hib OM/SA	15 (93.7%)	1 (6.3%)	0 (0%)	1.0
S.aureus OM/SA	44 (95.6%)	1 (2.2%)	1 (2.2%)	0.68

*** NT = not typable**
**** Statistical analysis of patient groups compared to controls**

10.6.1.2 *Hib OM/SA*

G1m(f) was present in 35.3% of Coloured patients and 52.4% of the controls (Chi-square value = 1.77, p = 0.18, OR = 0.50 (0.15 < OR < 1.55)) which was not a significant difference Table (10.8).

Coloured patients had a significantly lower frequency of the G2m(n) allotype (0%) than the controls (35.6%) (Chi-square value = 9.26, p = .002, OR = 0.002 (0.00 < OR < 0.44)) (Table 10.9).

Although no Black patients were either G1m(f) or G2m(n) positive, the frequency of these markers is so low in the control group that this was not statistically significant (Tables 10.8 and 10.9).

The frequency of the Km(3) allotype in the Black or Coloured Hib OM/SA patients was not significantly different to racially matched controls (Table 10.10).

10.6.1.3 *S.aureus OM/SA*

There were no statistically significant differences in the frequency of the G1m(f), G2m(n) or Km(3) allotypes in either the Black or coloured patients when compared to racially matched controls (Table 10.8, 10.9 and 10.10).

10.6.1.4 *Discussion*

The frequency of the G1m(f) allotype marker was not different between the patient and control groups. The frequency of the G2m(n) marker in the Hib meningitis and Hib OM/SA patient groups was significantly decreased. The Km(3) positive allotype was significantly decreased in Black and Coloured children with Hib meningitis. The findings, in Coloured children, are summarized in Figure 10.4. Both G2m(n) and G1m(f) positive allotypes are

rare in Black children thereby precluding analysis of these allotype frequencies in this ethnic group.

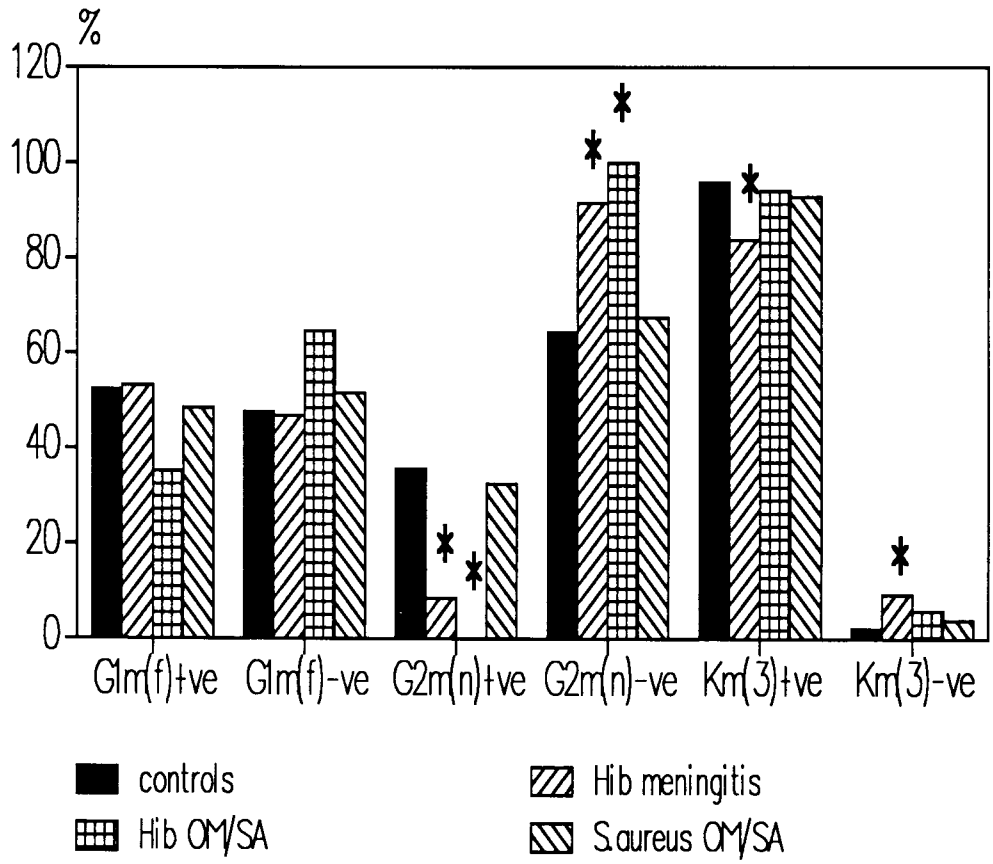


Figure 10.4: Frequency of the G1m(f), G2m(n) and Km(3) allotypes in healthy Coloured children and Coloured children with: Hib meningitis, Hib OM/SA or *S.aureus* OM/SA. A statistically significant difference ($p \leq 0.05$) between the patients compared to the controls is denoted by (*)

Of the 73 patients with Hib meningitis approximately 18 would have been expected to have been G2m(n) positive considering the frequency of the allotype in the Black and Coloured patients. There were only 4 Coloured and no Black patients positive for the G2m(n) allotype.

Of the 32 patients with Hib OM/SA, 7 patients would have been expected to be G2m(n) positive. No patients were positive for the G2m(n) allotype.

No significant difference in the frequency of the G2m(n) allotype was found in the *S.aureus* OM/SA patients when compared to the control subjects.

The results show a highly significant association between Hib meningitis and OM/SA and a decreased frequency of the G2m(n) marker and provides evidence that absence of the G2m(n) allotype is associated with an increased susceptibility to invasive Hib infection in Coloured children. The lack of association between G2m(n) and *S.aureus* OM/SA adds further weight to this observation.

Significant associations have been demonstrated between the Km(1) allotype and the post immunization antibody levels to *Haemophilus influenzae* and meningococcus C polysaccharides in White children but not in Black children and adults (Pandey et al 1979, Pandey et al 1981). Granoff et al (1984) showed that both Black and White Km(1) positive children had a higher antibody response to *H.influenzae* type b vaccine than children lacking the allotype, although only Black children with the Km(1) allotype showed a decreased risk of meningitis (Granoff et al 1984). There are no reports in the literature on the frequency of the Km(3) allotype in haemophilus infections. The finding that the Km(3) negative allotype is more frequent in patients with Hib meningitis is therefore a new observation.

10.6.2 Effect of G1m(f), G2m(n) and Km(3) allotypes on IgG and IgG subclasses in patients

This analysis was only done on the Coloured male patients for the following reasons:

i. The G1m(f) and G2m(n) allotypes are uncommon in South African Blacks. There were no G1m(f) or G2m(n) positive Black patients in the Hib meningitis or Hib OM/SA groups and only three G1m(f) and one G2m(n) positive patients in the *S.aureus* OM/SA group.

ii. There were only five Coloured female patients with Hib OM/SA which is too few for meaningful statistical analysis. Since the IgG, IgG1 and IgG3 and IgG4 levels of the Coloured male and female healthy controls had been shown to be statistically different the Coloured male and female patients had to be analysed separately.

10.6.2.1 *G1m(f)*

No effect of the G1m(f) allotype on the IgG or IgG subclass levels of patients with Hib meningitis, Hib OM/SA and *S.aureus* OM/SA patients was found. This may be due to smaller numbers when compared to the control study.

10.6.2.2 *G2m(n)*

The G2m(n) allotype had a positive influence on the IgG2 levels of the *S.aureus* OM/SA patients. A similar positive effect of the G2m(n) allotype on IgG2 had been noted in control subjects (Section B: Chapter 9.3.6.2).

The influence of this allotype on IgG2 in Hib meningitis and Hib OM/SA patients could not be assessed as there were only 4 G2m(n) positive Hib patients and no G2m(n) positive Hib OM/SA patients (Table 10.10).

10.6.2.3 *Km(3)*

There was no effect of the Km(3) allotype on IgG subclass concentrations in any of the three patient groups.

10.6.2.4 *Discussion*

In Coloured male patients the G1m(f) had no effect on IgG subclass levels. In the control group the G1m(f) had a negative influence on IgG and IgG1 levels and a positive influence on IgG2 levels.

The G2m(n) had a positive influence on the IgG2 levels of *S.aureus* OM/SA patients.

10.7 C4 PROTEIN TYPING

10.7.1 Samples

C4 typing was done in 39 (44.8%) of the children with Hib meningitis. Twenty seven of these children were Coloured and 12 were Black.

10.7.2 Frequency of C4A*QO and C4B*QO in Hib meningitis patients

The prevalence of C4A*QO or C4B*QO antigens in the children with meningitis did not differ from that in the Black and Coloured control subjects (Table 10.11). Distribution of homozygosity for these alleles in the study group conformed with the Hardy-Weinberg equilibrium.

TABLE: 10.11: FREQUENCY OF C4A*QO AND C4B*QO IN Hib MENINGITIS PATIENTS

PATIENTS		CA4*QO	(p =)**	C4B*QO	(p =)**
Coloured	n = 27	0.09	(0.7)	0.01	(0.8)
Black	n = 12	0.07	(0.2)	0.25	(0.1)
All	n = 39	0.07	(0.3)	0.21	(0.2)

** p = By Fisher's exact test with Yates correction

10.7.3 Discussion

Rowe et al (1989) have reported an increase in C4B deficiency in 46 patients with meningitis. Bishof et al (1990) found that in White patients but not in Black patients a complete C4B deficiency (e.g. C4B*QO homozygosity) is a risk factor for invasive disease with encapsulated organisms. Cates et al (1992) found no increase in C4B deficiency with bacteraemia or meningitis caused by encapsulated bacteria.

The present analysis was done on the Hib meningitis group as a whole as well as on an ethnic basis because of the relatively small numbers of Black (n = 12) and Coloured (n = 27) patients would have decreased the accuracy of the frequency analysis. Frequency of homozygosity of C4A*QO and CAB*QO was not increased in either the Black or Coloured children (results not shown). It is therefore concluded from this study that there is insufficient evidence that C4B deficiency is associated with disease caused by Hib encapsulated bacteria.

10.8 MANNOSE BINDING PROTEIN (MBP)

10.8.1 Patient samples

MBP was measured at the onset of illness in 76 (87.4%) of the children with Hib meningitis and in all of the children with Hib OM/SA.

10.8.2 MBP levels

The median levels and 95% confidence levels of the Black and Coloured children with Hib meningitis or Hib OM/SA are shown in Table 10.12. and compared to the Black adult controls.

TABLE 10.12: SERUM MBP LEVELS OF PATIENT AND CONTROL GROUPS

	RACE	NO	MEDIAN MBP (ng/ml)	RANGE Mean ± 2SD
Patients				
Hib meningitis	B	27	2068	≤ 10-8548
	C	49	2386	≤ 10-8379
Hib OM/SA	B	16	678	≤ 10-8166
	C	17	2703	≤ 10-9561
Controls				
South African Black adults	B	55	838	≤ 10-5779

B = Black **C = Coloured**

The values of the patient groups were compared to the adult controls as age does not appear to influence MBP levels (Lipscombe et al 1992).

The 95% confidence intervals of the median values of the patient groups had a much wider range than the South African Black adult controls or the reported range of adult controls (Lipscombe et al 1992).

The median values of the Black and Coloured patients were not significantly different from the controls although the median values of the Coloured patients tended to be higher than the Black patients and controls (Table 10.12). The latter findings may be due to a racial difference as MBP measurements were only done on Black and not Coloured adult controls.

10.8.3 Frequency of MBP deficiency in patients

There was no signficant difference in the frequency of MBP deficiency ($\leq$ 10 ng/ml) in either the Hib meningitis or Hib OM/SA patients when compared to the controls (Figure 10.5).

MBP levels were higher in the infected patient groups and this may be due to the fact that MBP is an acute phase protein. It would have been better to measure MBP levels after the patients had recovered and the acute phase response had settled. It is possible that if this were done additional cases of MBP deficiency as defined in the literature may have been uncovered.

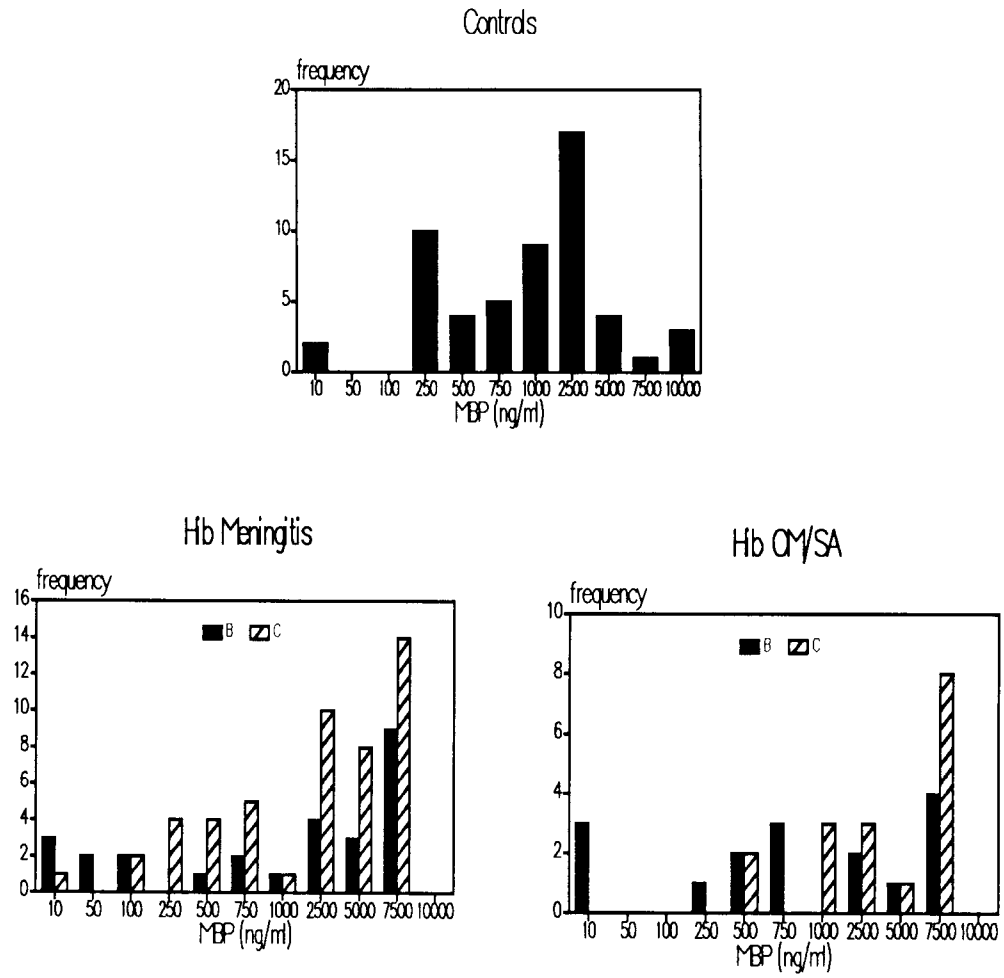


Figure 10.5: Serum MPB concentrations (ng/ml) in Black adult controls and in Black and Coloured patients with Hib meningitis and Hib OM/SA. B = Black patients and C = Coloured patients

10.9 SUMMARY OF MAIN FINDINGS

- Children with Hib infections are younger than those with *S.aureus* infections.
- There is a male predominance in patients with Hib and *S.aureus* OM/SA. This was not seen in Hib meningitis patients.

- Hib meningitis children are no more malnourished than control children.
- Black children with Hib meningitis had lower levels of IgG1, IgG2 and IgG4 than the control children.
- Coloured children with Hib meningitis only had lower IgG4 levels.
- The IgG and IgG subclass levels of the Hib OM/SA patients were no different to the controls.
- Children with *S.aureus* OM/SA had increased levels of IgG, IgG1 and IgG3.
- The IgG or IgG subclass levels did not increase during the illness in patients with Hib meningitis or Hib OM/SA whereas the IgG, IgG1 and IgG2 levels of *S.aureus* OM/SA patients did.
- Children with Hib meningitis or Hib OM/SA had low levels of Hib PRP antibodies and did not respond to Hib infection with a rise of Hib PRP specific antibodies.
- The tetanus toxoid antibody response of the patients was similar to that of child controls.
- IgG1 was the predominant anti-tetanus toxoid antibody.
- In Coloured patients with invasive haemophilus infections there was a decreased frequency of the G2m(n) allotype.
- In both the Coloured and Black Hib meningitis patients there was a decreased frequency of the Km(3) allotype.
- The frequency of G1m(f), G2m(n) and Km(3) allotypes of the *S.aureus* OM/SA patients were not significantly different to the controls.
- The C4A*QO and C4B*QO antigens in the Hib meningitis patients did not differ from the controls.
- MBP deficiency was not increased in the Hib meningitis or Hib OM/SA patients.

SUMMARY

The main objective of the experiments described in Section A of this thesis was to establish reliable and sensitive assays for the measurement of (i) IgG subclasses; (ii) IgG, IgG1 and IgG4 subclass specific antibodies to tetanus toxoid; (iii) IgG, IgG1 and IgG2 subclass specific antibodies to *H.influenzae* type b polyribosylribitol phosphate (Hib PRP); (iv) IgG, IgG1 and IgG2 subclass specific antibodies to *S.aureus* teichoic acid; and (v) G1m(f), G2m(n) and Km(3) allotypes. In Section B these assays were used to test the hypothesis that a lack of, or an impairment in IgG subclass or subclass specific antibody responses to carbohydrate antigens is a risk factor in the pathogenesis of *H.influenzae* type b (Hib) meningitis and Hib and *S.aureus* osteomyelitis/septic arthritis (OM/SA) in children. Invasive infections due to these organisms may be more likely to occur in children with decreased concentrations of IgG subclasses and/or IgG subclass specific antibodies. In addition the antibody responses of these patients may be influenced by host factors such as immunoglobulin allotypes, C4B alleles and mannose binding protein levels.

A solid phase capture non-competitive ELISA system using commercially available monoclonal antibodies was developed to quantitate IgG subclass levels. Each plate routinely included an in-house control as well as a dilution of the World Health Organization's primary reference serum for IgG, IgA and IgM (WHO 67/97). Results obtained with these assays were also evaluated in an international quality assurance scheme for the measurement of IgG subclasses. The sum of the four IgG subclasses measured by ELISA correlated well ($r = 0.89$) with the total IgG measured by nephelometry. These ELISAs were shown to be quantitative, sensitive and reproducible and are highly suitable for measurements in small children because ten microlitres of serum was sufficient to measure all four subclass levels.

The development of sensitive, specific IgG subclass ELISAs enabled measurement of IgG subclass concentrations in normal children from 3 months to 12 years of age. An analysis

of IgG subclass levels must take into account their non-Normal distribution, wide Normal ranges, and ethnic and sex differences. Age related percentile charts from healthy children in the Western Cape were established using the Box Cox transformation to construct smooth age-related percentile charts. IgG and IgG1 subclass levels were found to be higher than those reported from normal children in developed countries. In addition, the proportion of IgG1 of the total IgG in 2 year and 12 year old children was higher (90-95%) than in matched adults (65-75%). These age and race related differences in IgG subclass levels make it imperative to establish local normal values.

Black children with Hib meningitis had lower levels of IgG1, IgG2 and IgG4 than control children whereas in Coloured children only the IgG4 level was decreased. The IgG and IgG subclass levels of the Hib OM/SA patients were no different from the controls whereas children with *S.aureus* OM/SA had increased levels of IgG, IgG1 and IgG3.

Because of adsorption induced denaturation of coated antigen it is important to show that specificity has been maintained. This specificity was demonstrated in all the subclass specific ELISAs developed in this study by using soluble antigen to inhibit antibody binding to the immobilized antigen.

IgG, IgG1 and IgG4 tetanus toxoid specific antibodies were measured by ELISAs. The values of the IgG tetanus toxoid antibodies were calibrated against a commercially available reference standard. Although the assigned IgG1 and IgG4 tetanus toxoid values are arbitrary, they allow for comparison of values between pre- and post-tetanus toxoid immunization and also between different groups of children. All patient groups responded to the tetanus toxoid antigen suggesting normal immunocompetence to protein antigens.

ELISA is widely used to determine antibodies to protein antigens but there have been difficulties in its application to measure antibodies to polysaccharide antigens. In this study a method to measure IgG, IgG1 and IgG2 Hib PRP antibodies is described. These

assays have proved to be simple, specific, sensitive and reproducible. Serum anti-Hib PRP antibodies were measured to evaluate the antibody response to Hib PRP after invasive Hib infections.

Hib PRP antibodies were found to be low in young children with Hib meningitis and Hib OM/SA and did not increase significantly in the 2-3 weeks following the disease. In young children the Hib PRP antibodies were predominantly IgG1 and were not restricted to the IgG2 isotype as suggested from earlier studies. It would be informative to assess whether children with previous Hib disease responded to immunization with Hib vaccine to the same extent as children who had not had Hib disease especially with respect to the isotype and magnitude of the IgG subclass specific response. This may provide information on whether Hib disease arises as a result of a predisposed inability to mount a specific antibody response.

Antibodies to teichoic acid were determined by ELISA in patients with *S.aureus* OM/SA and in patients with OM/SA due to other organisms (who served as controls). The levels of IgG and IgG1 teichoic acid antibodies were significantly higher in patients with *S.aureus* OM/SA but no rise of specific teichoic acid antibody level was seen during *S.aureus* infections. Because variation of teichoic acid antibody levels has been reported in healthy children (Granström et al 1983) it would provide further information if normal concentrations in various age groups were determined. This may add weight to the argument that defective antibody production during invasive staphylococcal disease does not appear to be a major causative factor.

Immunological assays reflect the affinity as well as the concentration of the antibody. A predominantly low affinity antibody response may be considered an expression of immunodeficiency and may be an important factor in susceptibility to infection. Various IgG subclasses may show different affinities for an antigen (Persson et al 1988; Devey et al 1989). Diethylamine ELISAs which are practical and easy to perform were used to assess

antibody affinity. In the tetanus toxoid ELISAs the IgG1 antibody was not only the predominant class of antibody but it also had a higher affinity than the IgG4 anti-tetanus toxoid antibody. The IgG1 and IgG2 Hib PRP antibodies had a similar affinity. Although all patients had low levels of Hib PRP antibody, most had detectable levels of IgG1 Hib PRP antibody but few had detectable IgG2 PRP antibody levels. In the *S.aureus* teichoic acid ELISAs the IgG1 antibody had a higher affinity than the IgG2 antibody. While most patients had IgG1 teichoic acid antibody, very few had measurable levels of IgG2 teichoic acid antibody. No evidence was found for an association between the production of low affinity antibodies and Hib meningitis, Hib OM/SA and *S.aureus* OM/SA.

IgG subclass measurements are assuming an increasing importance in paediatrics. The significance of incorporating the measurement of antibody responses to protein as well as to polysaccharide antigens in the assessment of humoral immunodeficiency is also recognised. The assays developed in this study are suitable for the evaluation of patients referred for investigation of immunodeficiency.

There is evidence that allotypic markers influence IgG subclass levels and have an effect on disease susceptibility. ELISA methods for the quantitation of G1m(f) and G2m(n) allotypes in serum were established and are simple, quick, specific, reproducible and sensitive. The G1m(f) allotype was present in 52% of Coloured children but in only 2.2% of Black children. The G2m(n) allotype occurred in 34.4% of Coloured children and 2.9% of Black children. This difference in allotype frequency in Black and Coloured populations of the Western Cape has not been previously reported. The G1m(f) allotype in the patients with Hib meningitis, Hib OM/SA or *S.aureus* OM/SA did not differ significantly from the controls. There was a significantly decreased frequency of the G2m(n) positive allotype in Coloured patients with Hib meningitis and Hib OM/SA which was not present in patients with *S.aureus* OM/SA. This finding has not been previously reported. In Coloured children the G1m(f) positive allotype was associated with lower IgG and IgG1 levels and higher IgG2 and IgG3 levels whereas G2m(n) positive allotype was

associated with higher IgG2 levels. In Black subjects meaningful statistical analysis was not possible due to the low frequency of these markers.

A qualitative ELISA was established for the Km(3) allotype using a commercially available polyclonal antibody. It clearly discriminated between Km(3) negative and positive individuals except in about 2% of samples which were recorded as 'not typable'. The assay is simple, quick and reproducible. This allotype was very common in both the Coloured (94%) and Black (93.4%) populations. The allotype had no influence on IgG subclass levels, but in both the Coloured and Black children with Hib meningitis there was a significantly decreased frequency of the Km(3) allotype.

There have been several recent reports of an association between C4B deficiency and bacterial meningitis (Rowe et al 1989; Bishof et al 1990). Mannose binding protein deficiency has also been associated with increased infections in children. The frequency of $C4A^{*}Q0$ and $C4B^{*}Q0$ antigen in the Hib meningitis patients did not differ from the controls. MBP deficiency was not found to be increased in Hib meningitis or Hib OM/SA patients.

This study has clearly shown an association between a decrease in frequency of the G2m(n) allotype and Km(3) allotype and susceptibility to invasive infections caused by *H.influenzae*. G2m(n) positive individuals had higher IgG2 levels than G2m(n) negative individuals. The biological and genetic regulation of IgG subclass antibody induction and the roles that allotypic markers play in this process needs further investigation. The role of cytokines in isotype switching would be of particular interest because of their capacity to regulate B-cell terminal differentiation and immunoglobulin production.

APPENDIX A

ANTI-HUMAN IgG MONOCLONAL ANTIBODIES

CLONE NO	IUIS/WHO# STUDY NO	MOUSE ISOTYPE	HUMAN IgG SPECIFICITY	BIOTINY-LATED	CATALOGUE NO
8a4	HP6064	IgG1	IgG-Fc	No	O* MO6014
HP6017	HP6017	IgG_{2a-k}	IgG-Fc	Yes	Z**05-4201
JL512	HP6007	IgG1	IgG1	No	Z**05-0201
NL16	HP6012	IgG1	IgG1	No	O* M15015
HP6069	HP6069	IgG1-k	IgG1-Fc	No	Z** 05-3301
HP6069	HP6069	IgG1-k	IgG1- Fc	Yes	Z** 05-3340
HP6070	HP6070	IgG1	IgG1-Fc	No	Z** 05-3401
SG-16	-	IgG1	IgG1-Fab	No	S*** I-5385
GOM1	HP6008	IgG1	IgG2-Fc	No	O* M10015
GOM2	HP6009	IgG1	IgG2-Fc	No	Z**05-0301
HP6014	HP6014	IgG1-k	IgG2-Fd	No	O* M73013
HP6002	HP6002	IgG1	IgG2-Fc	Yes	Z** 05-3540
SH-21	-	IgG1	IgG2	No	S*** I-7010
ZG4	HP6010	IgG1	IgG3 hinge	No	O* M08010
HP6047	HO6047	IgG1	IgG3 hinge	Yes	Z** 05-3640
GB7B	HP6013	IgG1	IgG4-Fc	No	O* M16013
HP6025	HP6025	IgG1-k	IgG4-Fc	Yes	Z** 05-3840
RJ4	HP6011	IgG1	IgG4-Fc	No	O* M11013

 # Referred to in text by IUIS number only
*** Oxoid ** Zymed *** Sigma**

APPENDIX B

PREPARATION OF *H.INFLUENZAE* TYPE b (Hib) PRP ANTIGEN

Polyribose phosphate (PRP) is a linear polymer of ribose, ribitol and phosphate (ß -D-ribose (1Y) ribitol (5-phosphate)) found in the polysaccharide capsule of Haemophilus influenzae type b. PRP was prepared using a method adapted from Insel and Anderson (1986) as follows:

- Hemin (Sigma Catalogue No H2250)was added to 2 litres brain heart infusion medium (37 g/litre Difco Laboratories) in a Erlanmeyer flask to a final concentration of 1 mg/litre before autoclaving. After autoclaving ß-Nicotanamide Adenine Dinucleotide (Sigma, Catalogue No N-7004) was added to a final concentration of 1 mg/litre. Haemophilus influenzae (type b) previously cultured on a boiled blood agar plate in a CO_2 incubator was used to innoculate the broth.
- The culture was left on a shaker in a 37°C incubator until the late stationary phase of growth had been reached ($\pm$ 30 hours growth)
- The culture was centrifuged twice at 3 500 rpm (2 800 g) for 30 minutes. Unless otherwise stated, all centrifugation steps were performed at these settings.
- The supernatant was added to an equal volume of cold absolute ethanol and left overnight at 4°C.
- This solution was centrifuged in 50 ml conical tubes and the gummy brown deposit dissolved in approximately 100 ml distiled water and left overnight at 4°C.
- Hexadecyltri-methylammonium bromide (Cetavlon, Sigma, Catalogue No H-5882) was added to a final concentration of 0.01 M and left at 4°C for 30 minutes before centrifugation.
- The precipitate was extracted with 1 M NaCl and recentrifuged. The residual precipitate was extracted with 0.25 M NaCl, agitated and centrifuged.
- The supernatant was retained and tested for the presence of carbohydrate (400 μl PRP, 200 μl 5% phenol and 1 ml concentrated sulphuric acid were mixed together and carbohydrate presence was indicated by a strong orange colour).

- An equal volume of 90% phenol was added and stirred at room temperature for 30 minutes before centrifugation.
- The aqueous phase was retained and the phenol extraction was repeated until the interface was clear (usually 3-4 extractions). All phases were checked at this stage for carbohydrate presence.
- PRP was precipitated from the aqueous phase by adding NaCl to 0.1 M and the addition of an equal volume of cold absolute ethanol.
- PRP was dissolved in distilled water after centrifugation.
- The NaCl precipitation was repeated until the absorbance at 260 nm fell to a plateau, indicating removal of residual phenol.
- Final PRP was dissolved in 4 ml distilled water, dialysed against running tap water overnight, and freeze-dried.

Composition of the PRP was assessed by HPLC on derivatives of trifluoro acetic acid (TFA) hydrolysates. This was done by Dr E Merrifield, Department of Medical Biochemistry, University of Cape Town using an aminex HPX-87H (Biorad, Cat No 125.0140) which is on 8% crosslinked cation exchange resin optimized for the analysis of carbohydrates in solution. All laboratory preparations, the gift of PRP from Lederle and a standard of ribose were run on the HPLC. A single peak produced at 7.04 - 7.14 mins was produced by ribose. The peak at 4.2 to 4.4 minutes was produced by TFA.

All preparations were pure with no contaminants.

APPENDIX C

CONJUGATION OF POLY-L-LYSINE TO PRP

To optimise coating of antigen to the plates, the PRP was linked to poly-L-lysine (PLL) (Sigma, Catalogue No P9404) using a method adapted from Gray (1979).

- The freeze-dried PRP was reconstituted in sterile distilled water to a 1 mg/ml solution.
- A small beaker on ice on a magnetic stirrer was set up in a fume cupboard. 750 μl of 1 mg/ml PRP solution was pipetted and a micro pH probe inserted in the solution.
- 300 μl of a 25 mg/ml Cyanogen bromide (Merck, Catalogue No 820193) in 0.1 N NaOH was added and the pH monitored every 2 minutes for 10 minutes. The pH was controlled between 10.5 and 11.5 with the addition of 0.02 N NaOH.
- At 10 minutes 750 μl of a 0.5 mg/ml solution of PLL in 0.5 M $NaHCO_3$ (pH 8.5) containing 0.5 M NaCl was added. Although the pH started to decrease, a few drops of 0.5 M HCl were required to bring the pH down to the desired pH of 8.5.
- The solution was dialysed against several changes of phosphate buffered saline (pH 7.2) at 4°C overnight.
- The resulting volume was measured and the concentration of PRP in mg/ml was calculated and stored in aliquots at -70°C.

APPENDIX D

PREPARATION OF *S.AUREUS* RIBITOL TEICHOIC ACID ANTIGEN

The antigen was prepared from log phase cultures of fresh isolates of *S.aureus* Lafferty strain adopted from the method of White et al 1983. The Lafferty strain of *S.aureus* was obtained from Professor Koornhof (Department of Microbiology, SAIMR, Johannesburg).

A broth culture of *S.aureus* Lafferty strain was grown up in a tryptic soya broth for 24-48 hours at 37°C. The broth was centrifuged at 2 500 rpm for 30 minutes. The supernatant was decanted and the precipitate redissolved in sterile saline and centrifuged (3 000 rpm) for 35 minutes. The sterile saline washes were repeated three times. After the final wash the precipitate was resuspended in saline (5-10 mls). Lysostaphin was added at a concentration of 10 U/ml to the suspension. This was incubated at 37°C for 1½ hours with constant vigorous shaking. Then trypsin (0.1 mg/10 ml of staph suspension) was added. Shaking/agitating at 37°C was continued for a further 30 minutes to inactivate any autolysin as well as any residual trypsin. The suspension was then boiled for 30 minutes. This was followed by sonicating the suspension for 15 minutes (24 μ). The suspension was then spun at 10 000-12 000 rpm for 15-30 minutes. The supernatant was then aliquoted and lypohilized. Reconstituted preparations were stored at -20°C.

The partially purified preparations were characterized by immunodiffusion using Meridian Diagnostic Endo-Staph Kits (see Appendix E).

APPENDIX E

ENDO-STAPH IMMUNODIFFUSION ASSAY

The teichoic acid antibody assay materials and procedure are described in the package insert and are supplied by Meridian Diagnostics, Inc., Cincinnati, Ohio (Catalogue No H290100). The antigen is a partially purified teichoic acid prepared by phenylhydrazine extraction of the Lafferty strain of *S.aureus*. The test antigen used in the assay is standardized to contain 30 mg of teichoic acid per litre, as determined by radial immunodiffusion by reference to the purified ribitol teichoic acid. The positive control serum sample is prepared by immunizing rabbits with this partially purified ribitol teichoic acid. In immunodiffusion, the control serum sample forms a single precipitin line of identity with test antigen and the purified teichoic acid. A 40 μl portion of the purified antigen is placed in the central well, 40 μl of the rabbit anti ribitol teichoic acid (positive control) is placed in each of two of the surrounding wells and 40 μl of the twofold serial dilutions of sera is placed in each of the remaining wells. The plates are incubated at room temperature in 24 h in Parafilm-sealed petridishes. The teichoic acid titre is taken as the highest serum dilution forming a precipitin line of identity with the positive control serum sample.

APPENDIX F

RECOMMENDED ALLOTYPE DESIGNATIONS

(A) Immunoglobulin allotypes currently testable
Recommended designations

Location	Alphameric		Numeric	
IgG1	G1m	(a)	G1m	(1)
		(x)		(2)
		(f)		(3)
		(z)		(17)
IgG2	G2m	(n)	G2m	(23)
IgG3	G3m	(b0)	G3m	(11)
		(b1)		(5)
		(b3)		(13)
		(b4)		(14)
		(b5)		(10)
		(c3)		(6)
		(c5)		(24)
		(g)		(21)
		(s)		(15)
		(t)		(16)
		(u)		(26)
		(v)		(27)
IgA_2	A2m	(1)	A2m	(1)
	A2m	(2)	A2m	(2)
κ-chain	Km	(1)	Km	(1)
		(2)		(2)
		(3)		(3)

(B) Specificities no longer testable due to a lack of reagents

Gm	(r) = 7	R	O3 = 19
	(p) = 9		20 = 20
R	02 = 18		y = 22

Reference:

WHO. Review of the notation for the allotypic and related markers of human Immunoglobulins. Eur J Immunol 1976; 6:599-601.

APPENDIX G

DETERMINATION OF Km ALLOTYPES BY HAEMAGGLUTINATION INHIBITION METHOD

(Ref: Dr G de Lange, CLB Laboratory, Netherlands)

MATERIALS AND METHODS

a. Materials

i. Kappa marker sera: anti-Km(1), anti-D Km(1), anti-Km(3) and anti-D Km(3) obtained from the Central Laboratory of Netherlands (CLB) Amsterdam.

ii. Red blood cells - Group OR_1R_2 or Group OR_2R_2 were obtained from a donor at the Western Province Blood Transfusion Service, Cape Town.

iii. Preservation fluid -

iv. Reference sera - R1117 = Km(1) pos
R1113 = Km(3) pos

b. Method

Antigen coated erythrocytes were prepared from either EDTA blood group OR_2R_2 (cDE/cDE) or blood group OR_1R_2 (CDe/cDE) washed four times in PBS pH 7.2. Cells were coated by incubating one volume of packed washed erythrocytes with four volumes of anti-Rh serum and 4 volumes of PBS at 37°C for 60 minutes. Every 15 minutes the cells were mixed by inverting the tubes. Cells were then washed four times in PBS and stored at 4°C in preservation fluid or PBS.

Coated erythrocytes can be stored at 4°C in PBS, for one week. If the coated erythrocytes are stored in preservation fluid, they can be used 4-6 weeks. Every 2-3 days a fresh 0.1% suspension has to be made.

The working dilution of each allotype-specific antiserum was determined by the antiserum in doubling dilutions from undiluted to 1:512 diluent.

Equal volumes (25 μl) of serum, in the appropriate dilution in PBS and antiserum in the optimal dilutions in PBS were mixed in V shaped microtitre plates followed by a 0.1% suspension in PBS of antigen coated erythrocytes. After mixing again the plates were left overnight at 4°C when they were read macroscopically after tilting the plate for 10 minutes at 60°C.

Results were interpreted as:

No agglutination = inhibition; allotype present

Agglutination = no inhibition; allotype absent.

Controls must include:

1. Antiserum and antigen coated erythrocytes: antigen coated erythrocytes with the diluted antiserum must show sufficiently strong agglutination.
2. Serum and antigen coated erythrocytes: the sample must not show agglutination after incubation with the coated erythrocyte only. This may occur when the sample contains antibodies against the erythrocytes or the antigen used for cell coating.
3. Positive and negative controls: known allotyped samples have to be included in at least the same dilutions as the samples to be tested.

APPENDIX H

A MINIATURISED METHOD FOR IMMUNOGLOBULIN ALLOTYPE SEROLOGY

(Young NT, Street J and Darke C. J Immunol Methods 1989; 122:143-147)

The antigen coated erythrocytes were prepared from a fresh group OR_1R_2 blood sample by the same method mentioned in Appendix G.

The working dilution of each allotype-specific antiserum was determined by titrating the antiserum in doubling dilutions from undiluted to 1:512 in diluent and dispensing a 2 μl aliquot of each dilution into the wells of a plastic 60 conical well Terasaki plate using a Hamilton syringe. 2 μl of antigen coated erythrocyte cell suspension was added to wells and the plates incubated at 22°C for 30 mins. The plates were then centrifuged at 130 x g for 1 minute and placed at an angle of 60° until the cells settled (about 20 minutes). Results were read on an inverted phase microscope at 10 x magnification. The working dilution of each antiserum was defined as the last doubling dilution which gave a definitive positive reaction.

Inhibition of agglutination was determined by testing one antigen positive and one antigen negative serum for each allotype in doubling dilutions from 1:4 to 1:512 in diluent. 2 μl aliquots of the working dilution of each antiserum were added to 2 μl of each dilution of reference serum in the wells of the Terasaki plate and incubated at 22°C for 20-30 minutes. 2 μl of appropriately coated cell suspension were added to each well and the plates incubated, centrifuged and read as before. Inhibition of agglutination by the antigen positive serum and agglutination of cells in the presence of the antigen negative serum indicated that the typing system was suitable for use.

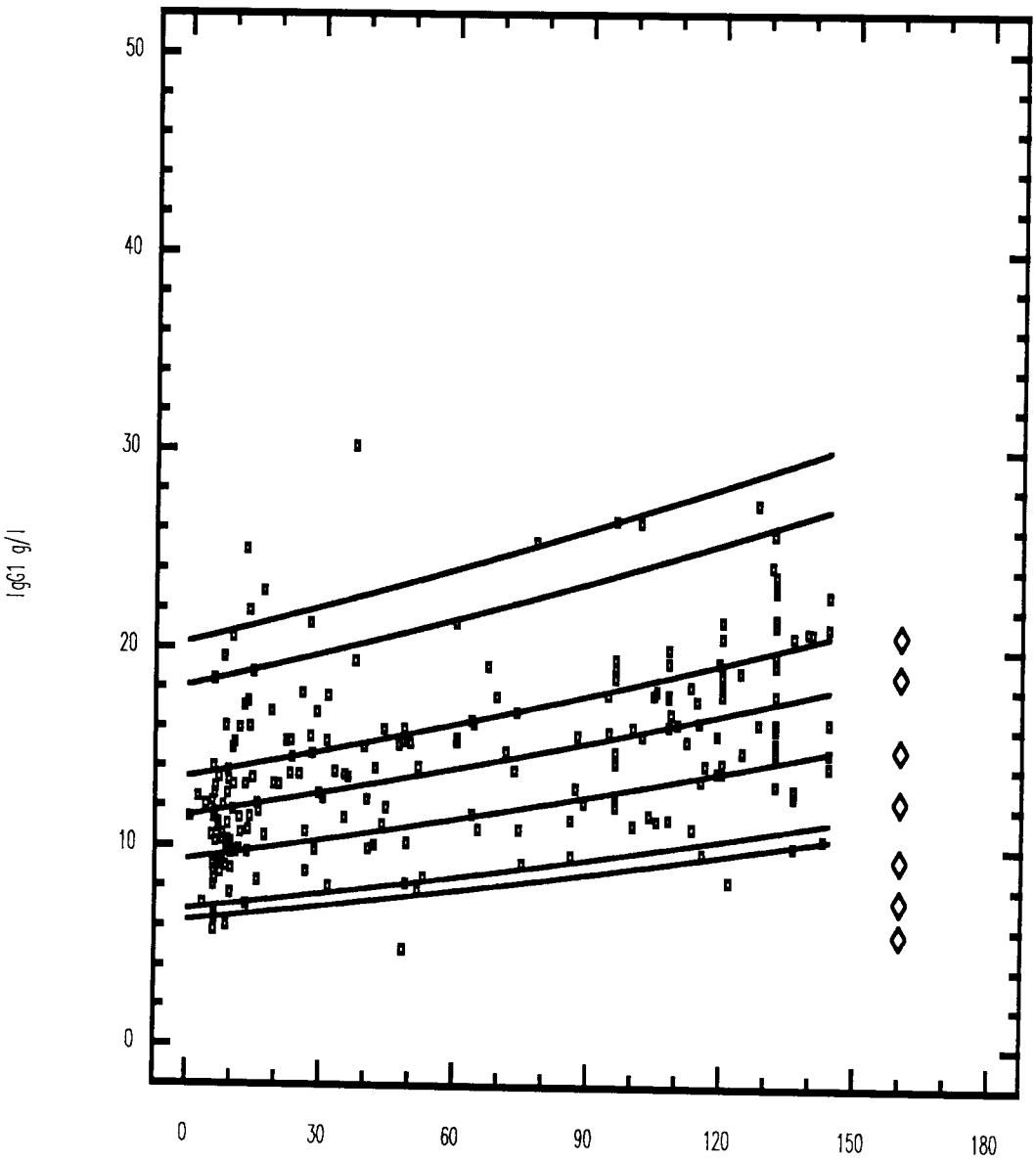
APPENDIX I

NORMAL PERCENTILE CHARTS FOR IgG SUBCLASSES

The following figures are the IgG1, IgG2, IgG3, IgG4 and IgG concentration percentile charts for Black, Coloured male and Coloured female children. The percentile lines shown are 2.5%, 5%, 25%, 50%, 75%, 95% and 97.5%. The equivalent adult percentile points are shown (◇). As there was no significant difference between the IgG2 values of the Coloured male and female children they are shown together on one chart.

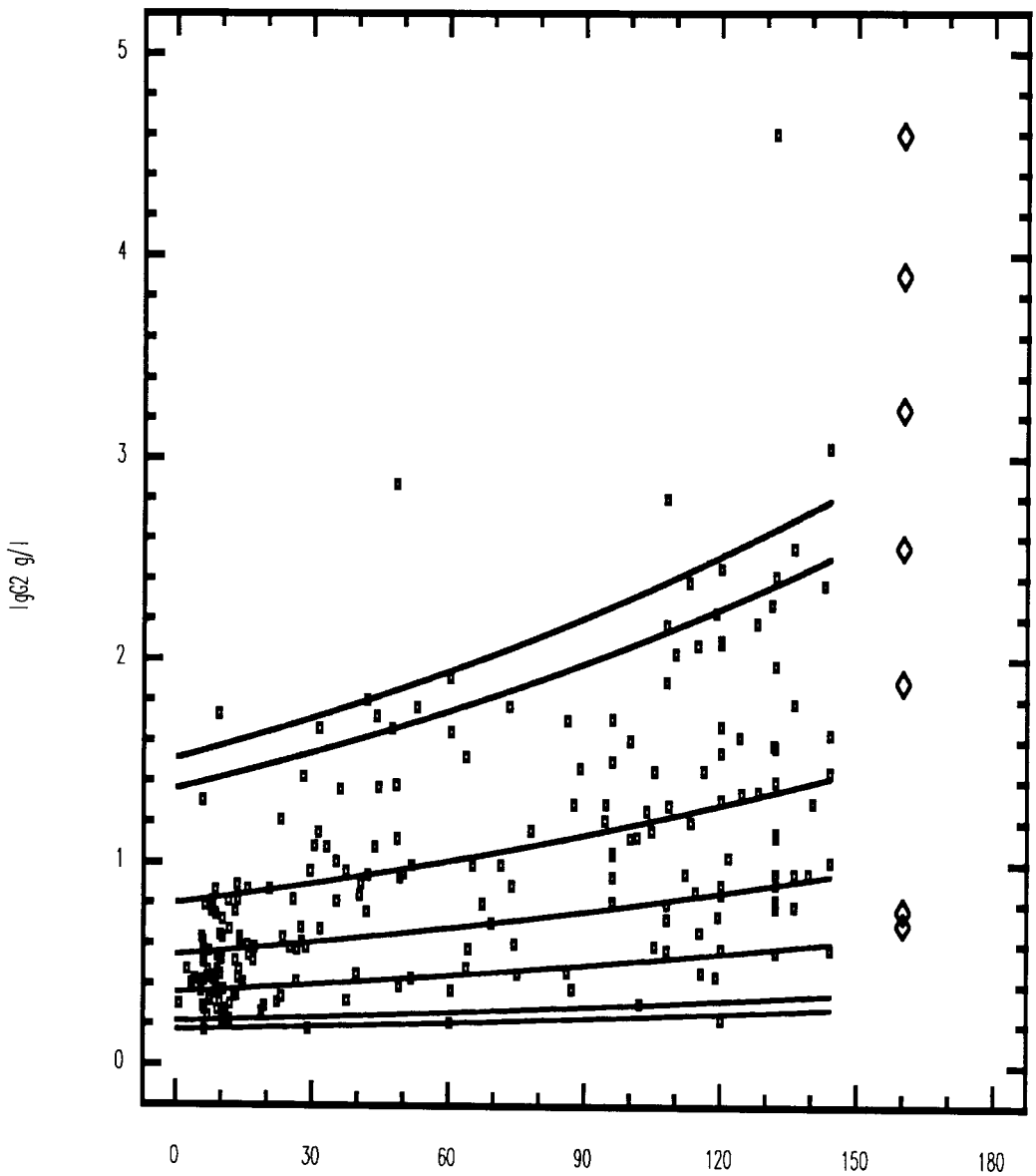
Black Controls

IgG1



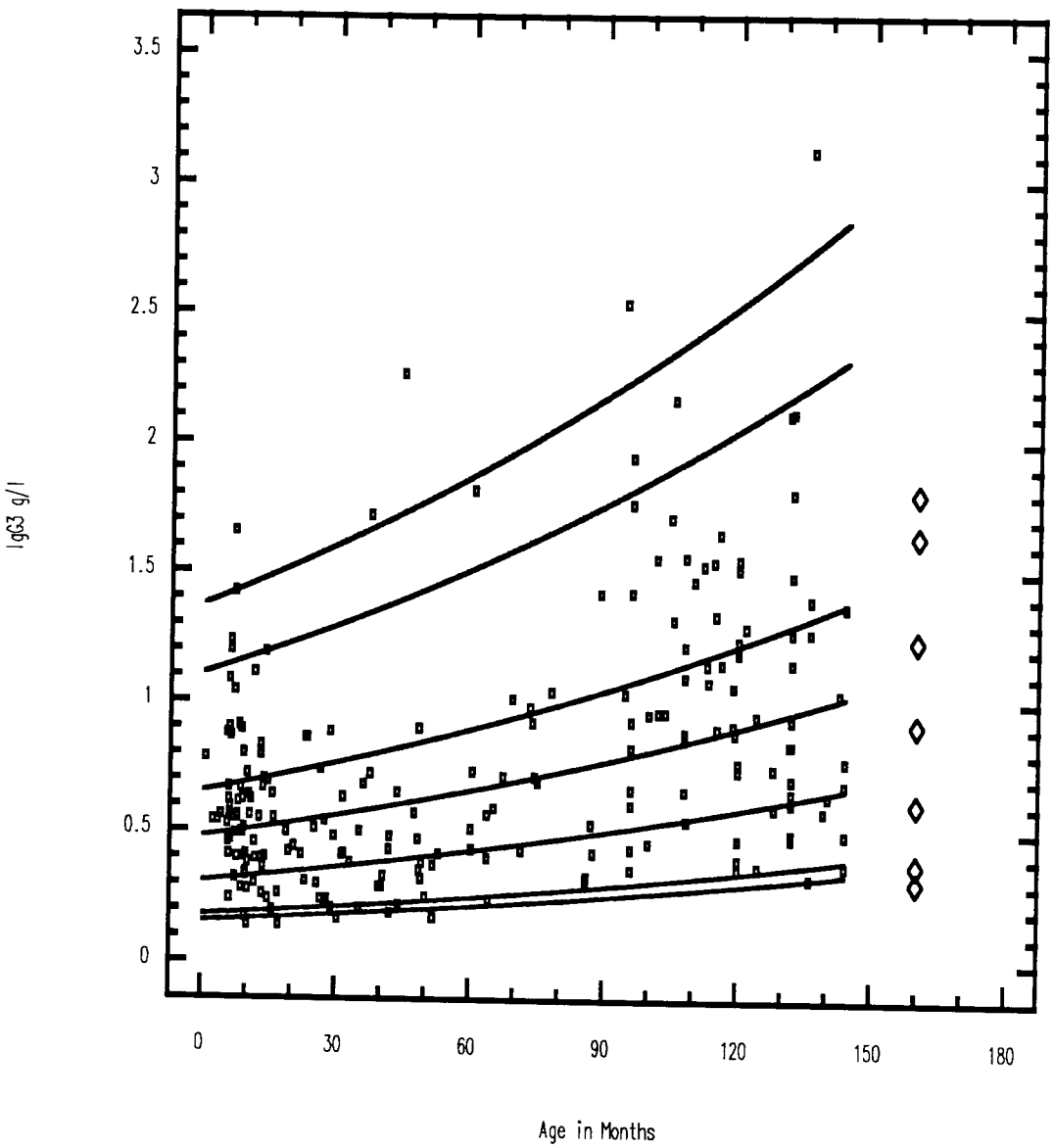
Black Controls

IgG2



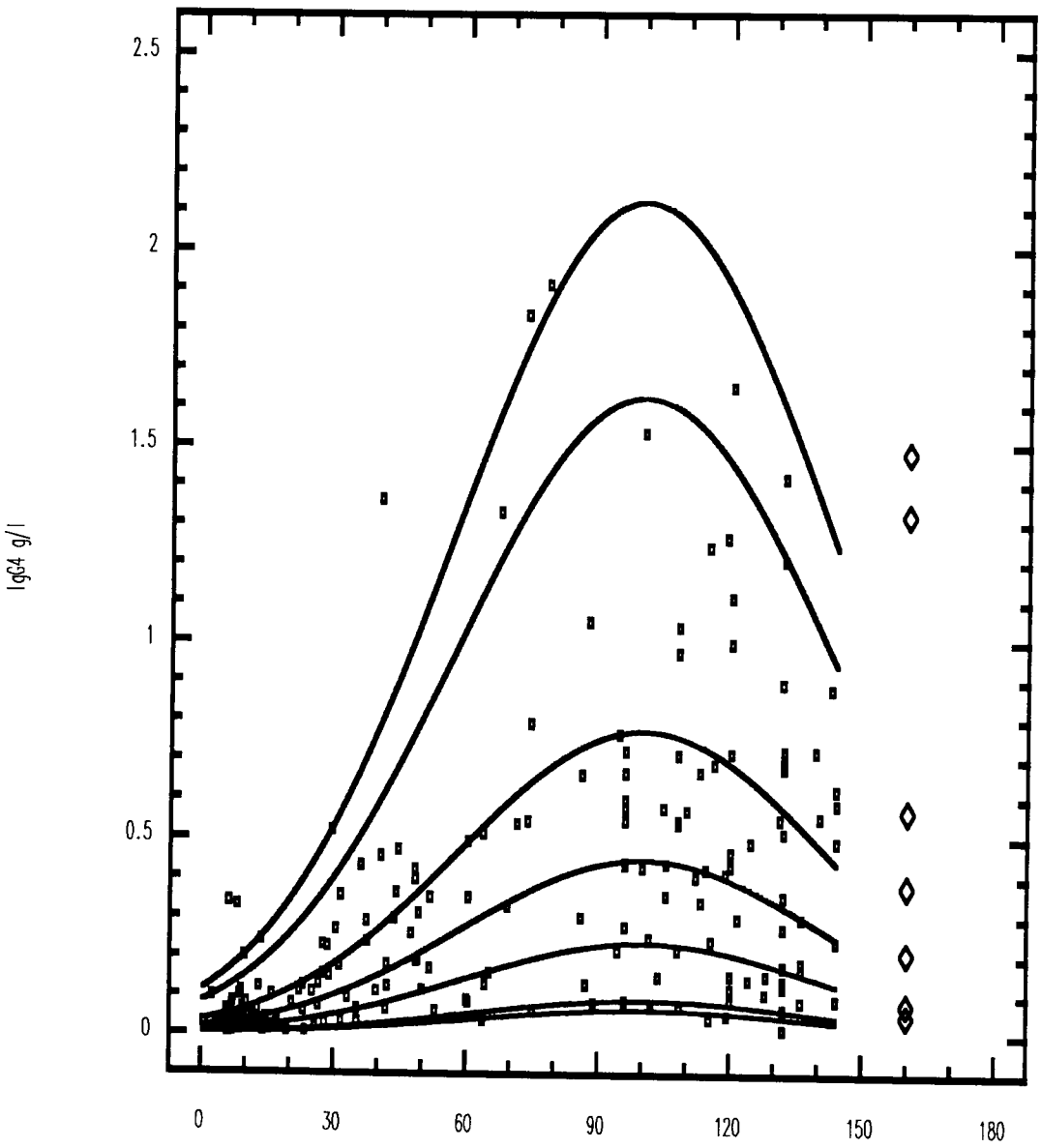
Black Controls

IgG3



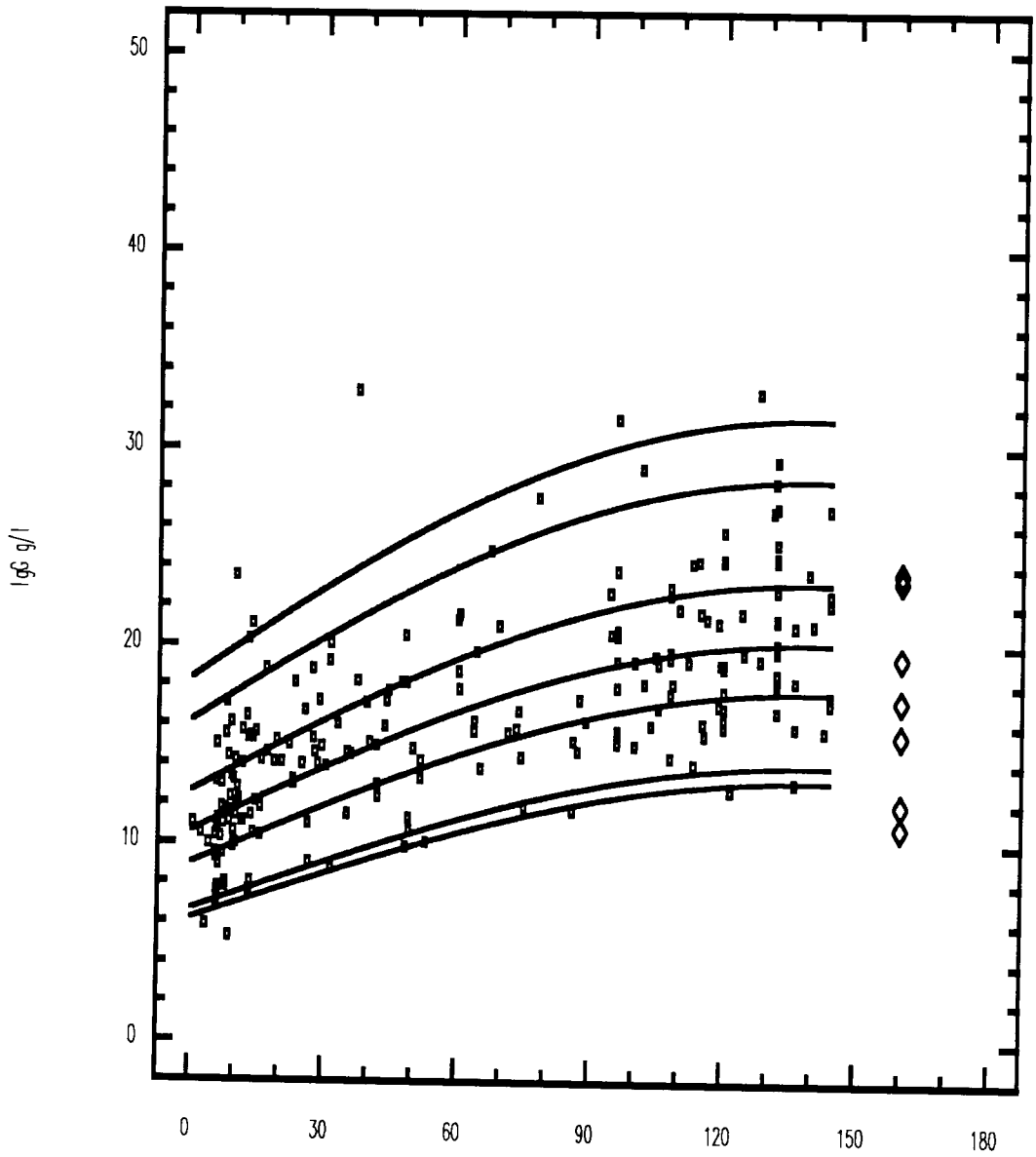
Black Controls

IgG4



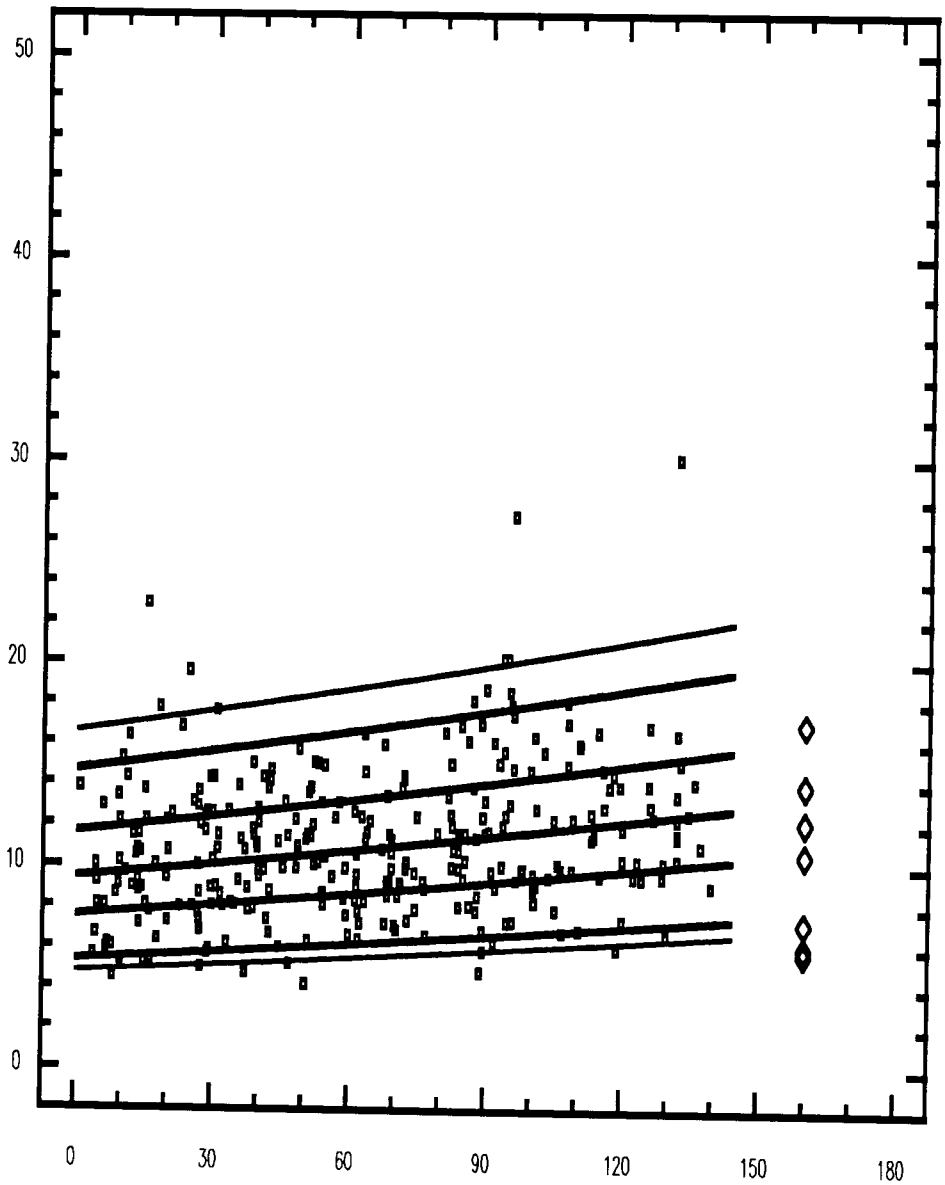
Black Controls

IgG



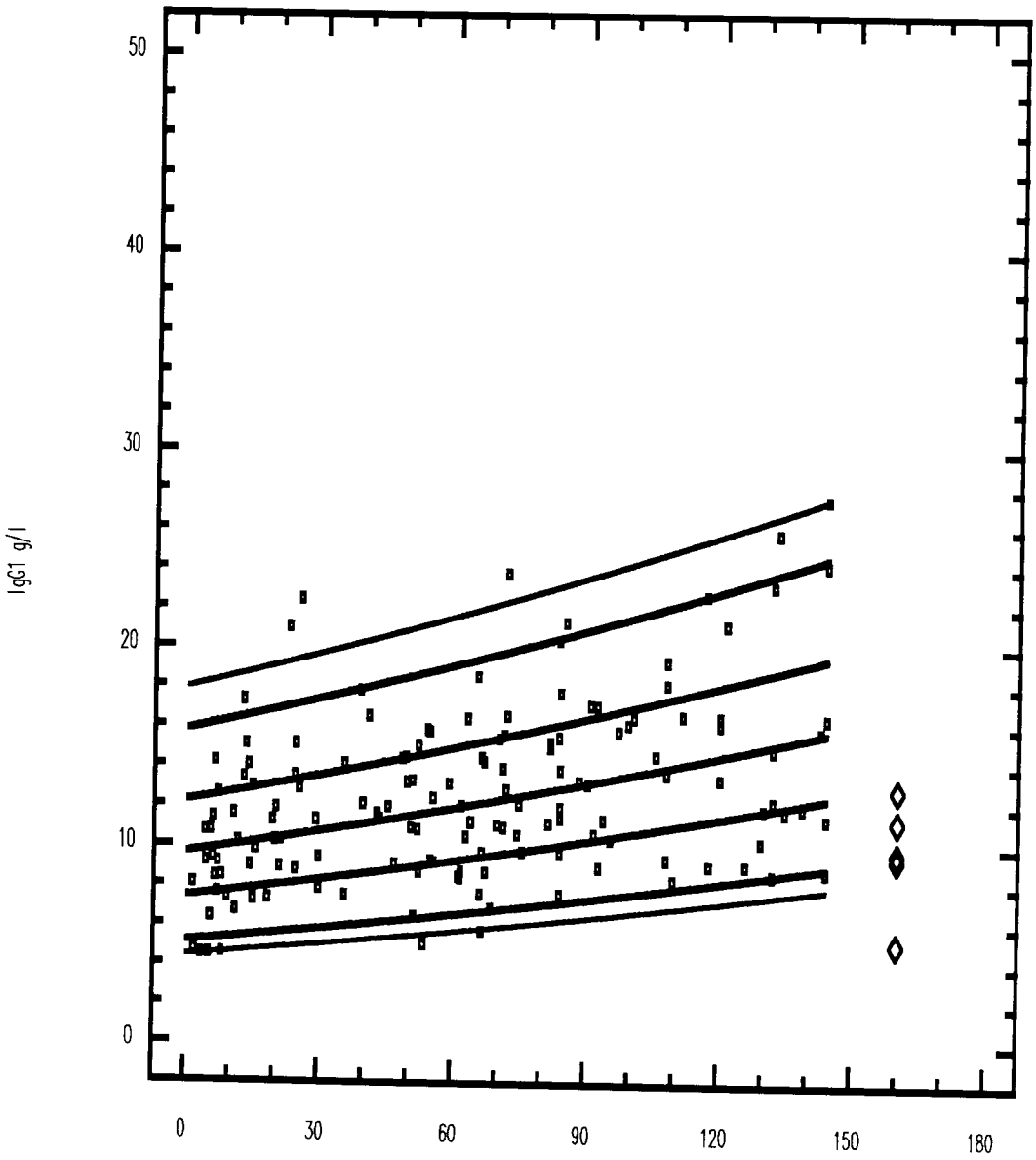
Coloured Male Controls

IgG1



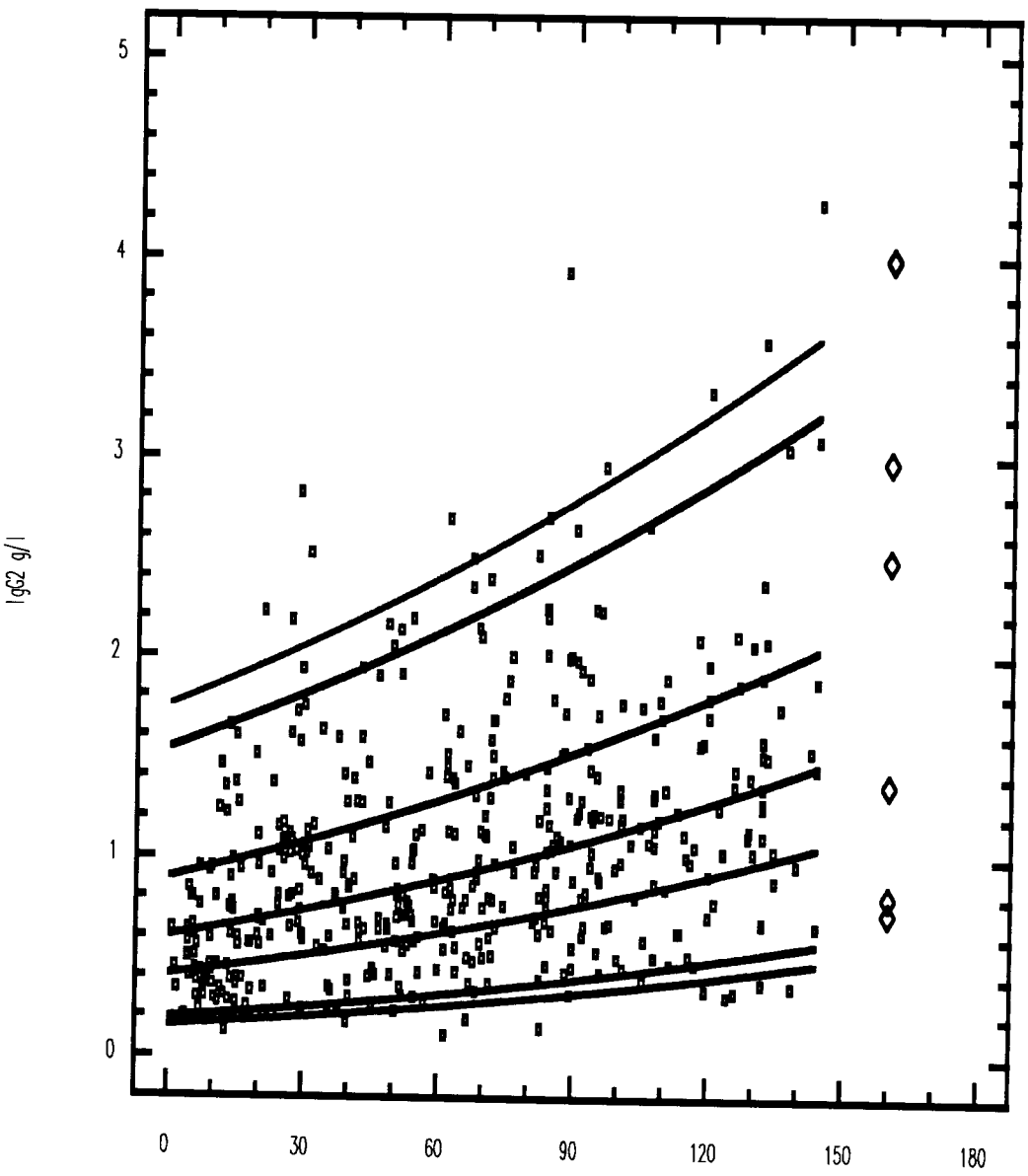
Coloured Female Controls

IgG1



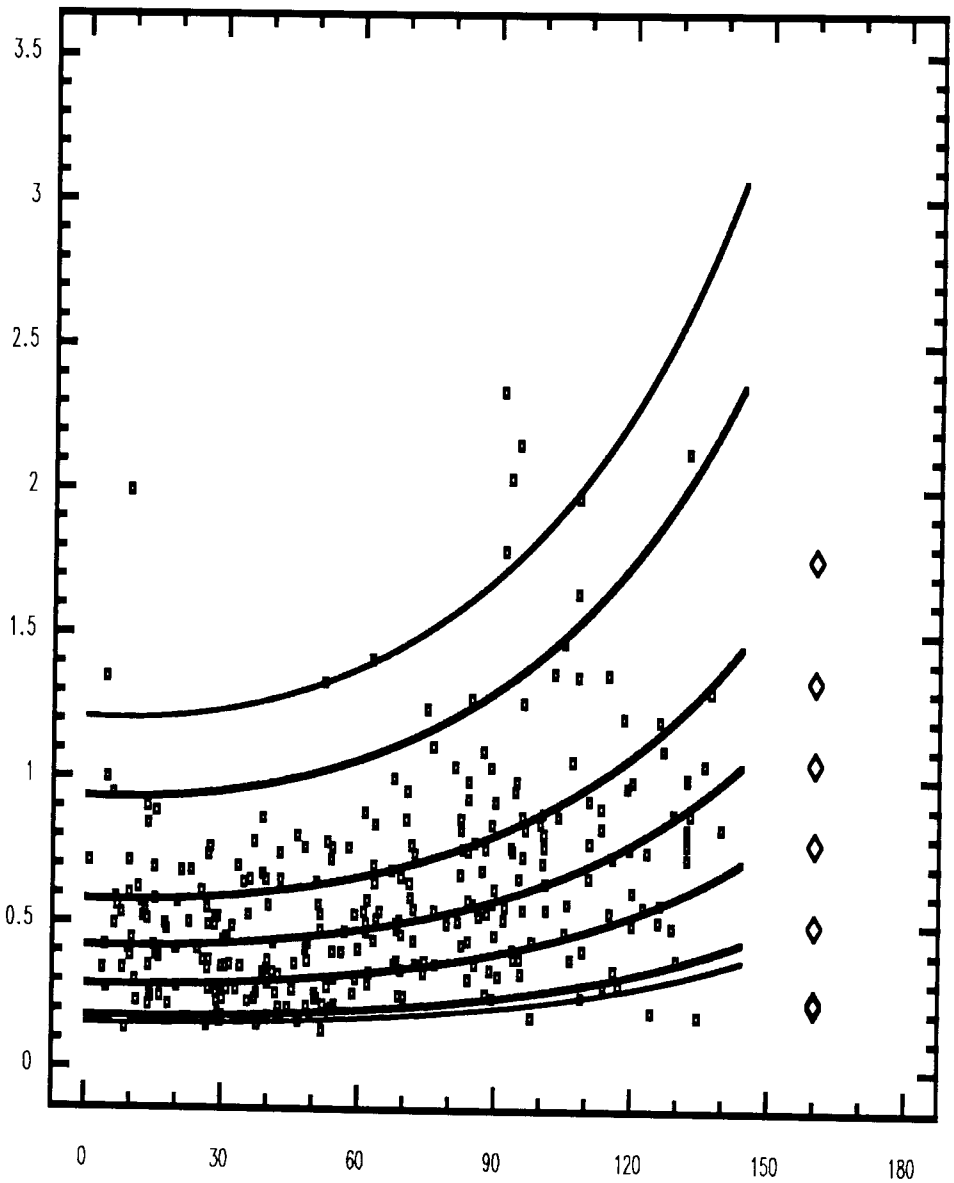
Coloured Male & Female Controls

IgG2



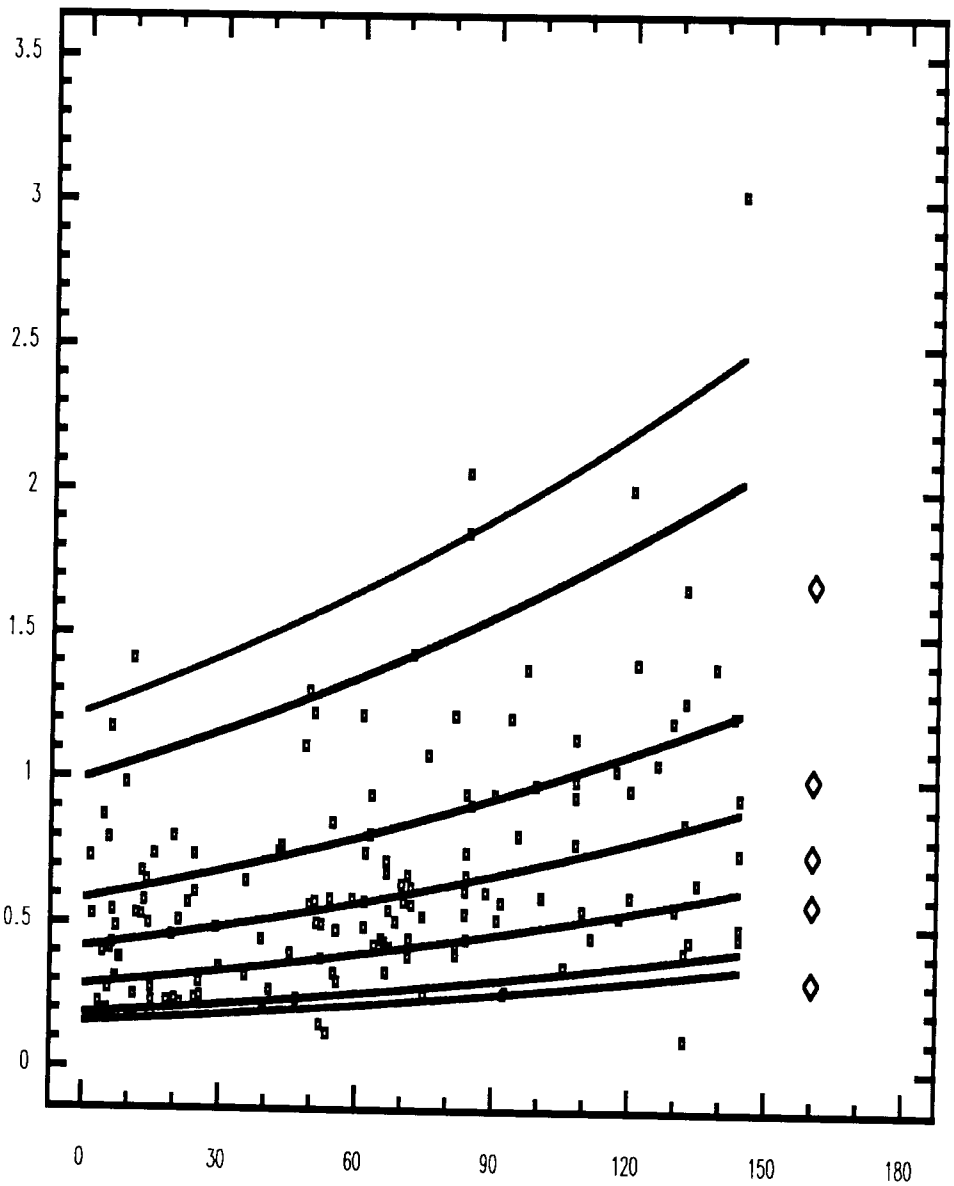
Coloured Male Controls

IgG3



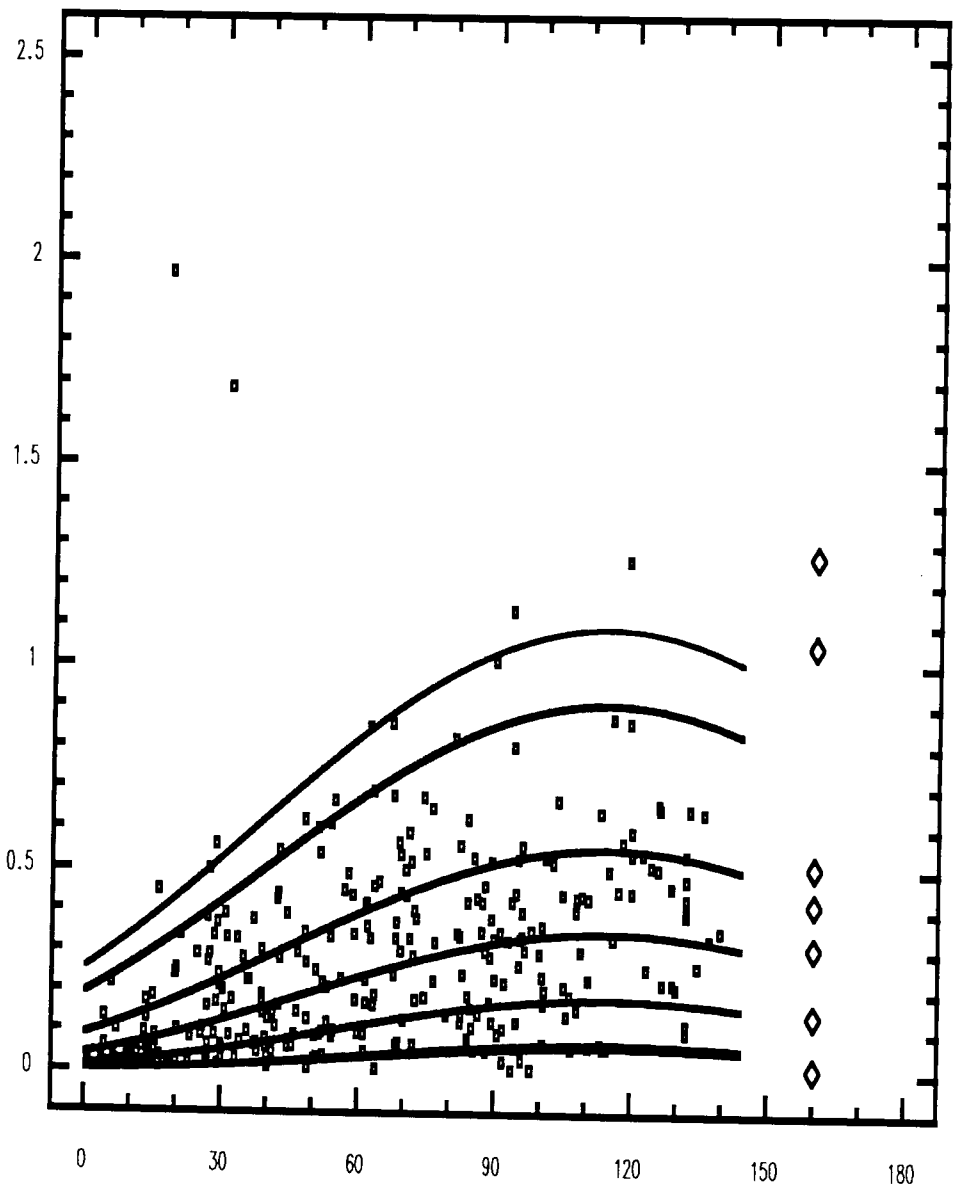
Coloured Female Controls

IgG3



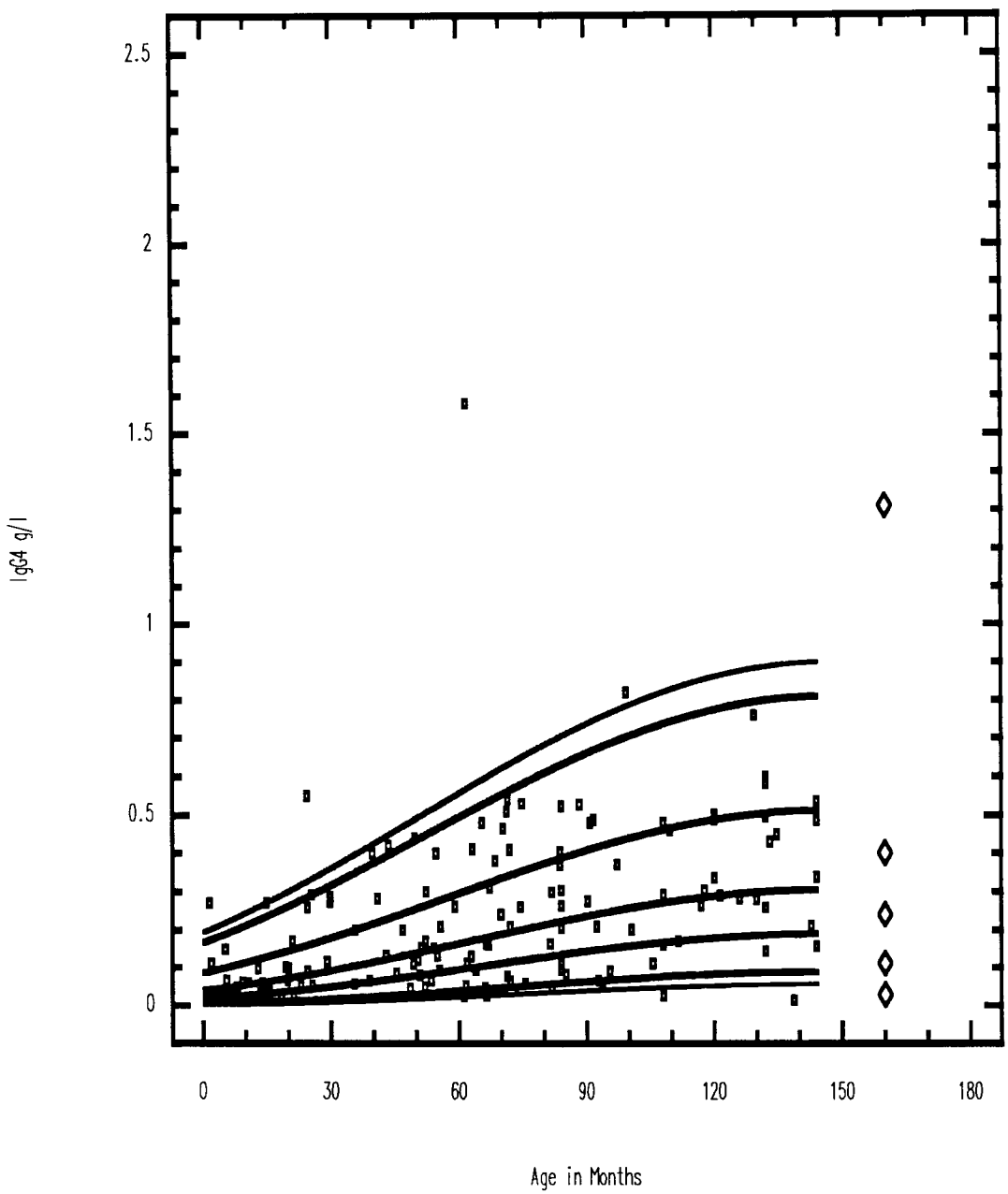
Coloured Male Controls

IgG4



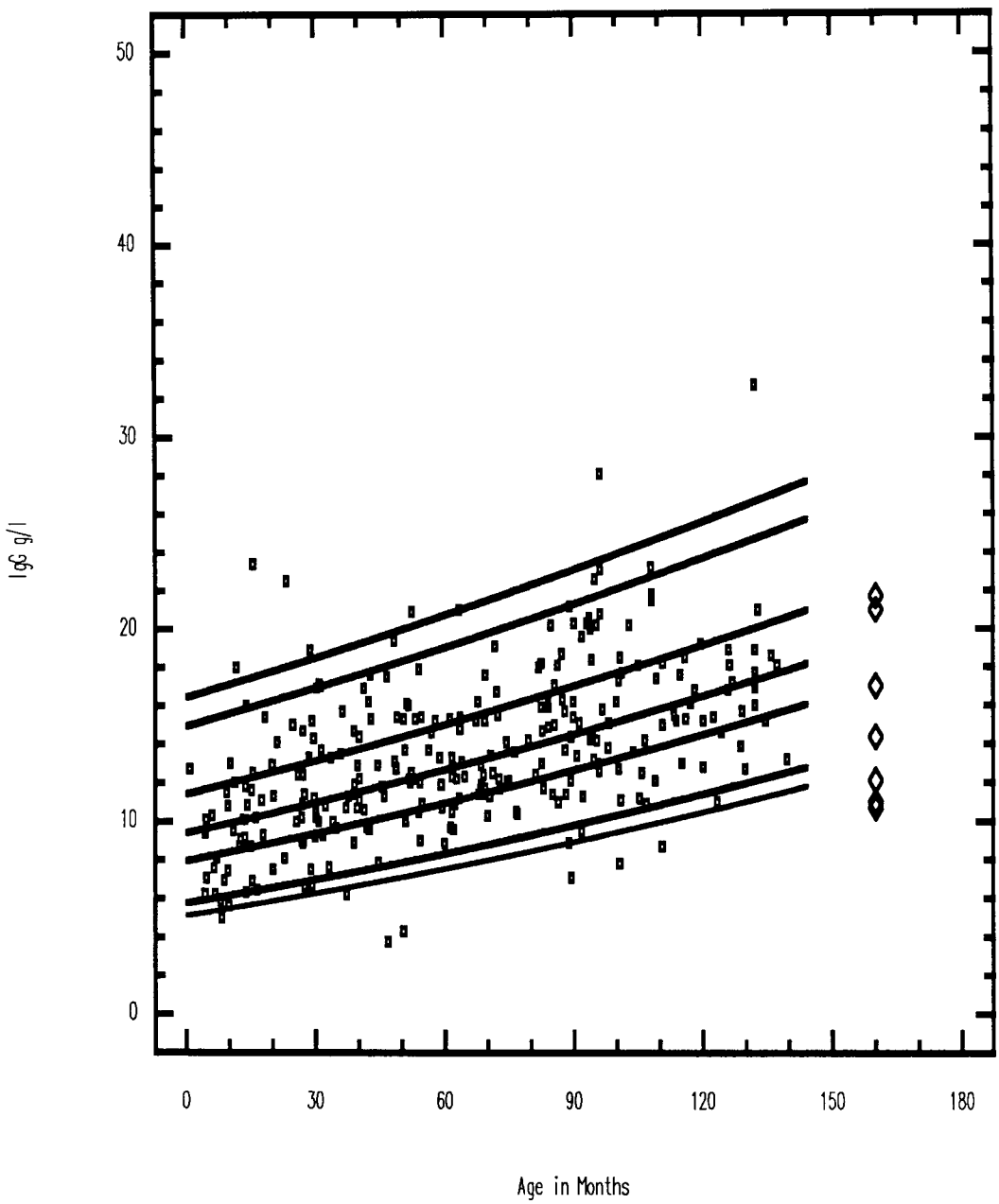
Coloured Female Controls

IgG4



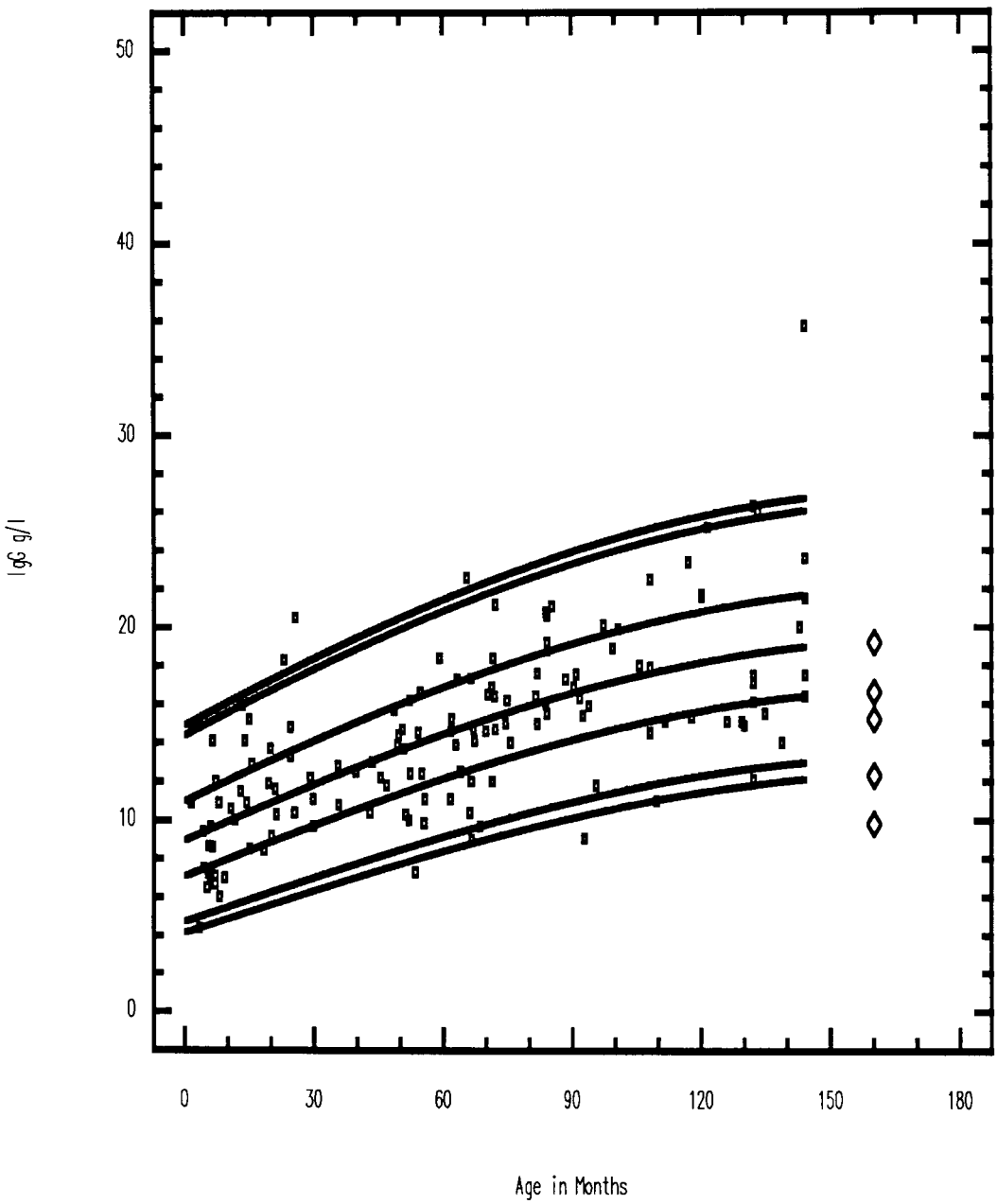
Coloured Male Controls

IgG



Coloured Female Controls

IgG



APPENDIX J

NORMAL RANGES FOR IgG SUBCLASSES

AGE GROUP PERCENTILES IgG : BLACK MALE AND FEMALE CONTROLS

AGE	2.5%	5%	25%	50%	75%	95%	97.5%
6 mths	6.632	7.121	9.547	11.219	13.303	16.977	19.205
1 yr	7.098	7.610	10.138	11.877	14.038	17.839	20.139
2 yrs	8.022	8.577	11.302	13.166	15.475	19.518	21.956
3 yrs	8.919	9.513	12.422	14.403	16.849	21.116	23.681
4 yrs	9.770	10.401	13.478	15.565	18.136	22.607	25.289
5 yrs	10.557	11.221	14.449	16.632	19.315	23.968	26.753
6 yrs	11.265	11.957	15.317	17.584	20.365	25.178	28.052
7 yrs	11.878	12.594	16.067	18.405	21.268	26.216	29.167
8 yrs	12.384	13.121	16.684	19.080	22.011	27.067	30.080
9 yrs	12.774	13.526	17.158	19.597	22.579	27.719	30.778
10 yrs	13.039	13.801	17.480	19.949	22.964	28.160	31.251
11 yrs	13.174	13.941	17.644	20.127	23.160	28.384	31.491
12 yrs	13.176	13.943	17.646	20.130	23.163	28.387	31.495
Adults	10.900	12.000	15.500	17.250	19.400	23.300	23.700

AGE GROUP PERCENTILES IgG1 : BLACK MALE AND FEMALE CONTROLS

AGE	2.5%	5%	25%	50%	75%	95%	97.5%
6 mths	6.422	6.997	9.521	11.734	13.712	18.411	20.641
1 yr	6.568	7.153	9.717	11.964	13.971	18.734	20.995
2 yrs	6.867	7.472	10.199	12.434	14.500	19.395	21.715
3 yrs	7.176	7.801	10.534	12.919	15.044	20.073	22.453
4 yrs	7.496	8.142	10.961	13.417	15.603	20.769	23.211
5 yrs	7.826	8.494	11.402	13.931	16.178	21.483	23.988
6 yrs	8.167	8.857	11.855	14.458	16.770	22.216	24.784
7 yrs	8.520	9.231	12.323	15.001	17.377	22.967	25.601
8 yrs	8.884	9.618	12.804	15.560	18.001	23.738	26.437
9 yrs	9.259	10.017	13.299	16.134	18.642	24.528	27.294
10 yrs	9.647	10.428	13.809	16.724	19.300	25.338	28.173
11 yrs	10.047	10.852	14.333	17.330	19.976	26.168	29.072
12 yrs	10.459	11.289	14.872	17.953	20.669	27.019	29.994
Adults	5.730	7.420	9.460	12.350	14.960	18.680	20.730

AGE GROUP PERCENTILES IgG2 : BLACK MALE AND FEMALE CONTROLS

AGE	2.5%	5%	25%	50%	75%	95%	97.5%
6 mths	0.201	0.219	0.375	0.568	0.811	1.258	1.404
1 yr	0.211	0.230	0.392	0.593	0.846	1.311	1.462
2 yrs	0.231	0.252	0.428	0.646	0.919	1.421	1.584
3 yrs	0.253	0.276	0.467	0.703	0.999	1.540	1.716
4 yrs	0.278	0.302	0.510	0.765	1.085	1.669	1.858
5 yrs	0.304	0.330	0.556	0.832	1.178	1.807	2.001
6 yrs	0.332	0.361	0.606	0.905	1.278	1.957	2.175
7 yrs	0.363	0.395	0.660	0.984	1.386	2.117	2.352
8 yrs	0.397	0.431	0.718	1.068	1.503	2.289	2.543
9 yrs	0.434	0.471	0.782	1.160	1.628	2.475	2.747
10 yrs	0.473	0.514	0.850	1.259	1.764	2.675	2.967
11 yrs	0.516	0.560	0.924	1.365	1.909	2.889	3.203
12 yrs	0.563	0.610	1.004	1.480	2.066	3.119	3.456
Adults	0.700	0.770	1.888	2.553	3.240	3.900	4.593

AGE GROUP PERCENTILES IgG3 : BLACK MALE AND FEMALE CONTROLS

AGE	2.5%	5%	25%	50%	75%	95%	97.5%
6 mths	0.159	0.184	0.317	0.492	0.671	1.141	1.415
1 yr	0.164	0.191	0.328	0.508	0.693	1.177	1.459
2 yrs	0.176	0.204	0.350	0.542	0.738	0.253	1.552
3 yrs	0.188	0.218	0.374	0.578	0.787	1.333	1.650
4 yrs	0.201	0.233	0.399	0.616	0.838	1.418	1.755
5 yrs	0.215	0.249	0.426	0.657	0.893	1.508	1.866
6 yrs	0.230	0.266	0.454	0.700	0.951	1.604	1.983
7 yrs	0.246	0.284	0.484	0.746	1.012	1.706	2.108
8 yrs	0.262	0.303	0.517	0.795	1.078	1.814	2.240
9 yrs	0.280	0.324	0.551	0.847	1.147	1.928	2.380
10 yrs	0.300	0.346	0.588	0.902	1.221	2.050	2.529
11 yrs	0.320	0.369	0.626	0.961	1.299	2.178	2.686
12 yrs	0.342	0.394	0.668	1.023	1.382	2.315	2.853
Adults	0.314	0.380	0.610	0.915	1.239	1.640	1.804

AGE GROUP PERCENTILES IgG4 : BLACK MALE AND FEMALE CONTROLS

AGE	2.5%	5%	25%	50%	75%	95%	97.5%
6 mths	0.002	0.004	0.013	0.027	0.051	0.119	0.162
1 yr	0.004	0.006	0.019	0.039	0.073	0.168	0.227
2 yrs	0.009	0.012	0.037	0.075	0.136	0.307	0.411
3 yrs	0.016	0.023	0.065	0.128	0.229	0.506	0.672
4 yrs	0.026	0.037	0.101	0.198	0.350	0.758	1.001
5 yrs	0.038	0.053	0.144	0.277	0.485	1.038	1.365
6 yrs	0.050	0.069	0.185	0.354	0.615	1.306	1.712
7 yrs	0.059	0.082	0.218	0.414	0.717	1.512	1.979
8 yrs	0.064	0.089	0.234	0.444	0.768	1.616	2.112
9 yrs	0.063	0.087	0.231	0.438	0.757	1.593	2.083
10 yrs	0.057	0.078	0.208	0.396	0.686	1.450	1.899
11 yrs	0.046	0.064	0.171	0.328	0.572	1.217	1.597
12 yrs	0.034	0.047	0.129	0.249	0.437	0.940	1.237
Adults	0.050	0.080	0.210	0.380	0.568	1.320	1.480

AGE GROUP PERCENTILES IgG : COLOURED MALE CONTROLS

AGE	2.5%	5%	25%	50%	75%	95%	97.5%
6 mths	5.328	5.984	8.224	9.713	11.754	15.323	16.8334
1 yr	5.554	6.224	8.507	10.023	12.097	15.717	17.248
2 yrs	6.020	6.719	9.090	10.658	12.797	16.520	18.091
3 yrs	6.507	7.235	9.694	11.314	13.519	17.345	18.956
4 yrs	7.014	7.771	10.318	11.990	14.262	18.191	19.843
5 yrs	7.541	8.327	10.963	12.688	15.025	19.059	20.751
6 yrs	8.089	8.903	11.628	13.406	15.810	19.948	21.680
7 yrs	8.657	9.500	12.314	14.145	16.616	20.858	22.631
8 yrs	9.245	10.118	13.022	14.906	17.443	21.790	23.604
9 yrs	9.854	10.756	13.750	15.687	18.292	22.744	24.598
10 yrs	10.483	11.415	14.499	16.490	19.162	23.179	25.615
11 yrs	11.133	12.094	15.269	17.314	20.053	24.716	26.653
12 yrs	11.804	12.795	16.061	18.159	20.966	25.735	27.713
Adults	10.700	11.000	12.100	14.350	17.000	21.000	21.700

AGE GROUP PERCENTILES IgG : COLOURED FEMALE CONTROLS

AGE	2.5%	5%	25%	50%	75%	95%	97.5%
6 mths	4.582	5.179	5.629	9.554	11.633	15.101	15.626
1 yr	5.025	5.644	8.169	10.144	12.270	15.808	16.343
2 yrs	5.907	6.565	9.231	11.299	13.513	17.180	17.733
3 yrs	6.772	7.466	10.258	12.409	14.704	18.488	19.057
4 yrs	7.606	8.332	11.239	13.465	15.833	19.722	20.306
5 yrs	8.399	9.154	12.162	14.456	16.889	20.873	21.470
6 yrs	9.141	9.921	13.019	15.374	17.864	21.933	22.542
7 yrs	9.823	10.626	13.803	16.210	18.751	22.895	23.515
8 yrs	10.438	11.259	14.505	16.959	19.544	23.753	24.382
9 yrs	10.978	11.816	15.121	17.614	20.237	24.501	25.138
10 yrs	11.440	12.291	15.644	18.170	20.824	25.135	25.778
11 yrs	11.817	12.679	16.072	18.623	21.303	25.651	26.299
12 yrs	12.107	12.978	16.399	18.971	21.669	26.046	26.698
Adults	9.810	9.810	12.300	15.200	16.600	19.200	19.200

AGE GROUP PERCENTILES IgG1 : COLOURED MALE CONTROLS

AGE	2.5%	5%	25%	50%	75%	95%	97.5%
6 mths	4.812	5.411	7.641	9.544	11.754	14.800	16.739
1 yr	4.882	5.489	7.746	9.670	11.905	14.984	16.944
2 yrs	5.025	5.647	7.958	9.927	12.212	15.358	17.359
3 yrs	5.172	5.809	8.175	10.190	12.526	15.740	17.784
4 yrs	5.322	5.975	8.398	10.459	12.847	16.130	18.217
5 yrs	5.476	6.145	8.625	10.733	13.175	16.529	18.660
6 yrs	5.633	6.319	8.858	11.014	13.510	16.936	19.111
7 yrs	5.795	6.497	9.096	11.302	13.852	17.351	19.572
8 yrs	5.960	6.679	9.340	11.595	14.202	17.776	20.043
9 yrs	6.130	6.866	9.589	11.895	14.559	18.209	20.523
10 yrs	6.304	7.058	9.844	12.202	14.924	18.651	21.013
11 yrs	6.481	7.254	10.105	12.516	15.297	19.103	21.513
12 yrs	6.664	7.455	10.372	12.836	15.678	19.564	22.024
Adults	5.710	6.060	7.290	10.640	12.200	13.980	16.970

AGE GROUP PERCENTILES IgG1 : COLOURED FEMALE CONTROLS

AGE	2.5%	5%	25%	50%	75%	95%	97.5%
6 mths	4.527	5.241	7.558	9.830	12.453	16.077	18.238
1 yr	4.645	5.374	7.733	10.044	12.711	16.391	18.584
2 yrs	4.889	5.646	8.094	10.485	13.238	17.032	19.290
3 yrs	5.143	5.930	8.467	10.940	13.782	17.692	20.016
4 yrs	5.407	6.224	8.853	11.410	14.334	18.371	20.763
5 yrs	5.681	6.529	9.253	11.896	14.922	19.071	21.531
6 yrs	5.965	6.846	9.667	12.397	15.519	19.791	22.321
7 yrs	6.261	7.174	10.094	12.914	16.133	20.531	23.133
8 yrs	6.567	7.514	10.536	13.448	16.766	21.293	23.968
9 yrs	6.885	7.867	10.993	13.999	17.419	22.076	24.826
10 yrs	7.215	8.232	11.465	14.567	18.090	22.882	25.707
11 yrs	7.557	8.610	11.952	15.152	18.781	23.710	26.612
12 yrs	7.911	9.002	12.456	15.756	19.493	24.560	27.541
Adults	5.167	5.167	9.450	9.740	11.340	12.912	12.912

AGE GROUP PERCENTILES IgG2 : COLOURED MALE AND FEMALE CONTROLS

AGE	2.5%	5%	25%	50%	75%	95%	97.5%
6 mths	0.158	0.200	0.425	0.627	0.930	1.587	1.808
1 yr	0.167	0.210	0.444	0.652	0.965	1.640	1.867
2 yrs	0.185	0.232	0.484	0.706	1.038	1.751	1.989
3 yrs	0.205	0.256	0.526	0.763	1.115	1.867	2.118
4 yrs	0.227	0.282	0.572	0.824	1.197	1.989	2.253
5 yrs	0.251	0.310	0.620	0.888	1.283	2.118	2.395
6 yrs	0.276	0.340	0.672	0.956	1.374	2.253	2.543
7 yrs	O.303	0.372	0.726	1.029	1.470	2.394	2.699
8 yrs	0.333	0.406	0.785	1.105	1.572	2.543	2.863
9 yrs	0.365	0.444	0.847	1.186	1.678	2.699	3.034
10 yrs	0.399	O.483	0.913	1.272	1.791	2.862	3.213
11 yrs	0.435	0.526	0.982	1.363	1.909	3.033	3.400
12 yrs	0.474	0.571	1.056	1.458	2.034	3.212	3.595
Adults	0.730	0.810	1.368	2.488	2.980	3.993	4.000

AGE GROUP PERCENTILES IgG3 : COLOURED MALE CONTROLS

AGE	2.5%	5%	25%	50%	75%	95%	97.5%
6 mths	0.151	0.176	0.284	0.416	0.576	0.929	1.205
1 yr	0.151	0.176	0.285	0.416	0.577	0.930	1.206
2 yrs	0.153	0.178	0.288	0.421	0.584	0.941	1.220
3 yrs	0.157	0.183	0.296	0.433	0.599	0.966	1.253
4 yrs	0.163	0.190	0.308	0.450	0.624	1.006	1.305
5 yrs	0.172	0.201	0.325	0.476	0.659	1.064	1.380
6 yrs	0.185	0.215	0.348	0.510	0.707	1.141	1.480
7 yrs	0.201	0.234	0.379	0.555	0.769	1.241	1.610
8 yrs	0.221	0.258	0.418	0.612	0.848	1.370	1.778
9 yrs	0.247	0.288	0.467	0.685	0.950	1.534	1.992
10 yrs	0.281	0.327	0.530	0.777	1.079	1.744	2.265
11 yrs	0.323	0.376	0.611	0.896	1.243	2.012	2.614
12 yrs	0.377	0.439	0.714	1.047	1.454	2.355	3.061
Adults	0.230	0.240	0.500	0.780	1.057	1.338	1.758

AGE GROUP PERCENTILES IgG3 : COLOURED FEMALE CONTROLS

AGE	2.5%	5%	25%	50%	75%	95%	97.5%
6 mths	0.158	0.189	0.293	0.429	0.602	1.026	1.258
1 yr	0.164	0.196	0.303	0.443	0.622	1.057	1.297
2 yrs	0.176	0.210	0.324	0.473	0.662	1.124	1.377
3 yrs	0.189	0.225	0.346	0.505	0.706	1.194	1.461
4 yrs	0.202	0.241	0.370	0.538	0.751	1.268	1.550
5 yrs	0.217	0.258	0.395	0.574	0.800	1.346	1.644
6 yrs	0.233	0.277	0.422	0.612	0.851	1.429	1.744
7 yrs	0.249	0.296	0.451	0.652	0.905	1.516	1.849
8 yrs	0.267	0.317	0.481	0.694	0.963	1.609	1.959
9 yrs	0.285	0.338	0.513	0.740	1.024	1.706	2.076
10 yrs	0.305	0.362	0.547	0.787	1.088	1.809	2.199
11 yrs	0.327	0.387	0.583	0.838	1.156	1.917	2.329
12 yrs	0.349	0.413	0.622	0.891	1.228	2.032	2.466
Adults	0.310	0.310	0.580	0.750	1.005	1.680	1.680

AGE GROUP PERCENTILES IgG4 : MALE COLOURED CONTROLS

AGE	2.5%	5%	25%	50%	75%	95%	97.5%
6 mths	0.001	0.002	0.017	0.053	0.111	0.231	0.301
1 yr	0.003	0.004	0.024	0.068	0.137	0.274	0.353
2 yrs	0.007	0.009	0.042	0.104	0.195	0.368	0.467
3 yrs	0.013	0.017	0.063	0.145	0.259	0.469	0.586
4 yrs	0.022	0.027	0.088	0.189	0.326	0.571	0.706
5 yrs	0.031	0.038	0.113	0.233	0.390	0.668	0.819
6 yrs	0.041	0.049	0.137	0.273	0.449	0.754	0.918
7 yrs	0.049	0.059	0.157	0.306	0.496	0.824	0.999
8 yrs	0.055	0.066	0.171	0.330	0.531	0.874	1.056
9 yrs	0.059	0.070	0.179	0.343	0.549	0.900	1.086
10 yrs	0.059	0.070	0.180	0.344	0.550	0.901	1.088
11 yrs	0.056	0.066	0.173	0.332	0.533	0.877	1.061
12 yrs	0.050	0.059	0.159	0.309	0.501	0.830	1.006
Adults	0.012	0.143	0.311	0.419	0.51	1.050	1.270

AGE GROUP PERCENTILES IgG4 : COLOURED FEMALE CONTROLS

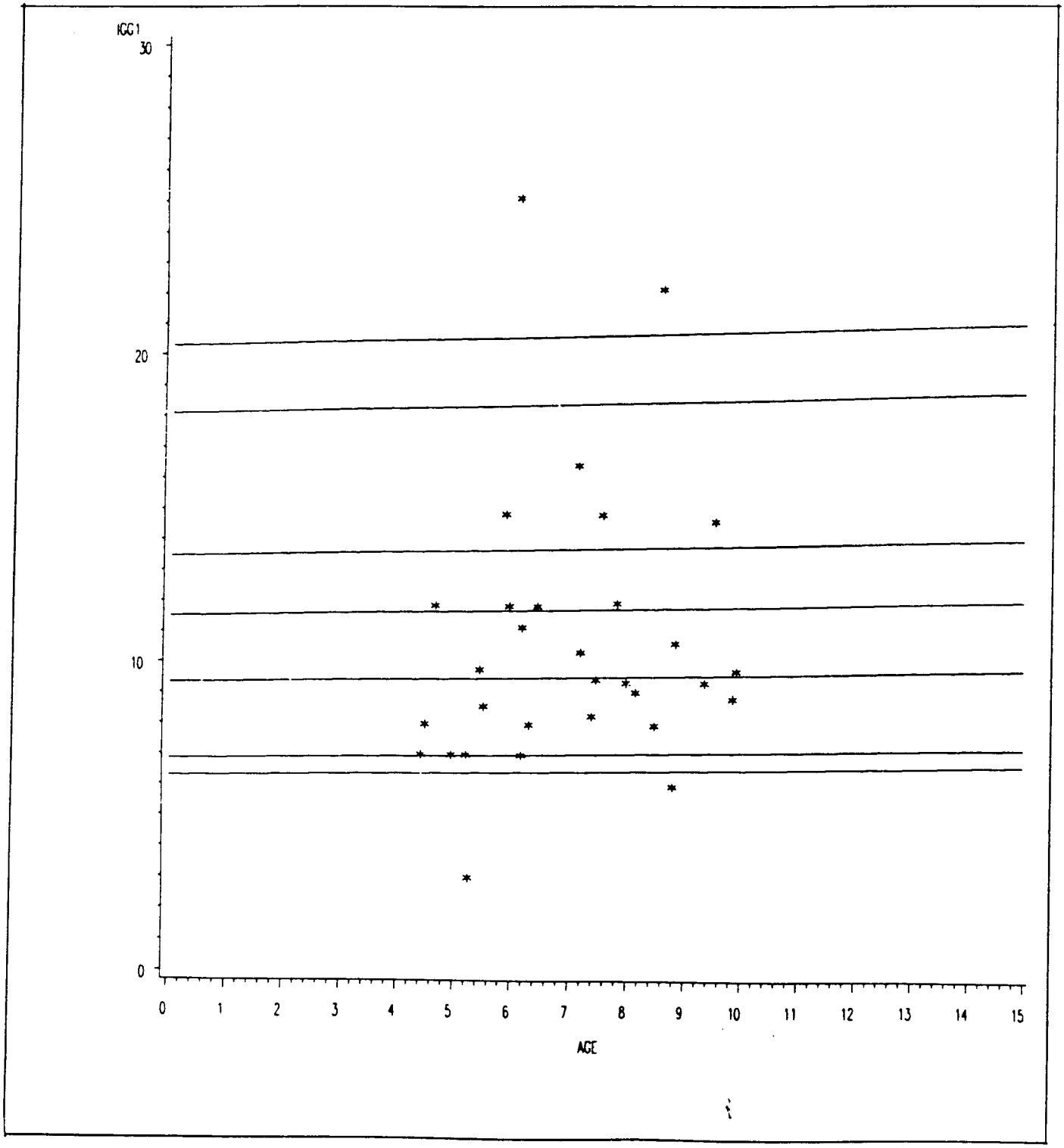
AGE	2.5%	5%	25%	50%	75%	95%	97.5%
6 mths	0.002	0.006	0.227	0.047	0.101	0.193	0.223
1 yr	0.003	0.008	0.028	0.057	0.119	0.221	0.255
2 yrs	0.006	0.013	0.041	0.079	0.158	0.284	0.324
3 yrs	0.011	0.020	0.057	0.105	0.202	0.352	0.400
4 yrs	0.016	0.028	0.075	0.134	0.248	0.423	0.478
5 yrs	0.022	0.038	0.094	0.164	0.296	0.495	9.557
6 yrs	0.029	0.048	0.114	0.194	0.343	0.565	0.634
7 yrs	0.035	0.058	0.133	0.223	0.388	0.631	0.705
8 yrs	0.042	0.067	0.151	0.249	0.428	0.689	0.769
9 yrs	0.047	0.075	0.166	0.271	0.462	0.738	0.822
10 yrs	0.052	0.081	0.178	0.288	0.487	0.775	0.862
11 yrs	0.055	0.086	0.186	0.299	0.504	0.799	0.888
12 yrs	0.056	0.087	0.189	0.304	0.511	0.809	0.899
Adults	0.028	0.028	0.110	0.240	0.401	1.310	1.310

APPENDIX K

The following figures are the IgG1, IgG2, IgG3 and IgG4 and IgG values of individual patients with Hib meningitis, Hib OM/SA and *S.aureus* OM/SA superimposed on age and sex matched percentile charts created from values from healthy children (Appendix I). The percentile lines shown are 2.5%, 5%, 25%, 50%, 75%, 95% and 97.5%. The individual patient values are shown (*). As the age distribution of the children with Hib meningitis, Hib OM/SA and *S.aureus* OM/SA are significantly different the data for the three patient groups is shown on separate graphs.

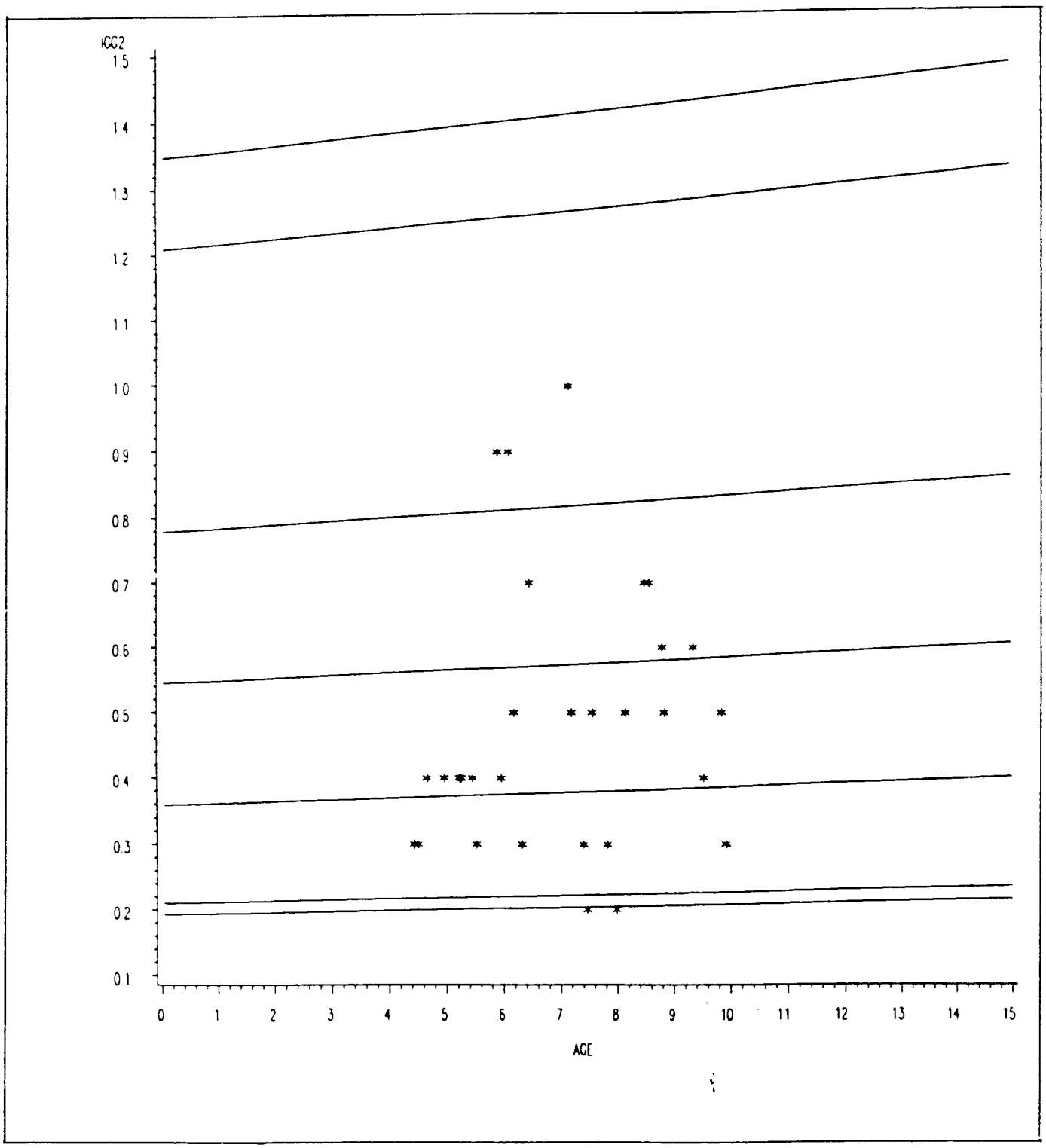
BLACK PATIENTS WITH Hib MENINGITIS

IgG1



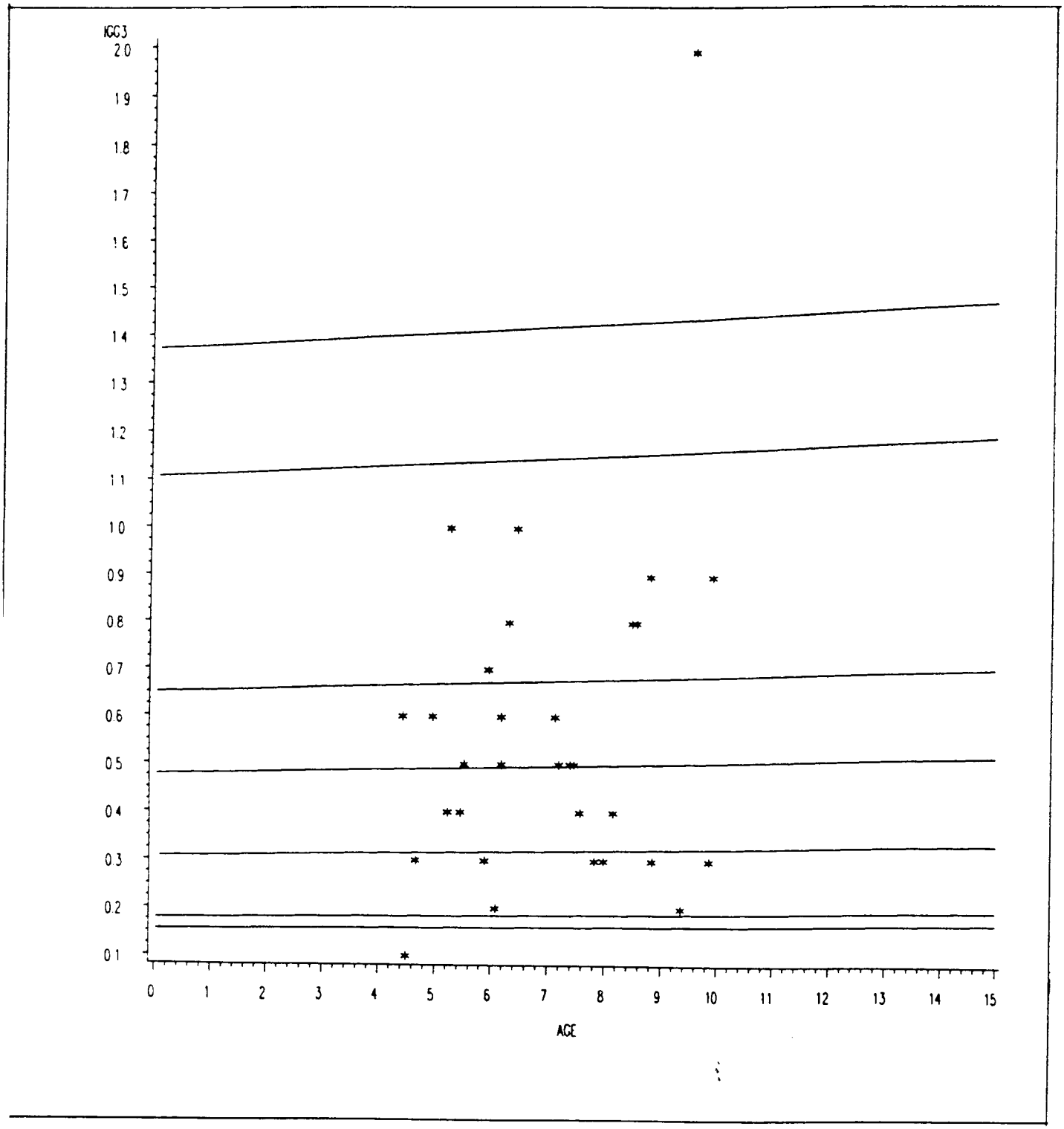
BLACK PATIENTS WITH Hib MENINGITIS

IgG2



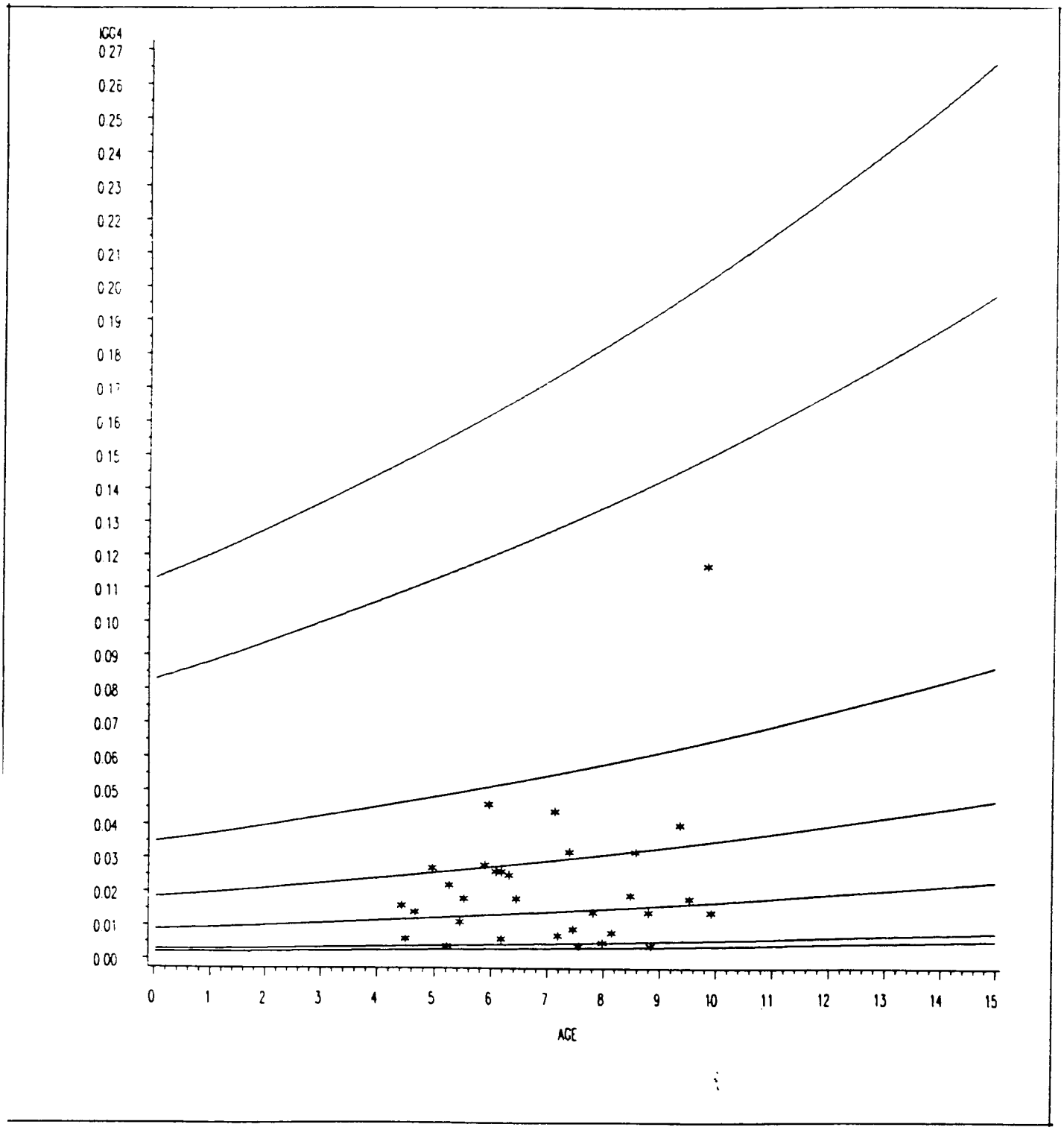
BLACK PATIENTS WITH Hib MENINGITIS

IgG3



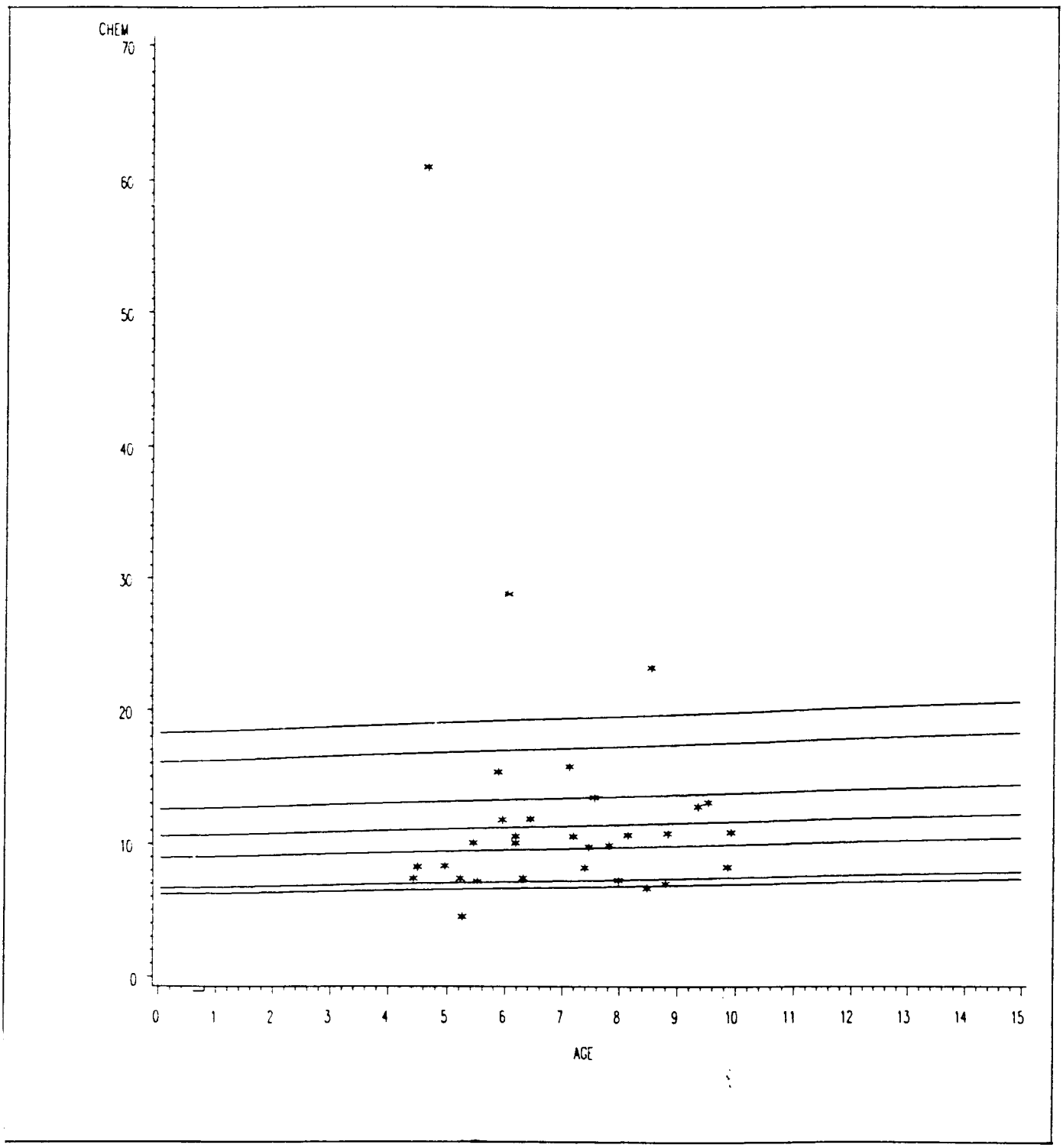
BLACK PATIENTS WITH Hib MENINGITIS

IgG4



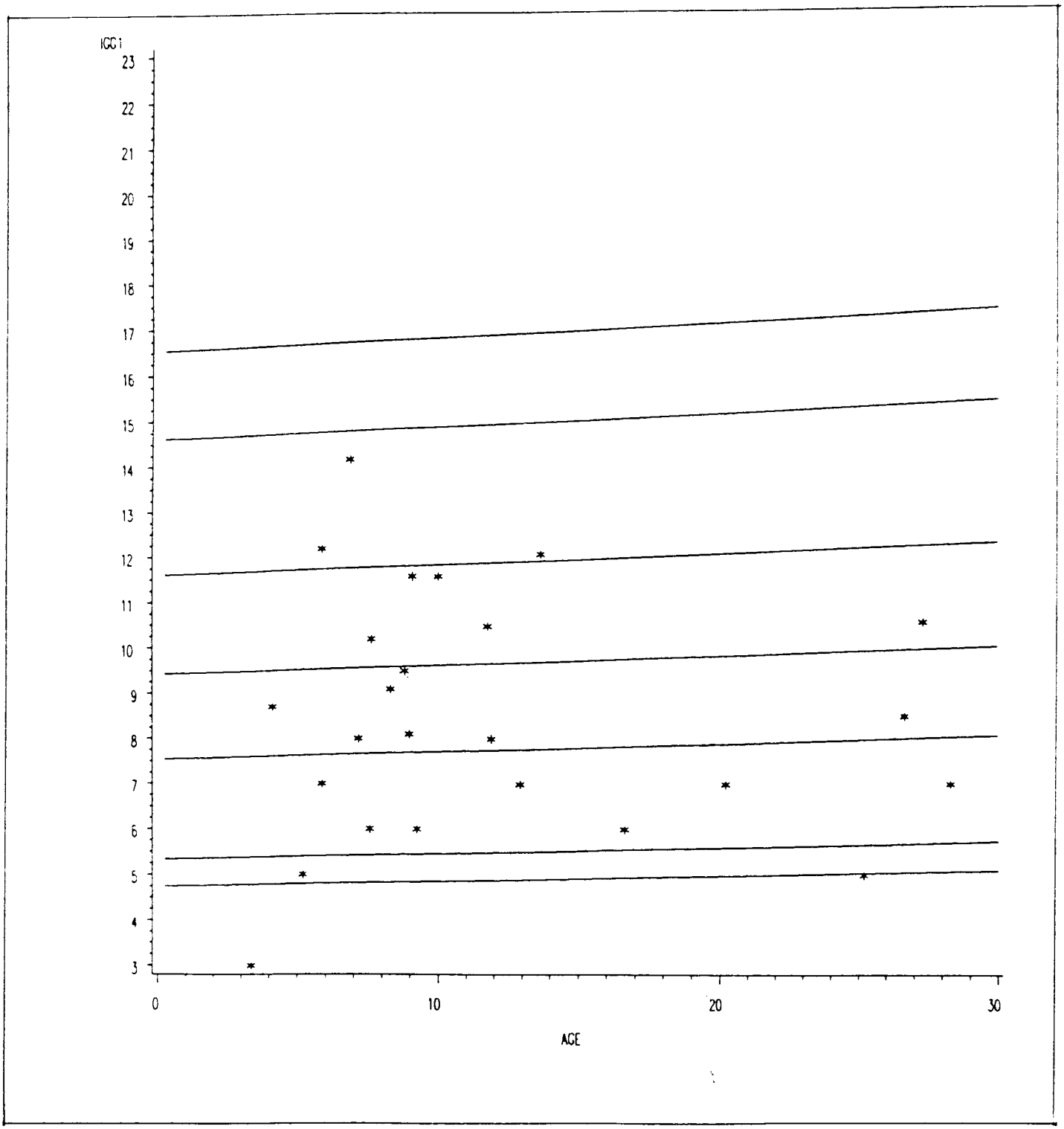
BLACK PATIENTS WITH Hib MENINGITIS

IgG



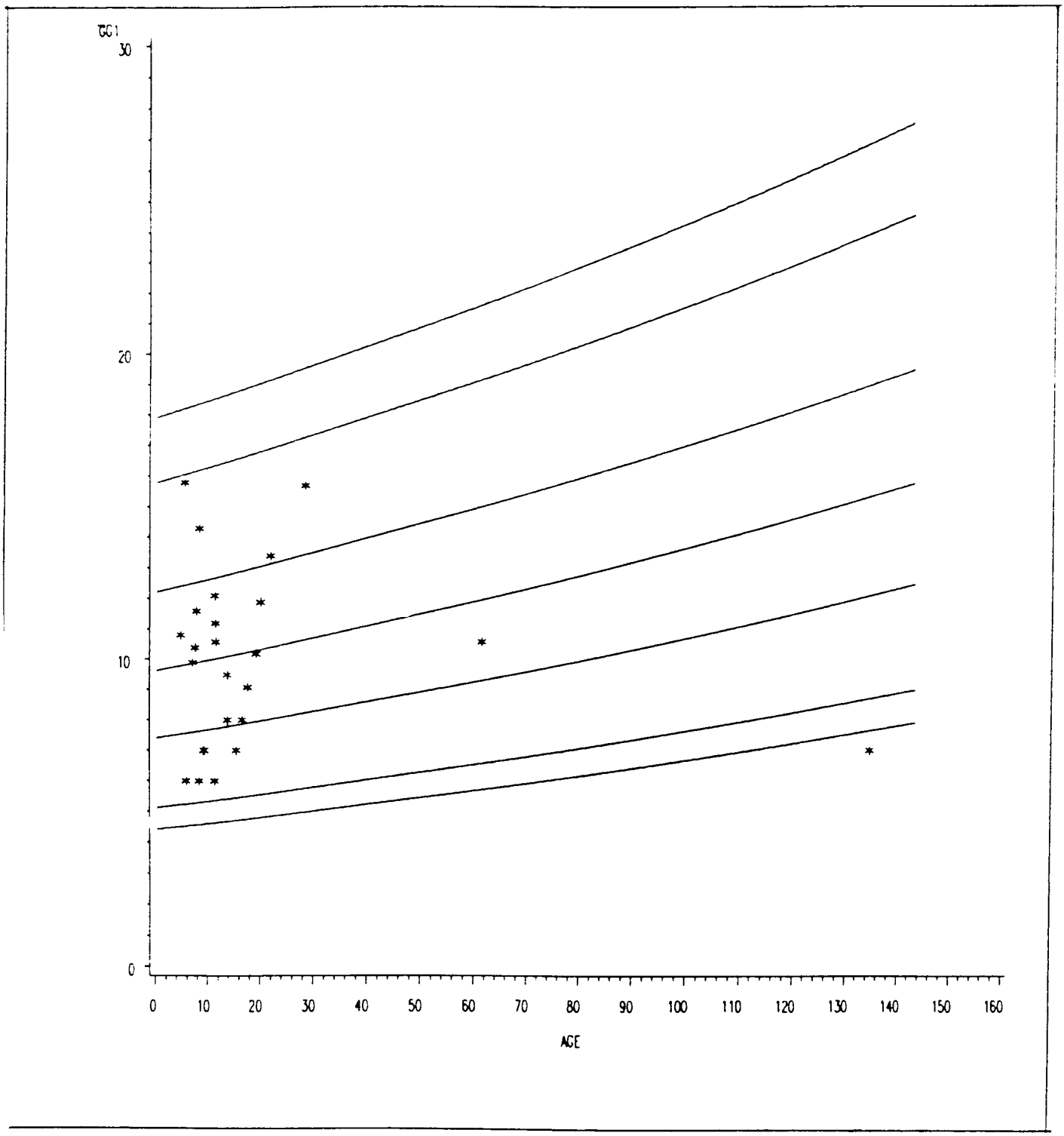
COLOURED MALE PATIENTS WITH Hib MENINGITIS

IgG1



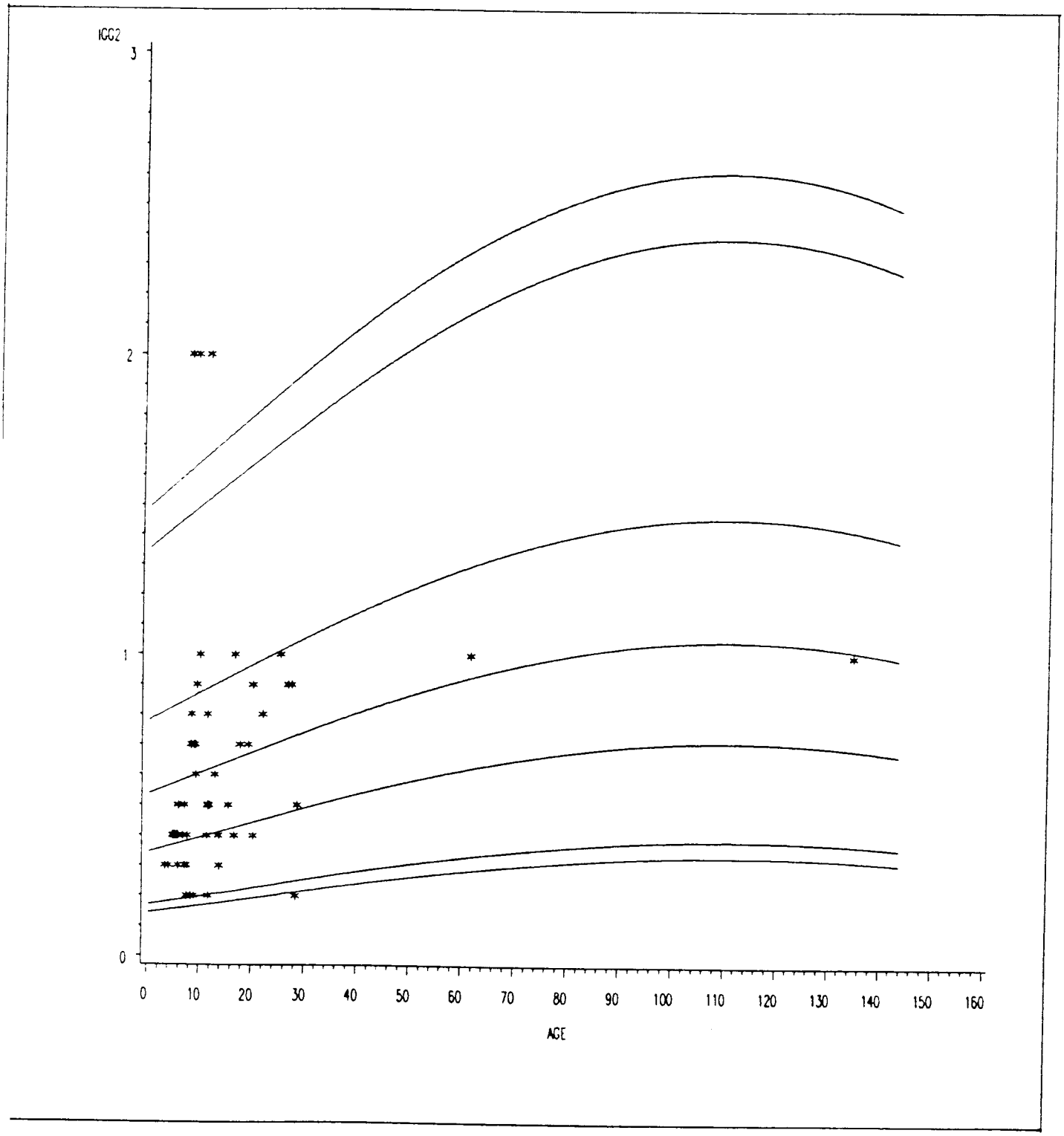
COLOURED FEMALE PATIENTS WITH Hib MENINGITIS

IgG1



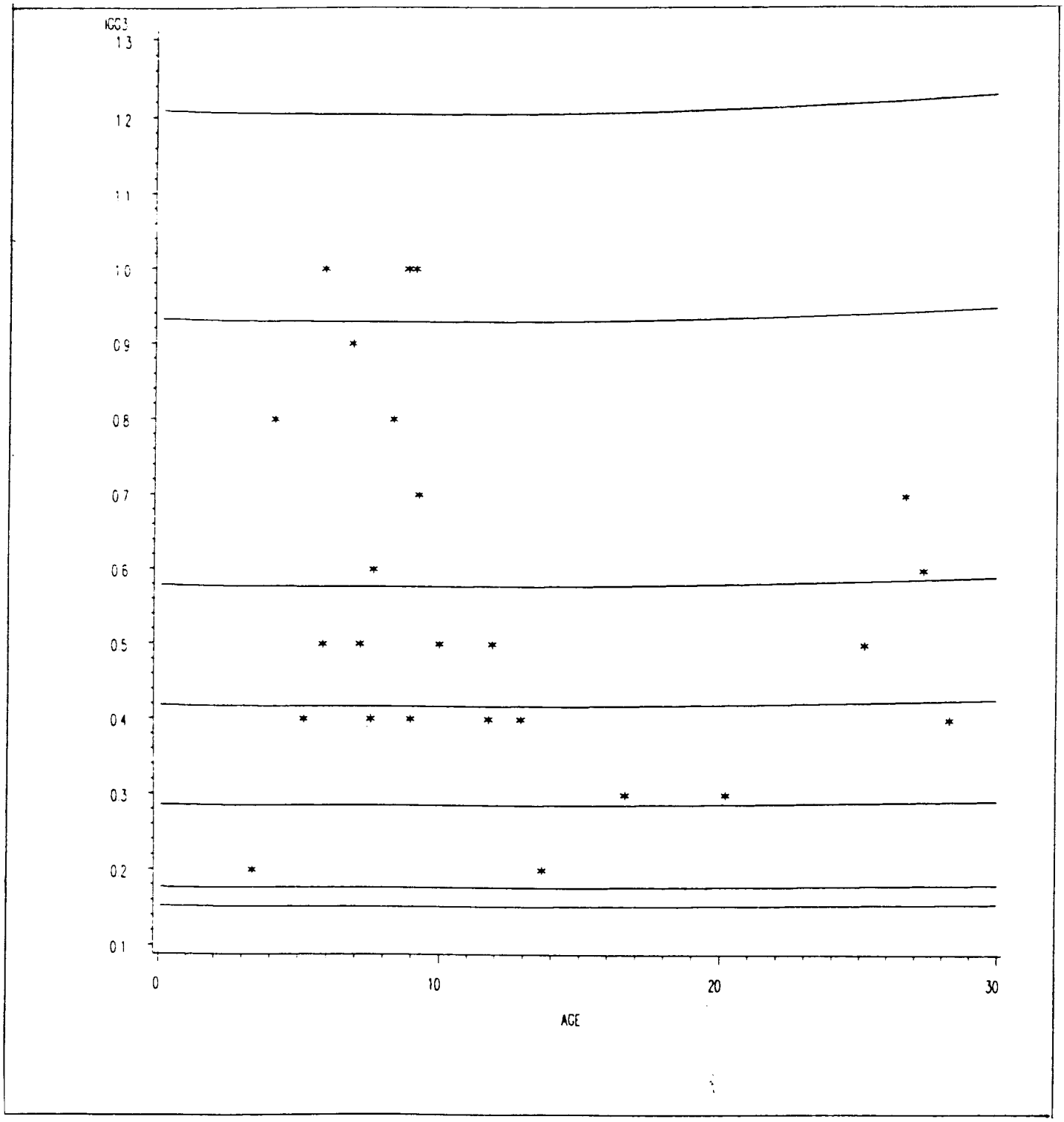
COLOURED MALE AND FEMALE PATIENTS WITH Hib MENINGITIS

IgG2



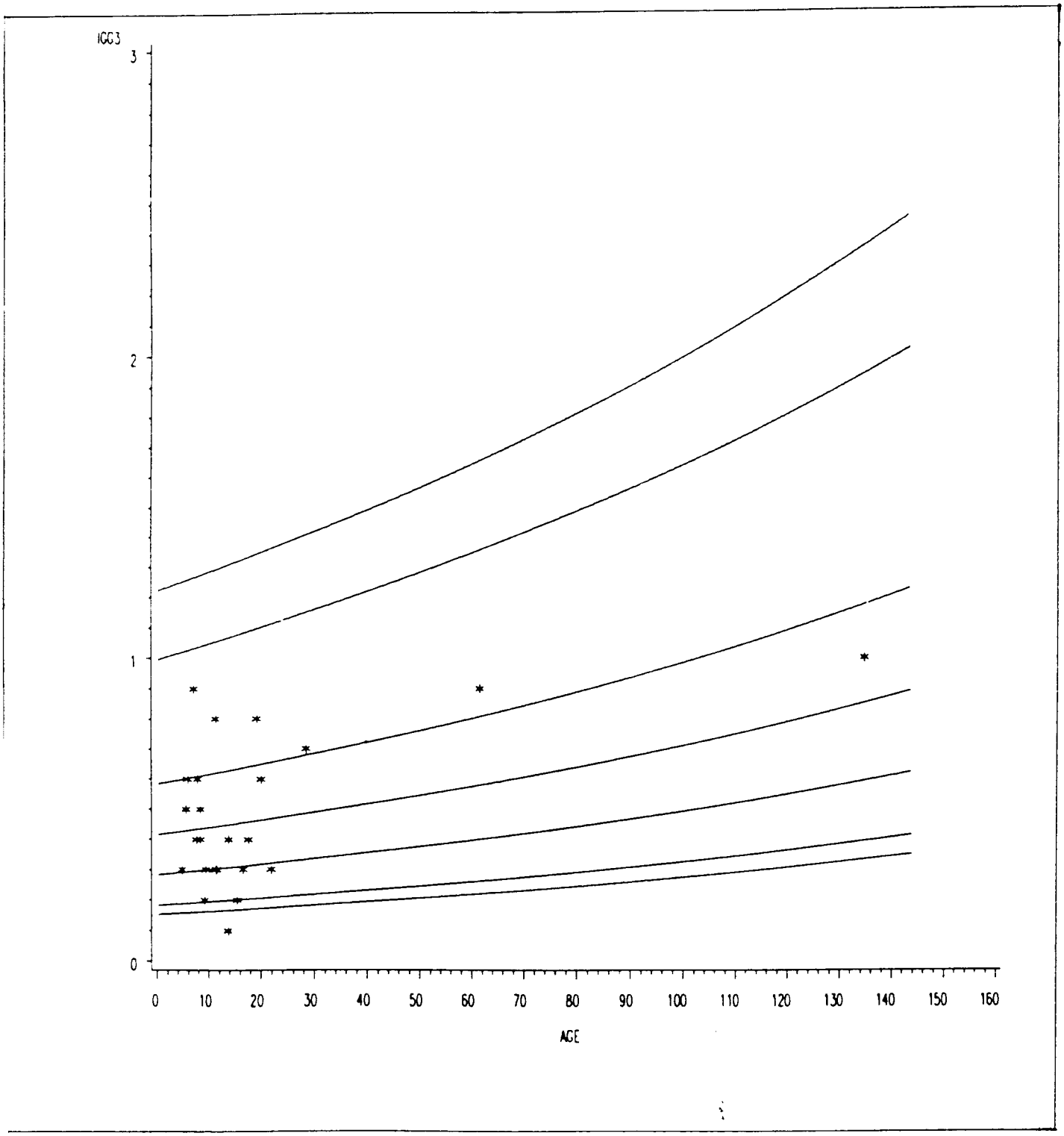
COLOURED MALE PATIENTS WITH Hib MENINGITIS

IgG3



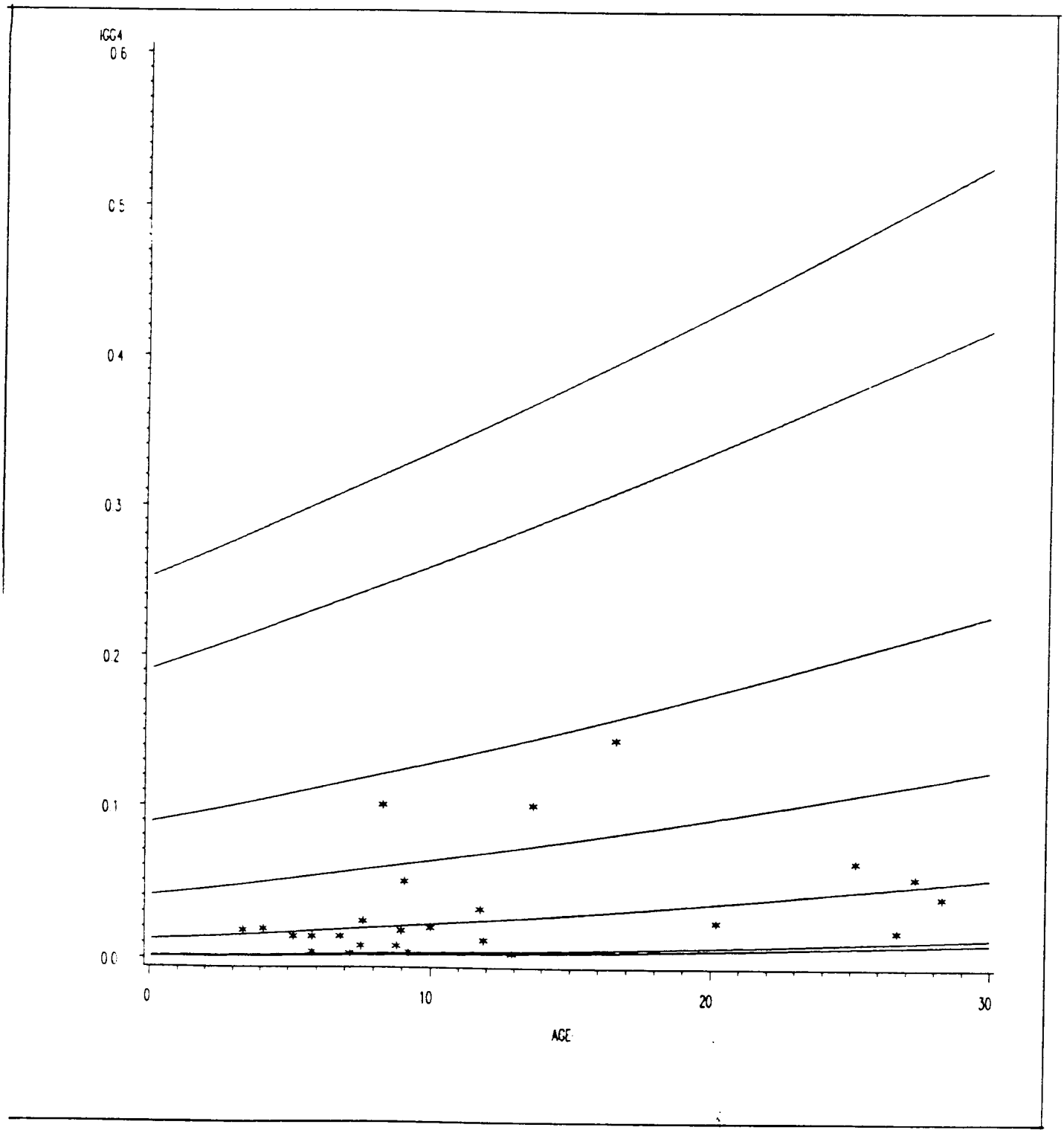
COLOURED FEMALE PATIENTS WITH Hib MENINGITIS

IgG3



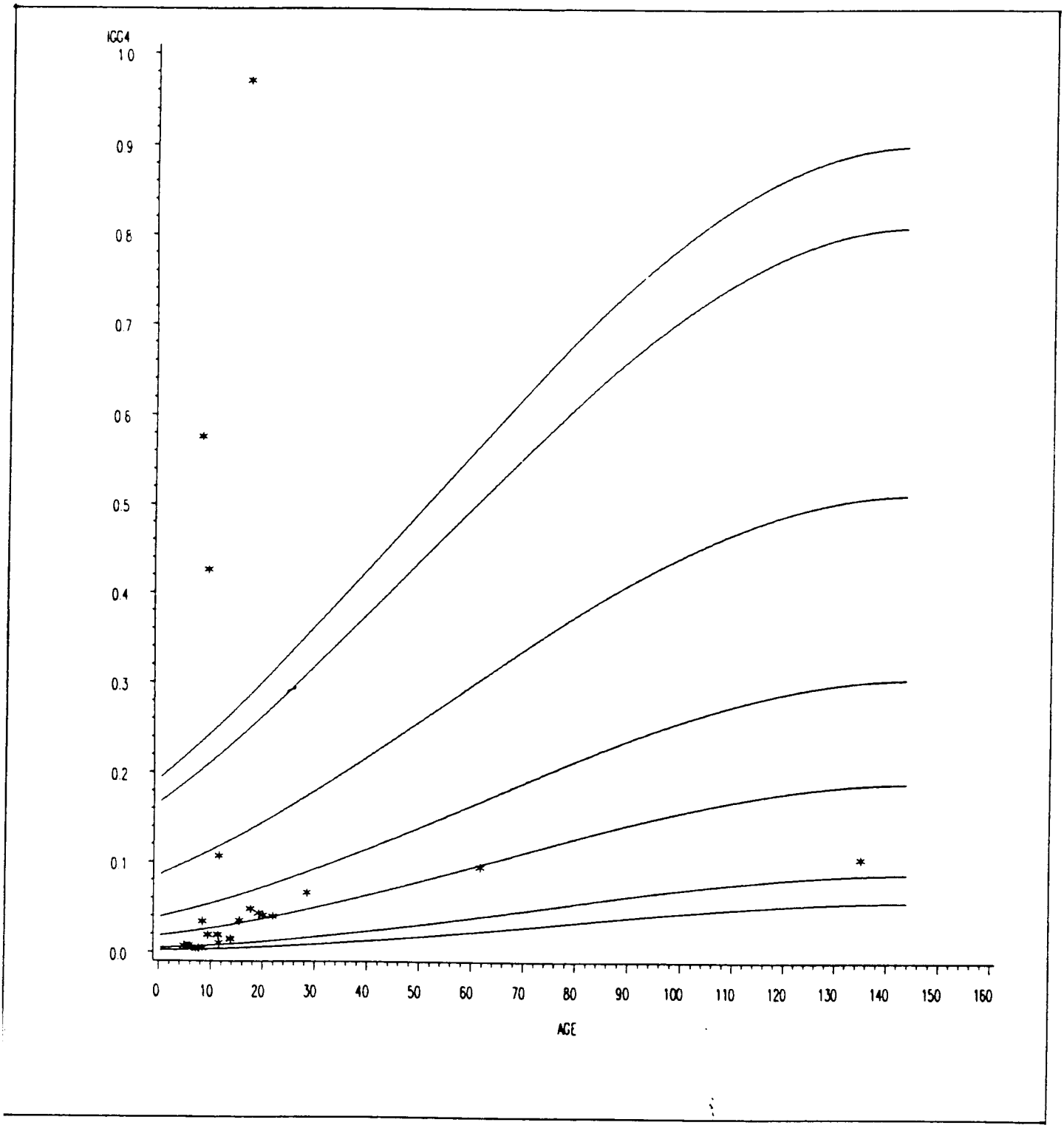
COLOURED MALE PATIENTS WITH Hib MENINGITIS

IgG4



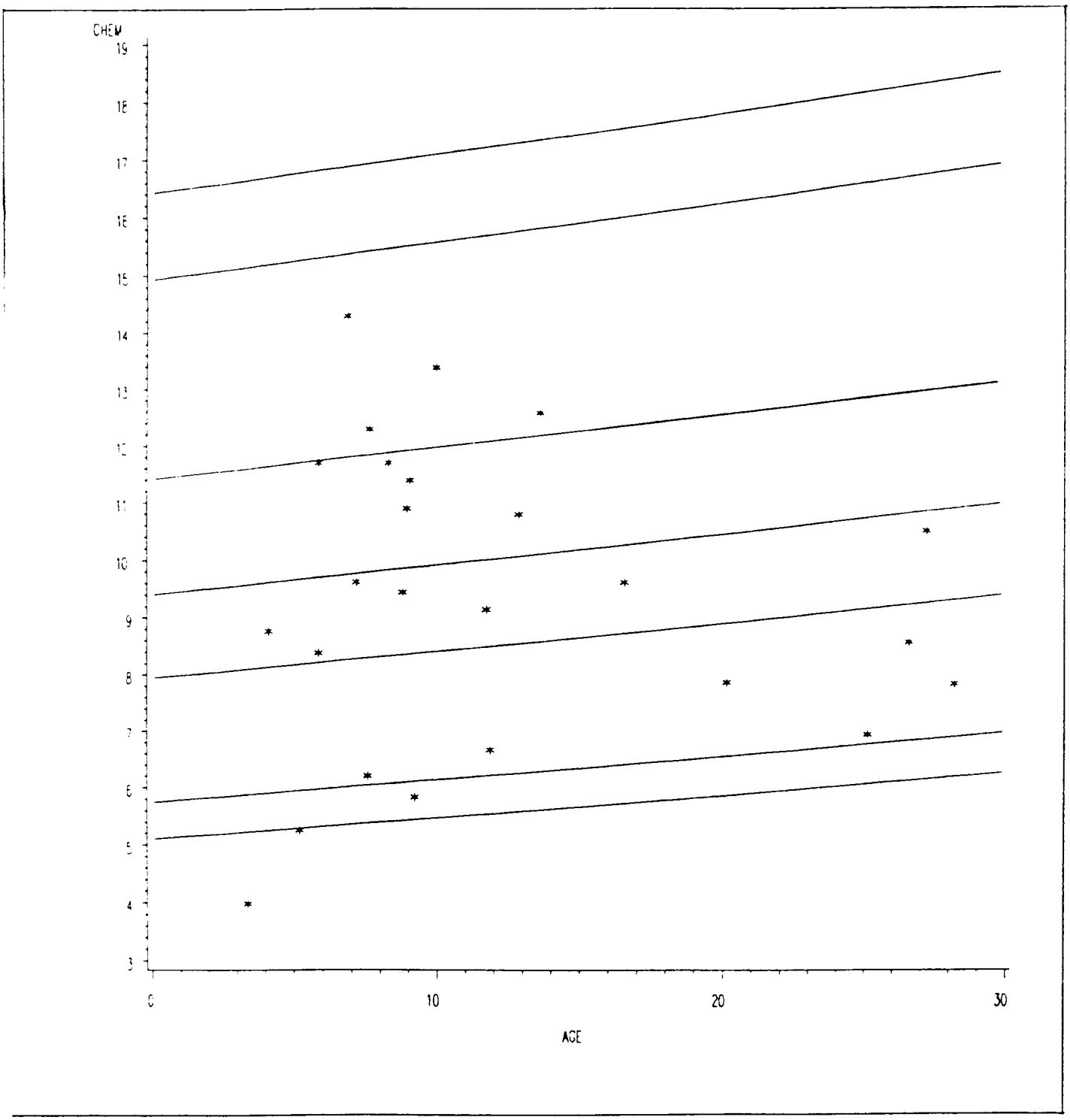
COLOURED FEMALE PATIENTS WITH Hib MENINGITIS

IgG4



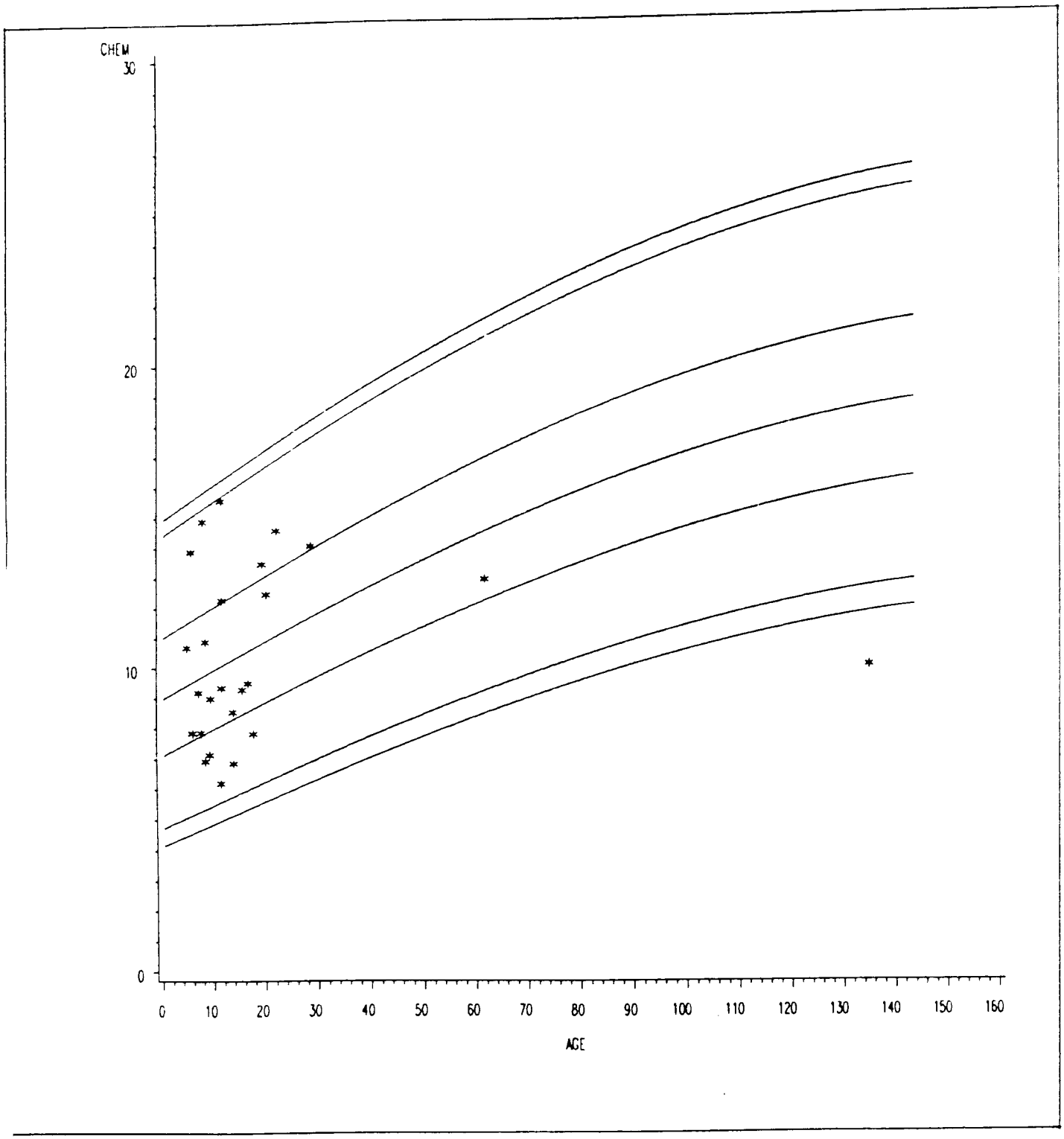
COLOURED MALE PATIENTS WITH Hib MENINGITIS

IgG



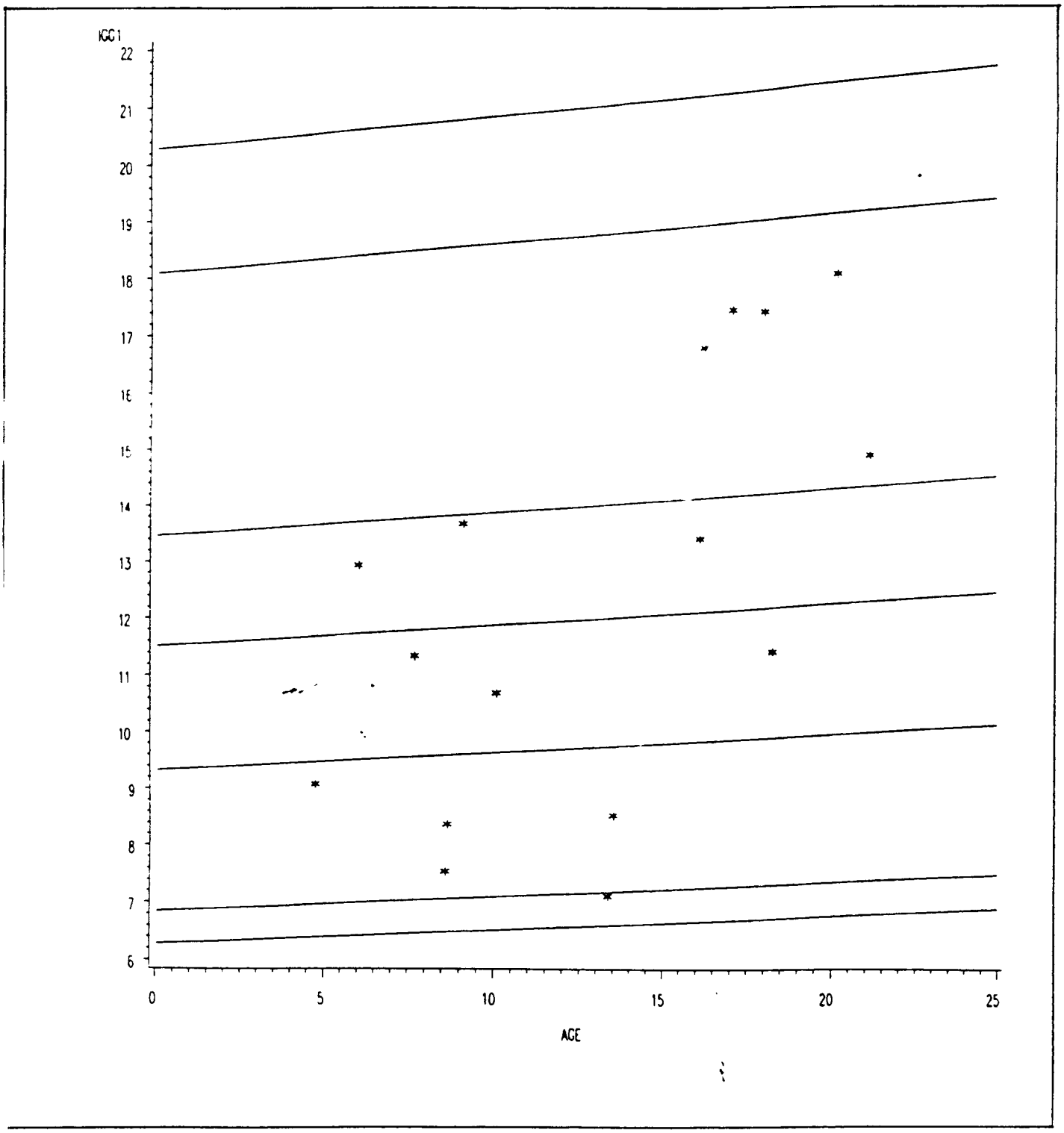
COLOURED FEMALE PATIENTS WITH Hib MENINGITIS

IgG



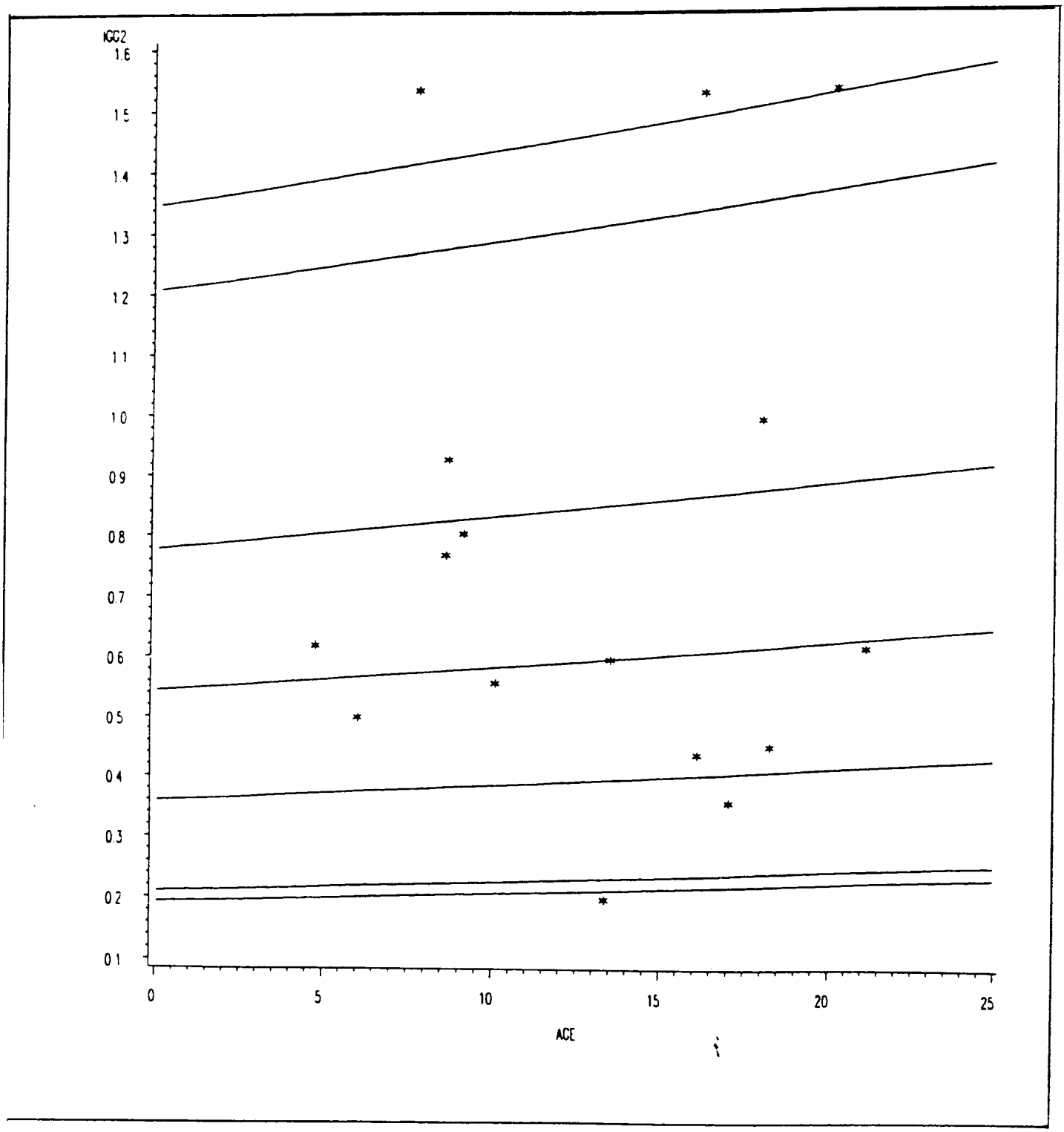
BLACK PATIENTS WITH Hib OM/SA

IgG1



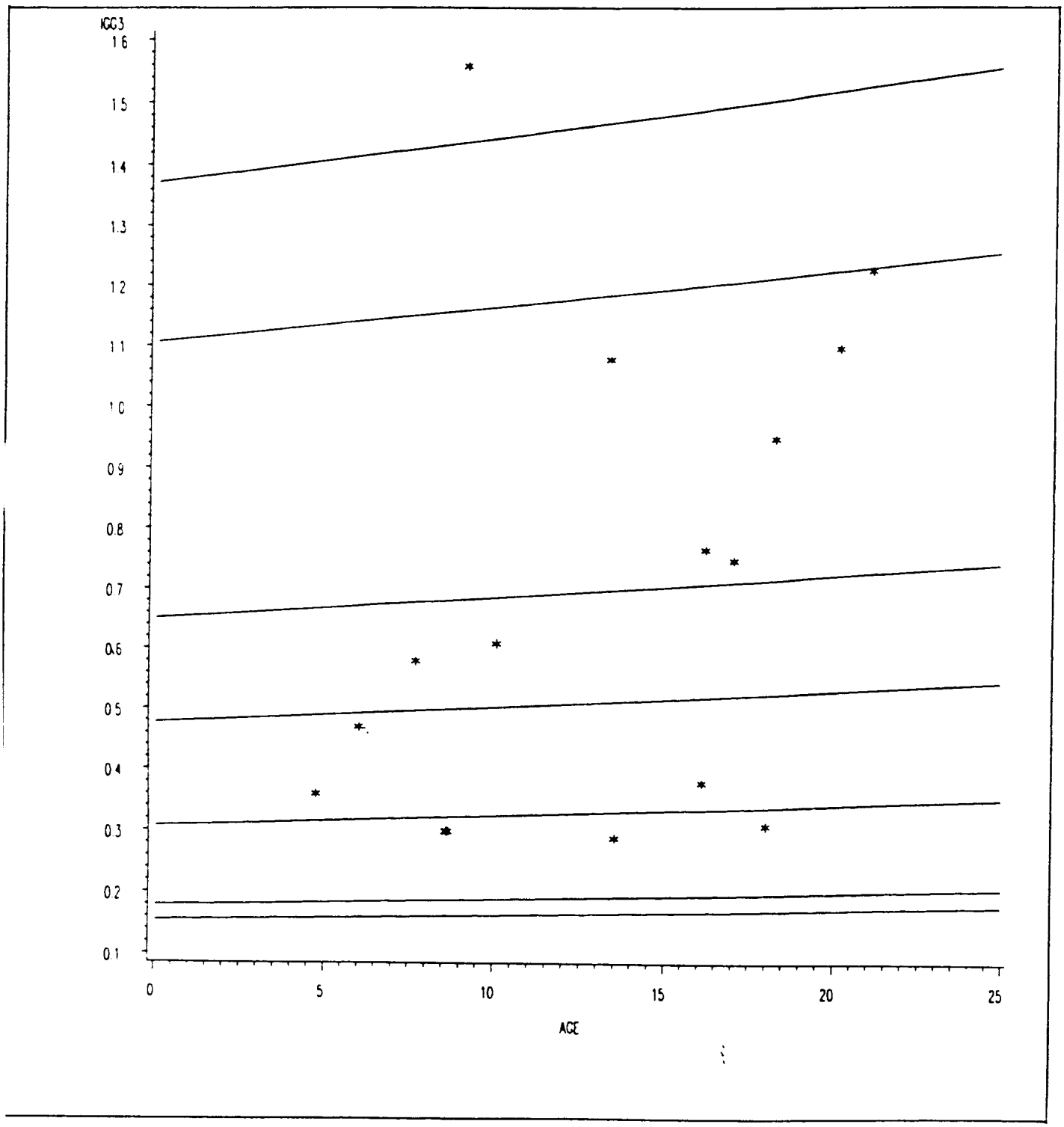
BLACK PATIENTS WITH Hib OM/SA

IgG2



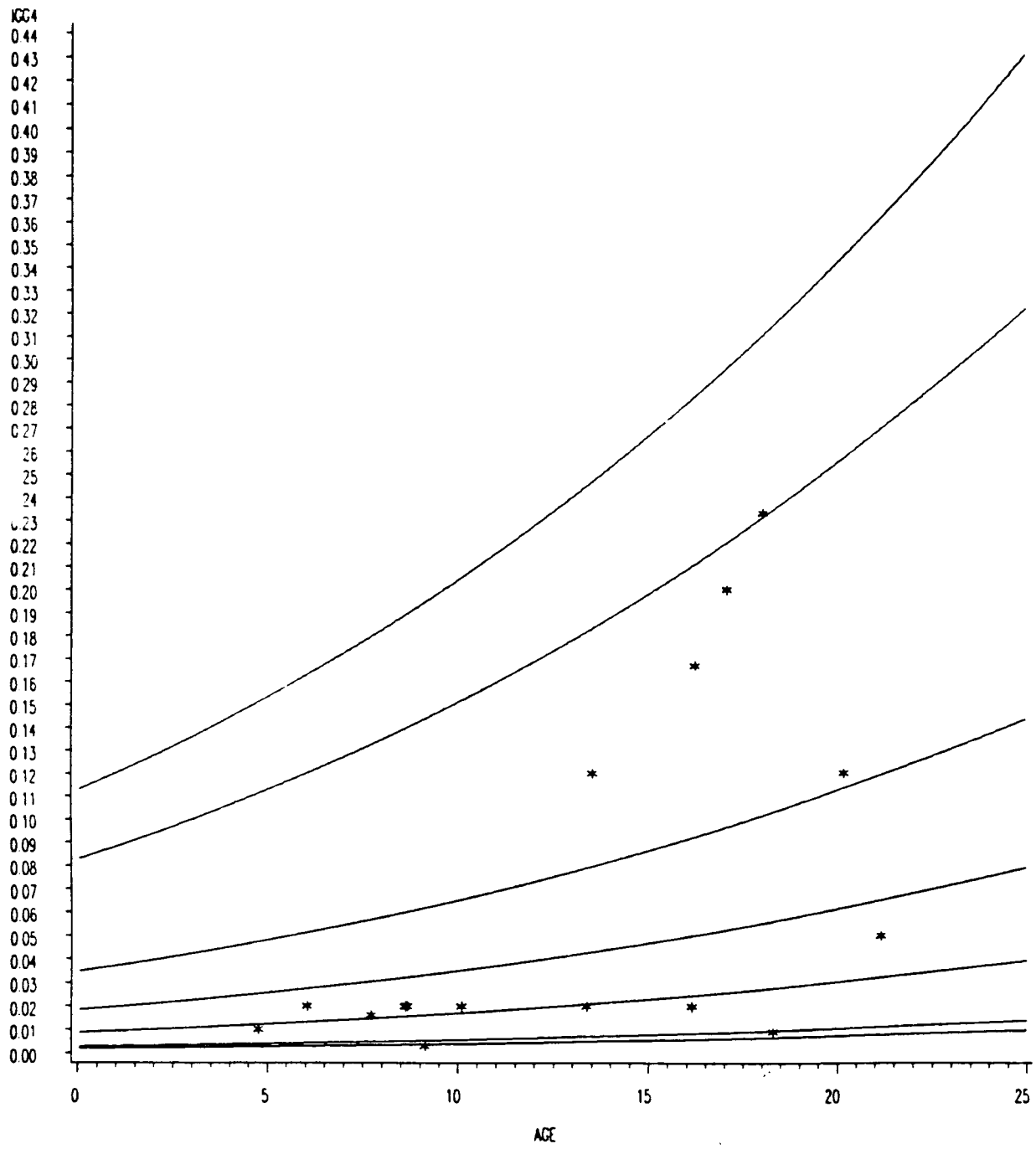
BLACK PATIENTS WITH Hib OM/SA

IgG3



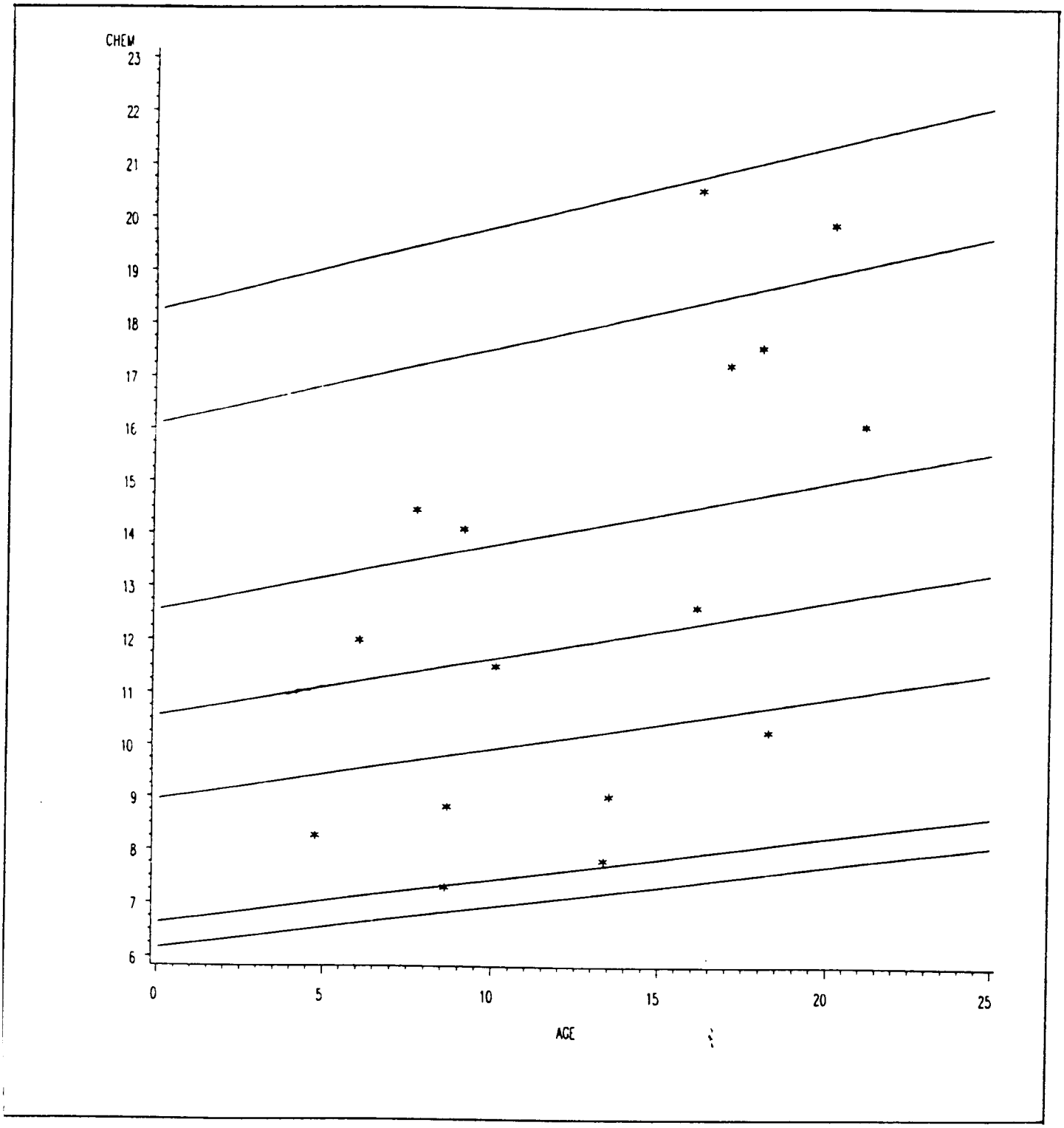
BLACK PATIENTS WITH Hib OM/SA

IgG4



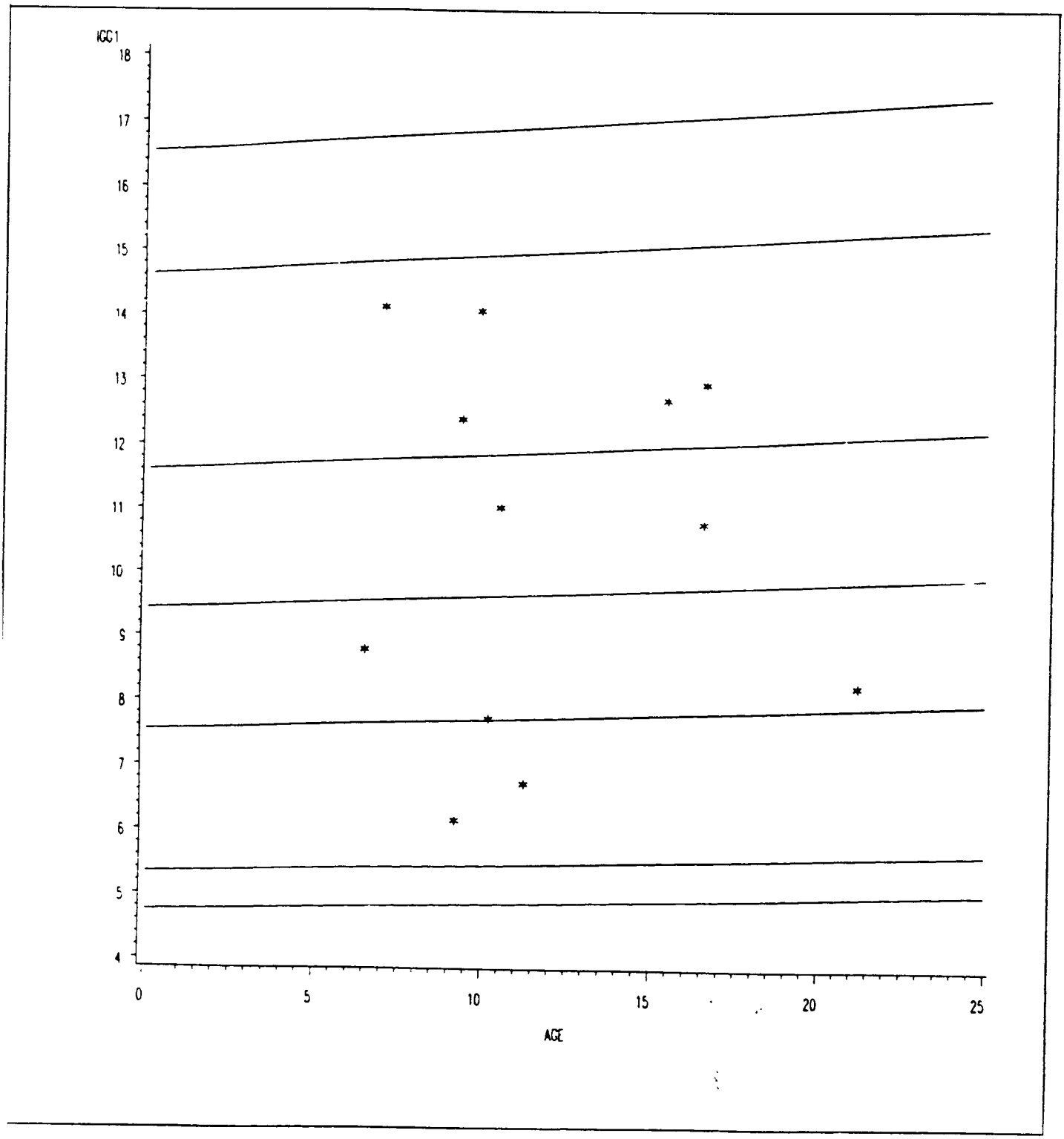
BLACK PATIENTS WITH Hib OM/SA

IgG



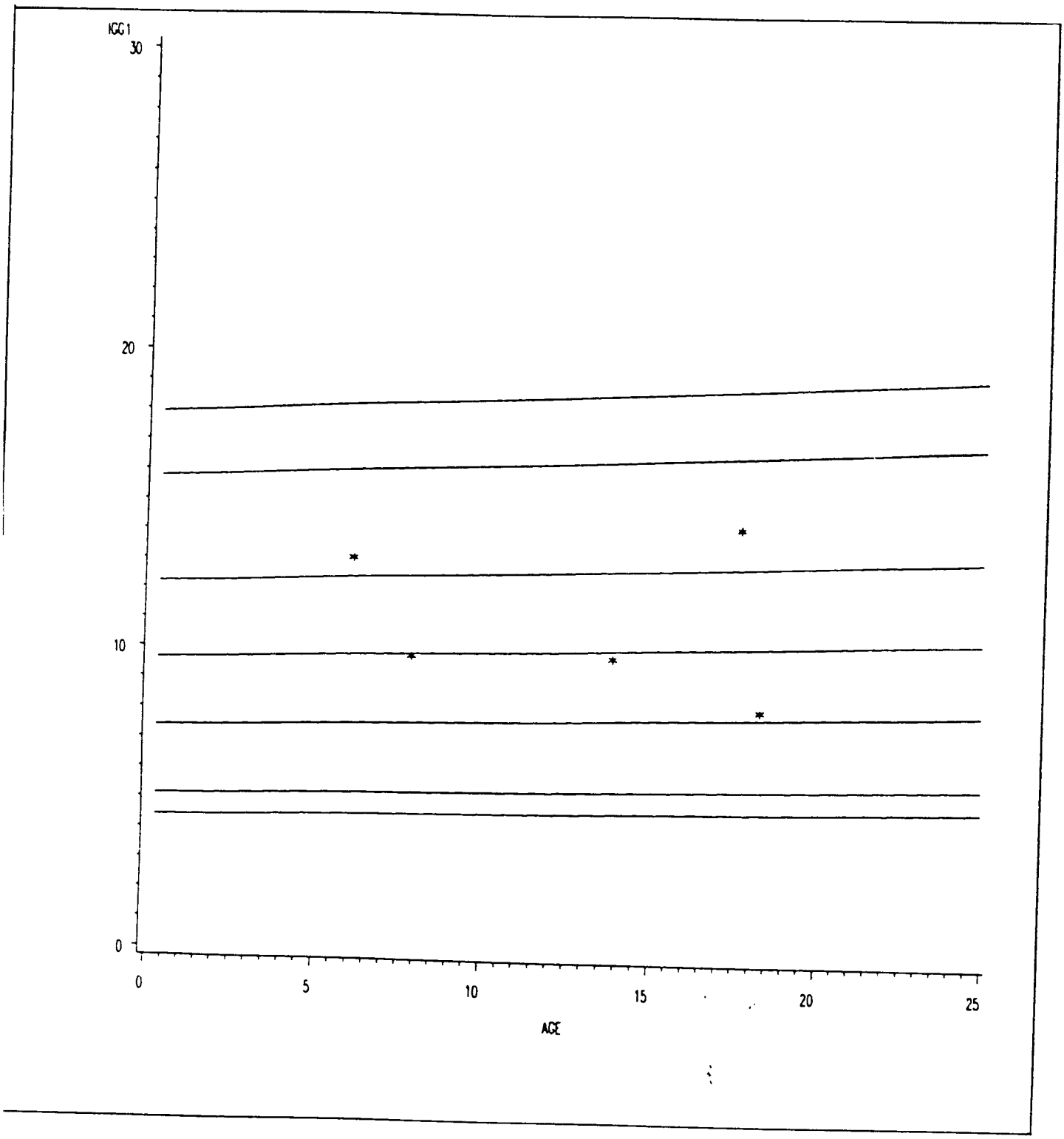
COLOURED MALE PATIENTS WITH Hib OM/SA

IgG1



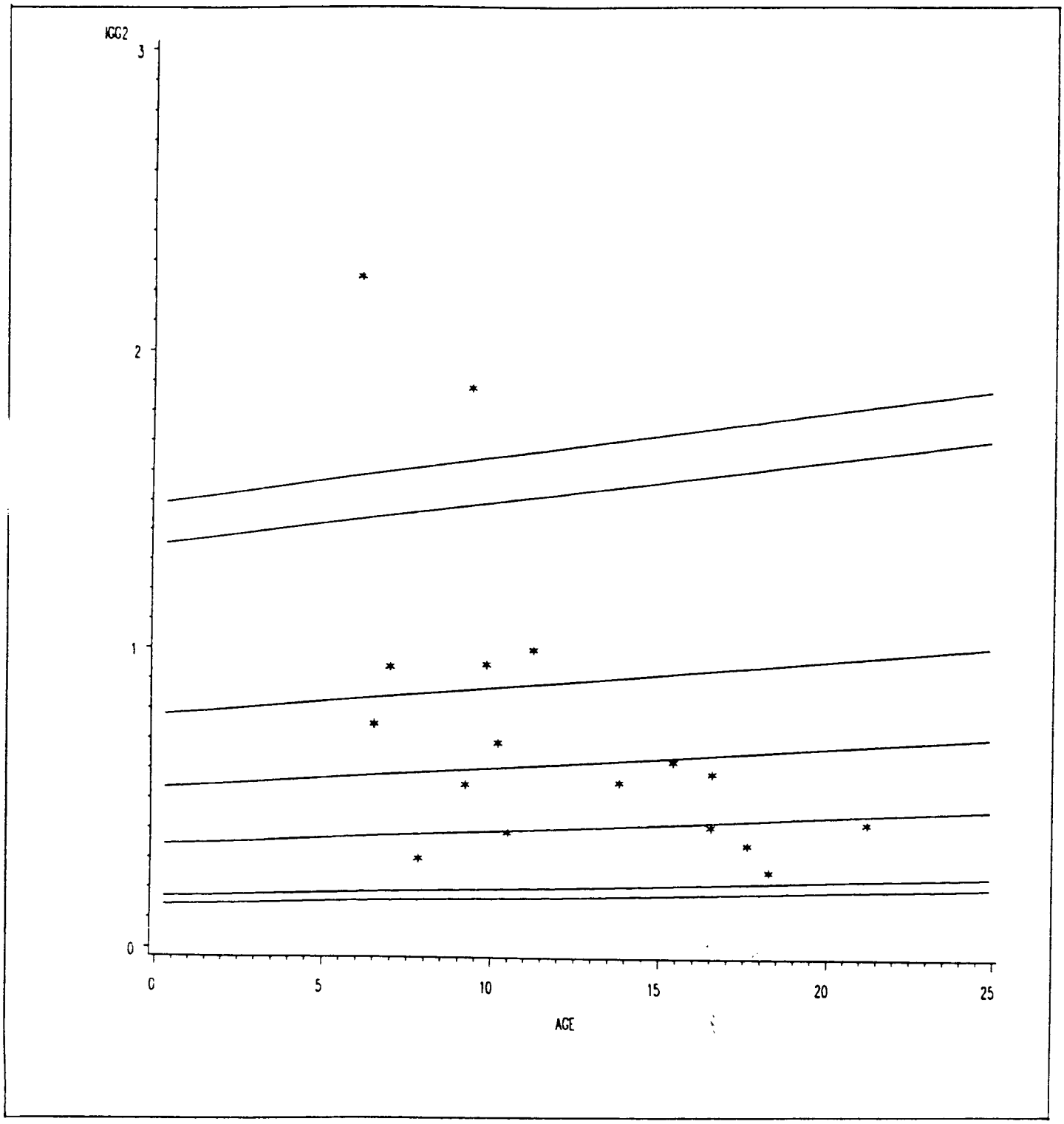
COLOURED FEMALE PATIENTS WITH Hib OM/SA

IgG1



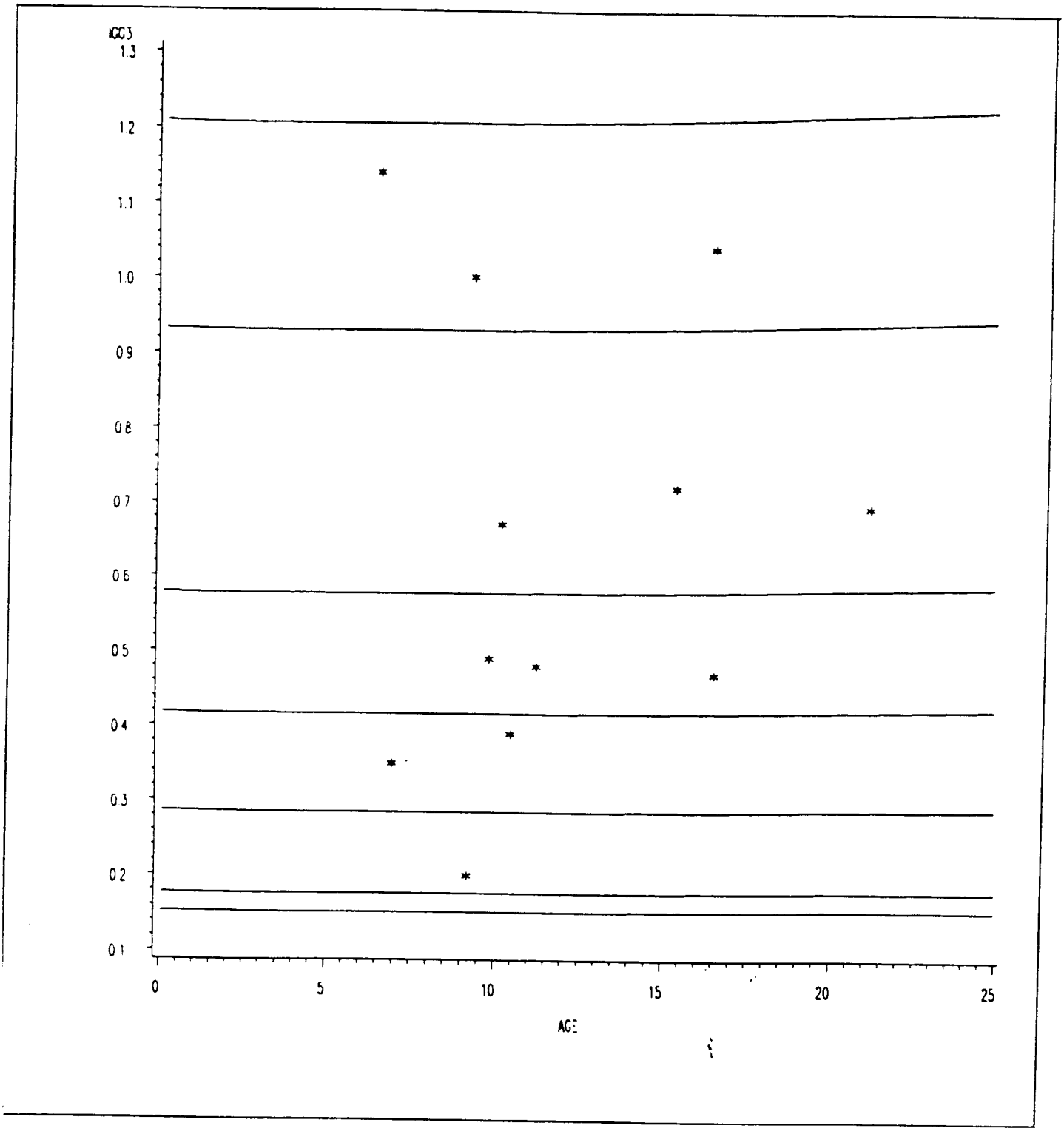
COLOURED MALE AND FEMALE PATIENTS WITH Hib OM/SA

IgG2



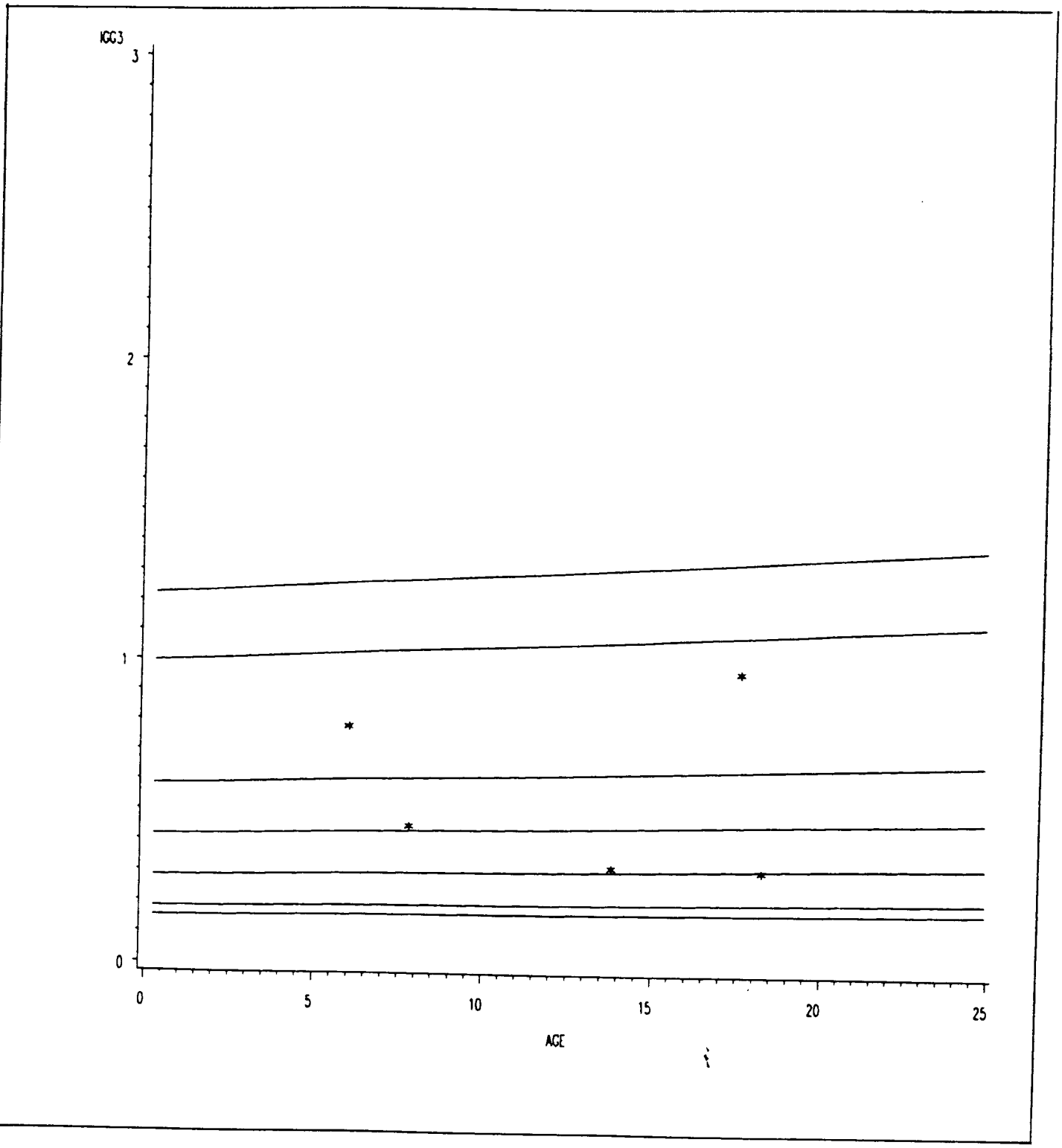
COLOURED MALE PATIENTS WITH Hib OM/SA

IgG3



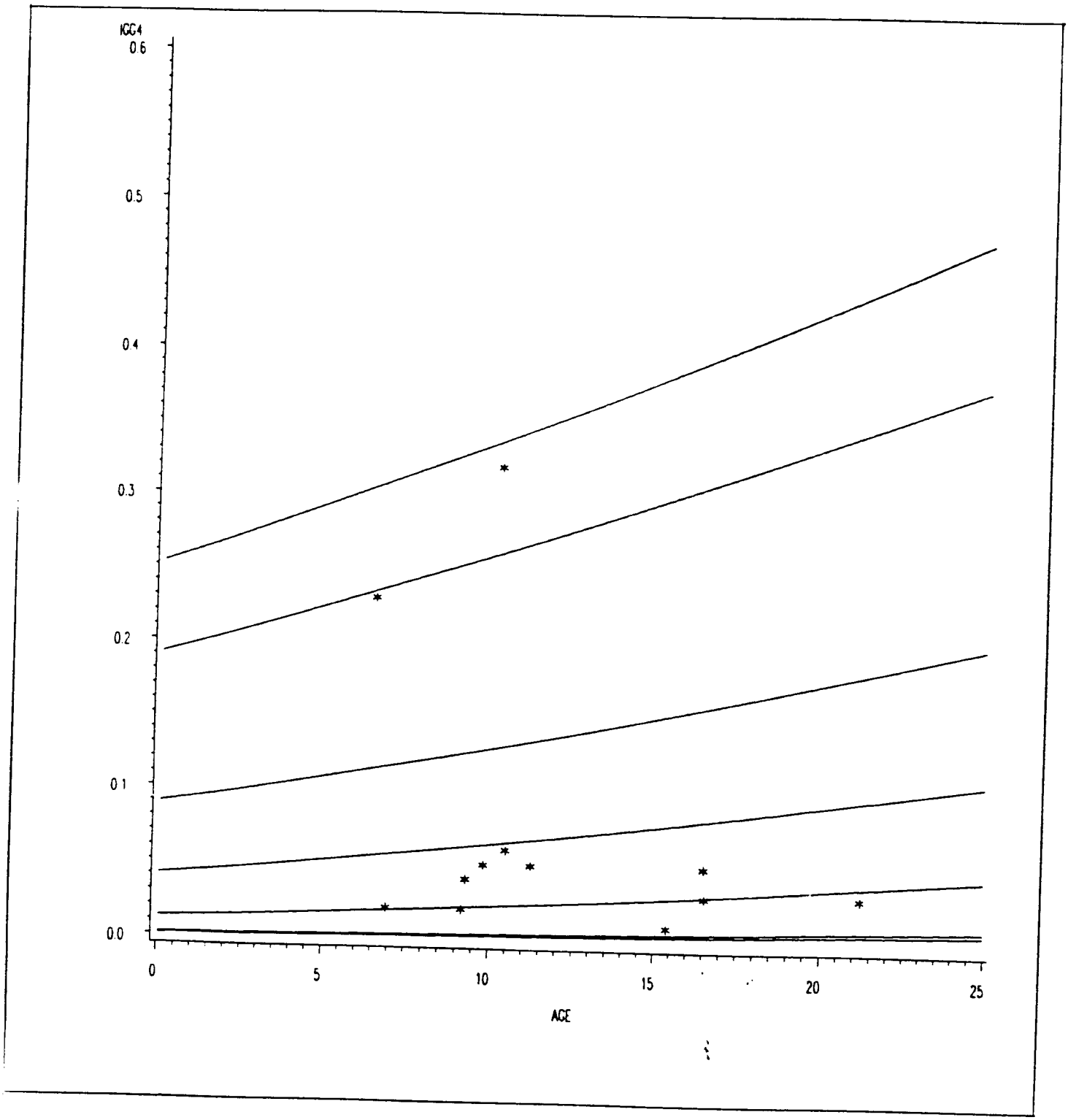
COLOURED FEMALE PATIENTS WITH Hib OM/SA

IgG3



COLOURED MALE PATIENTS WITH Hib OM/SA

IgG4



COLOURED FEMALE PATIENTS WITH Hib OM/SA

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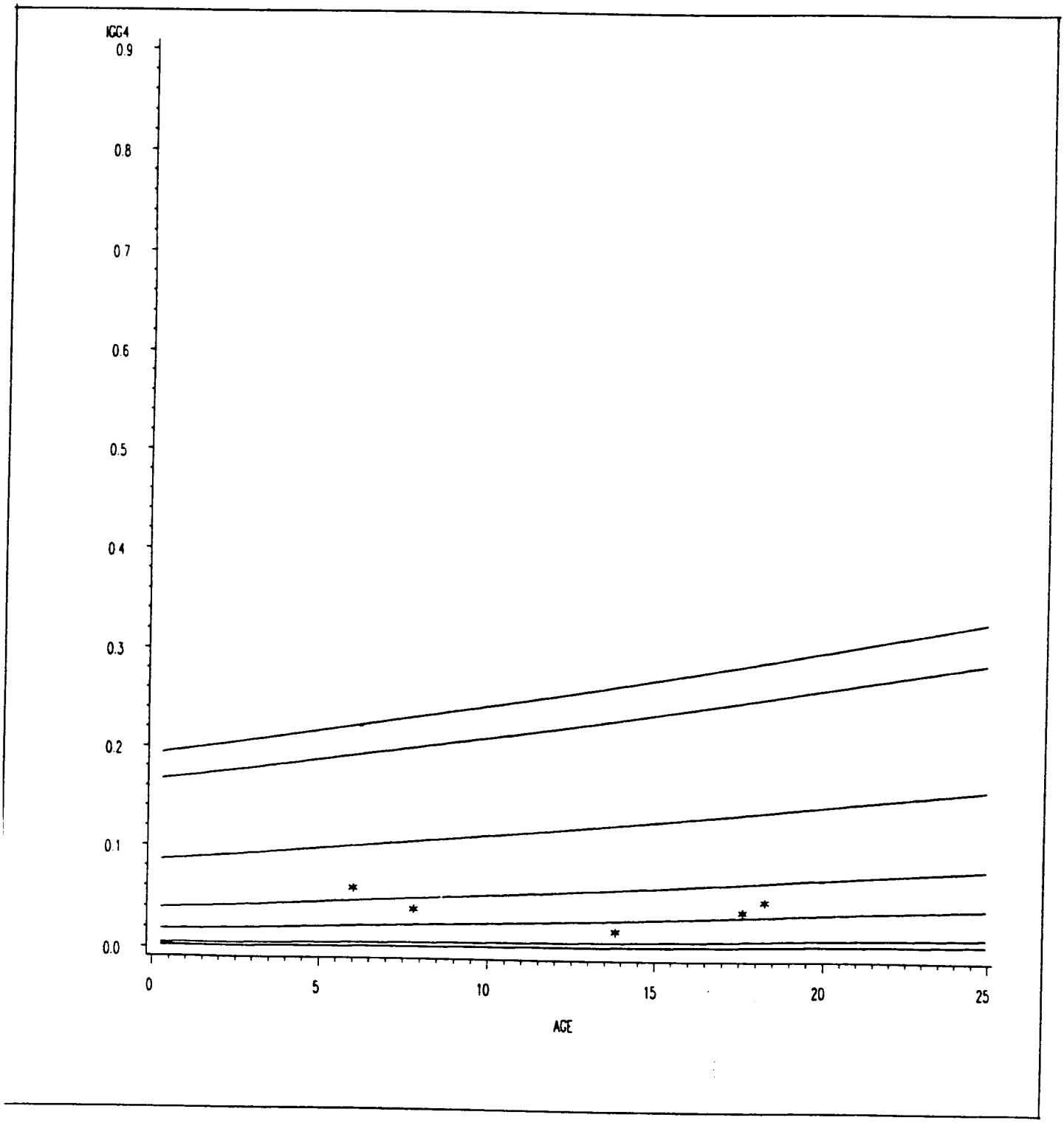
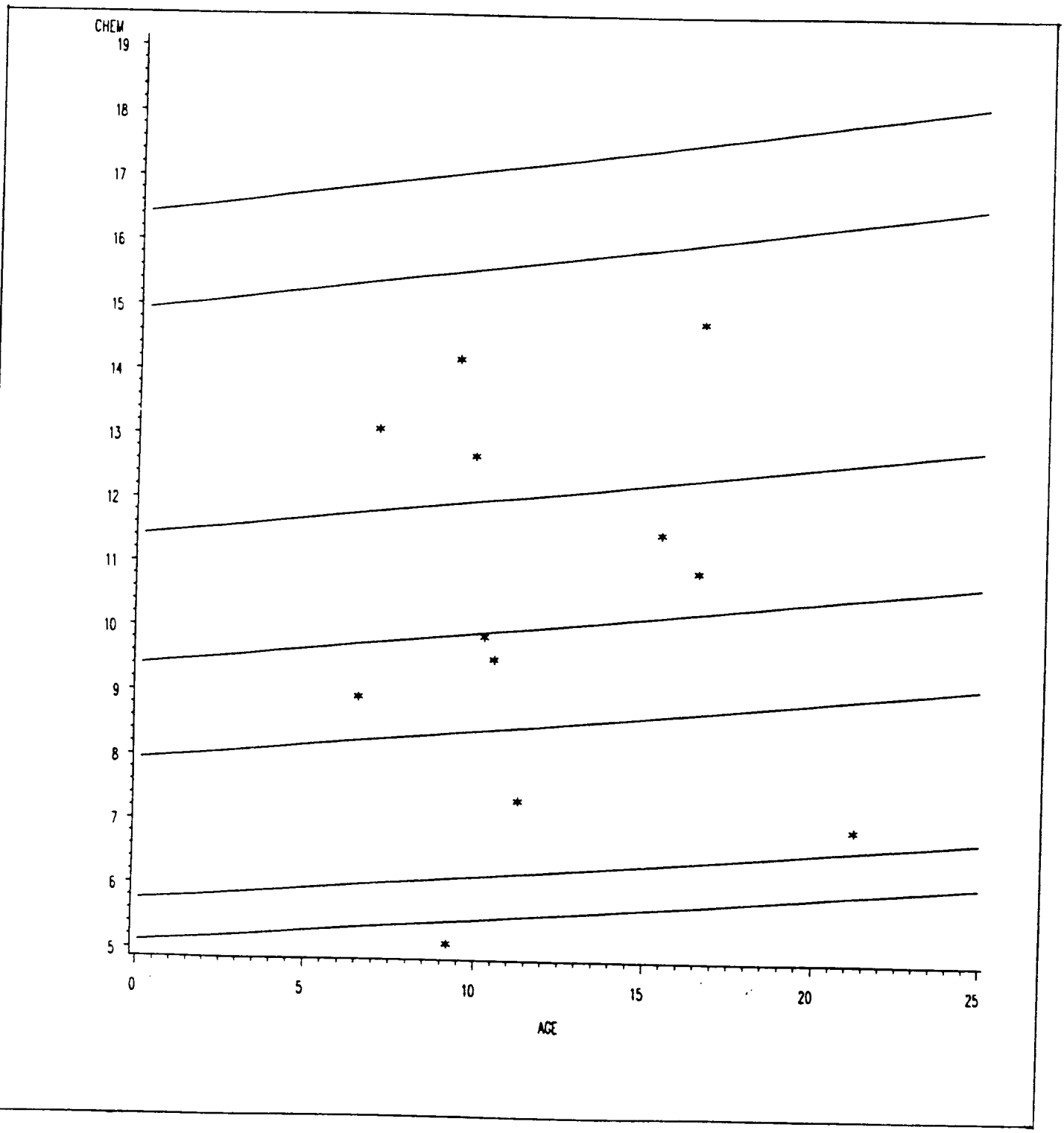


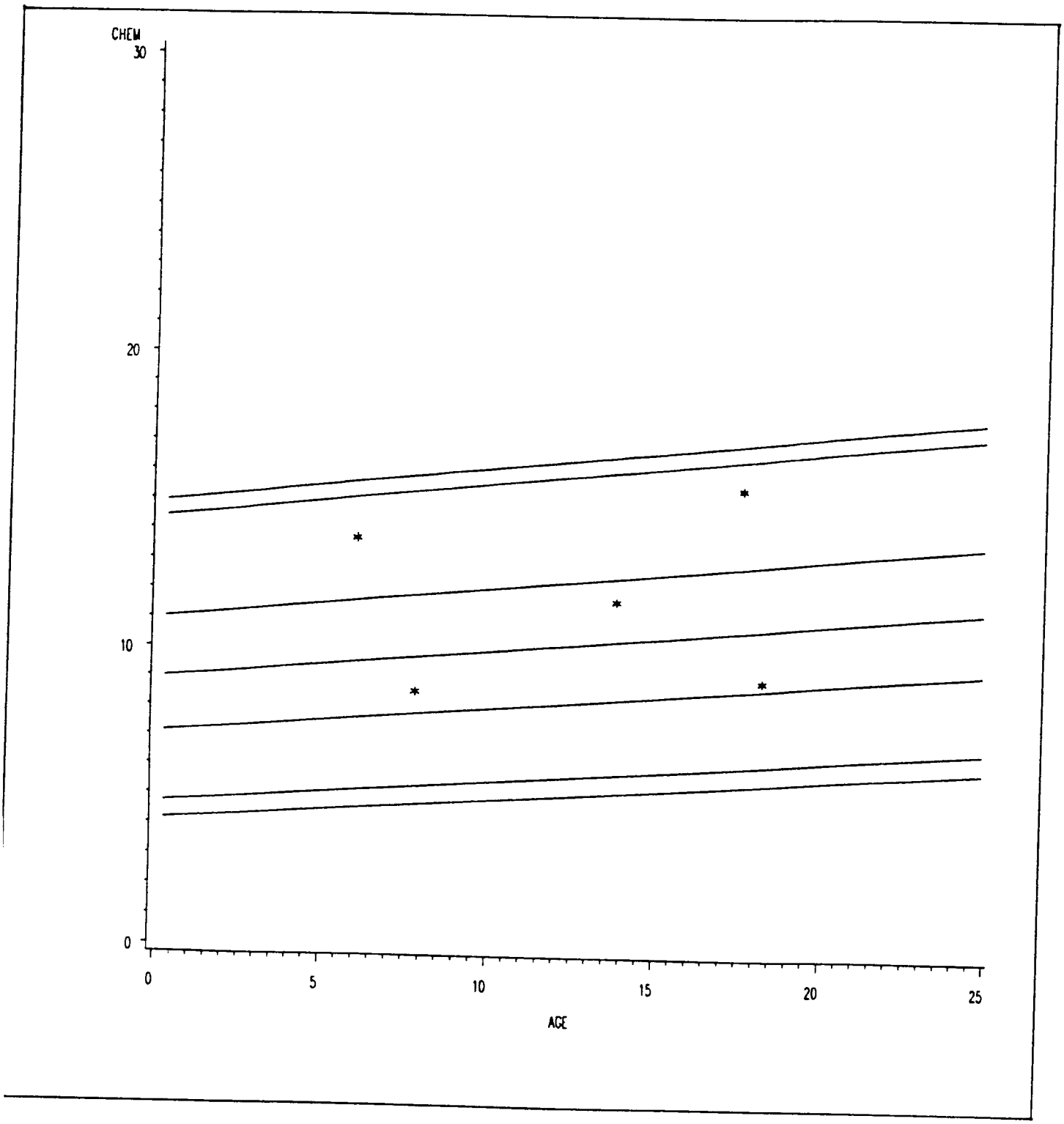
COLOURED MALE PATIENTS WITH Hib OM/SA

IgG



COLOURED FEMALE PATIENTS WITH Hib OM/SA

IgG



BLACK PATIENTS WITH *S.AUREUS* OM/SA

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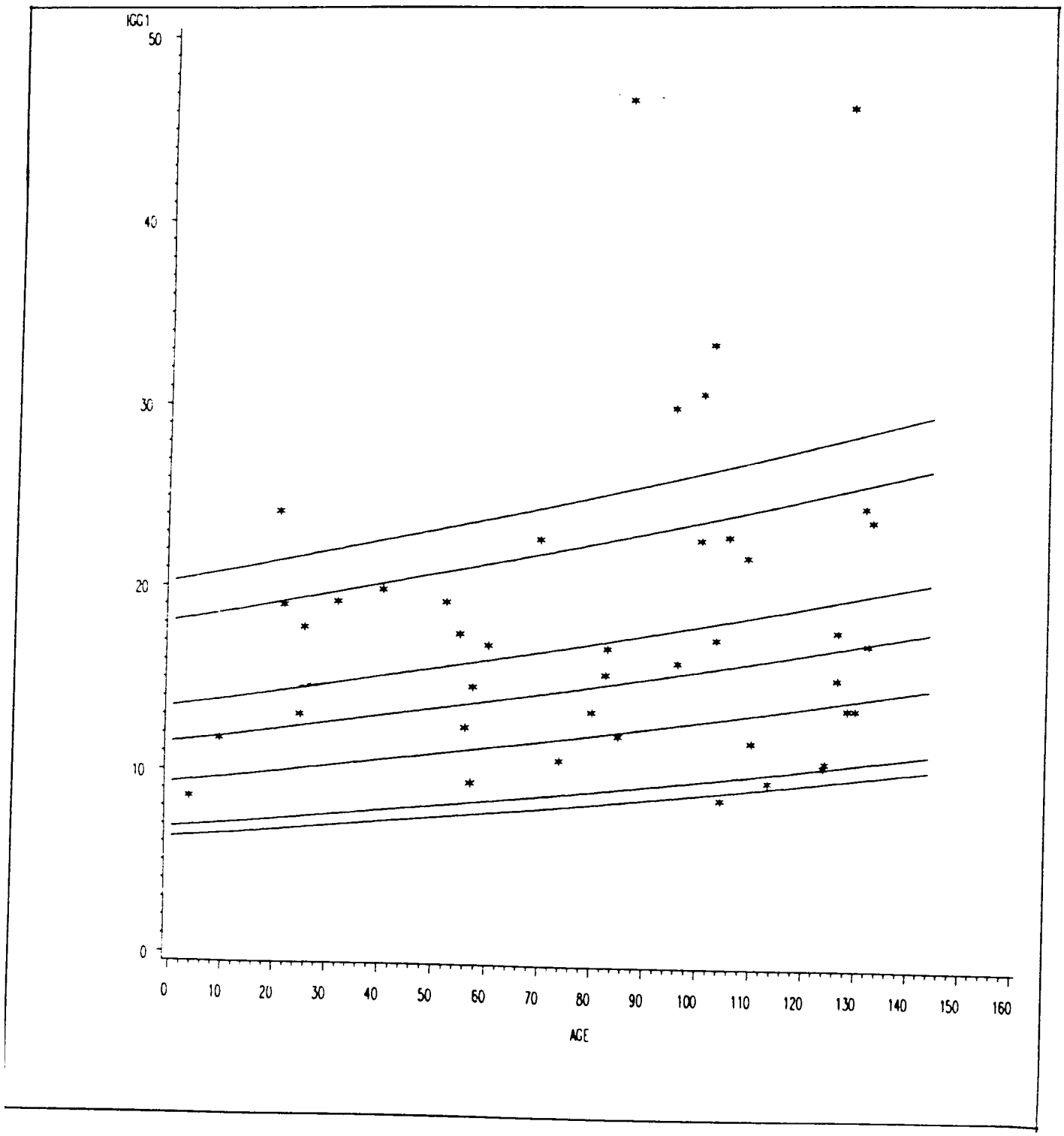

BLACK PATIENTS WITH *S.AUREUS* OM/SA

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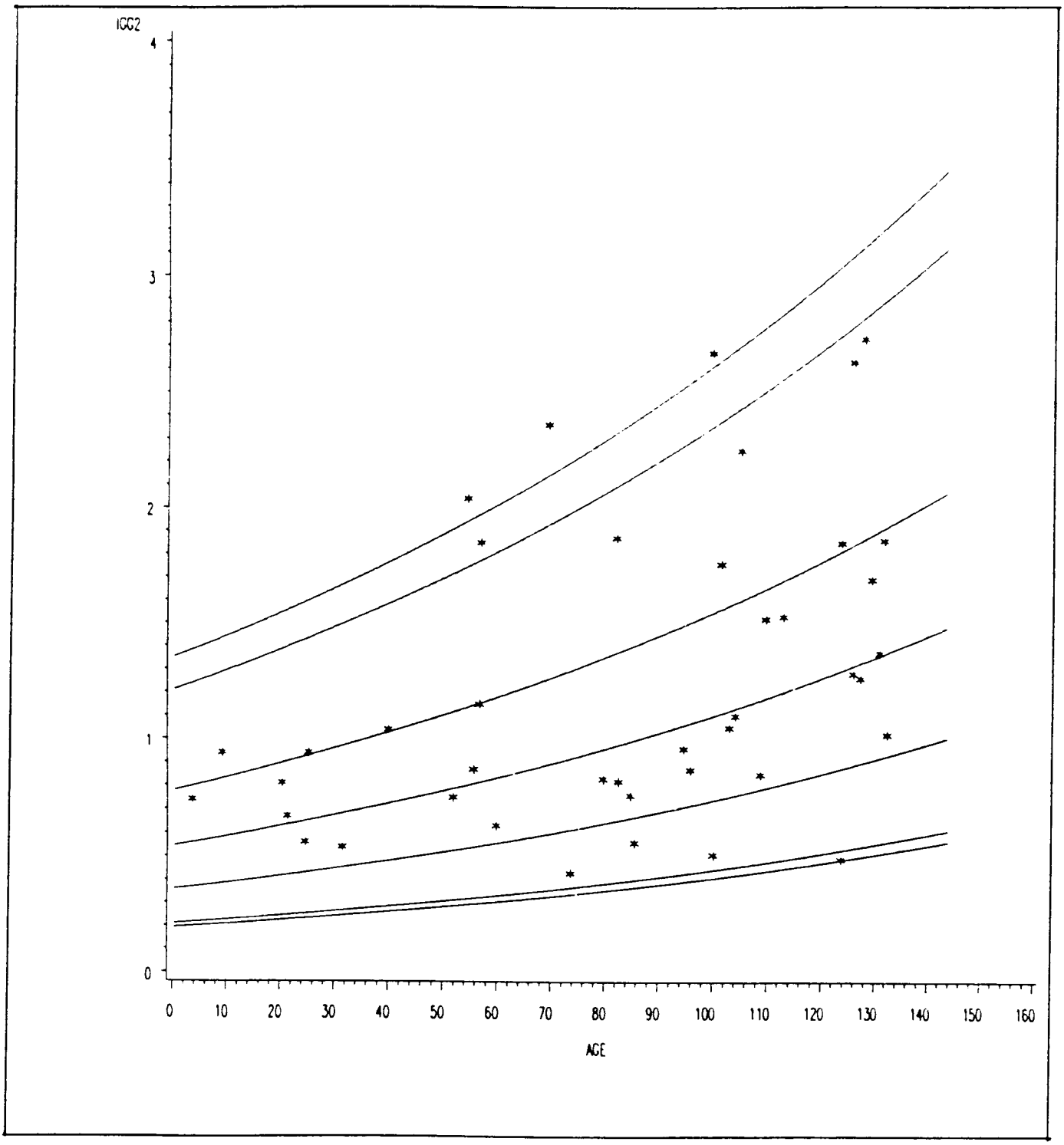

BLACK PATIENTS WITH *S.AUREUS* OM/SA

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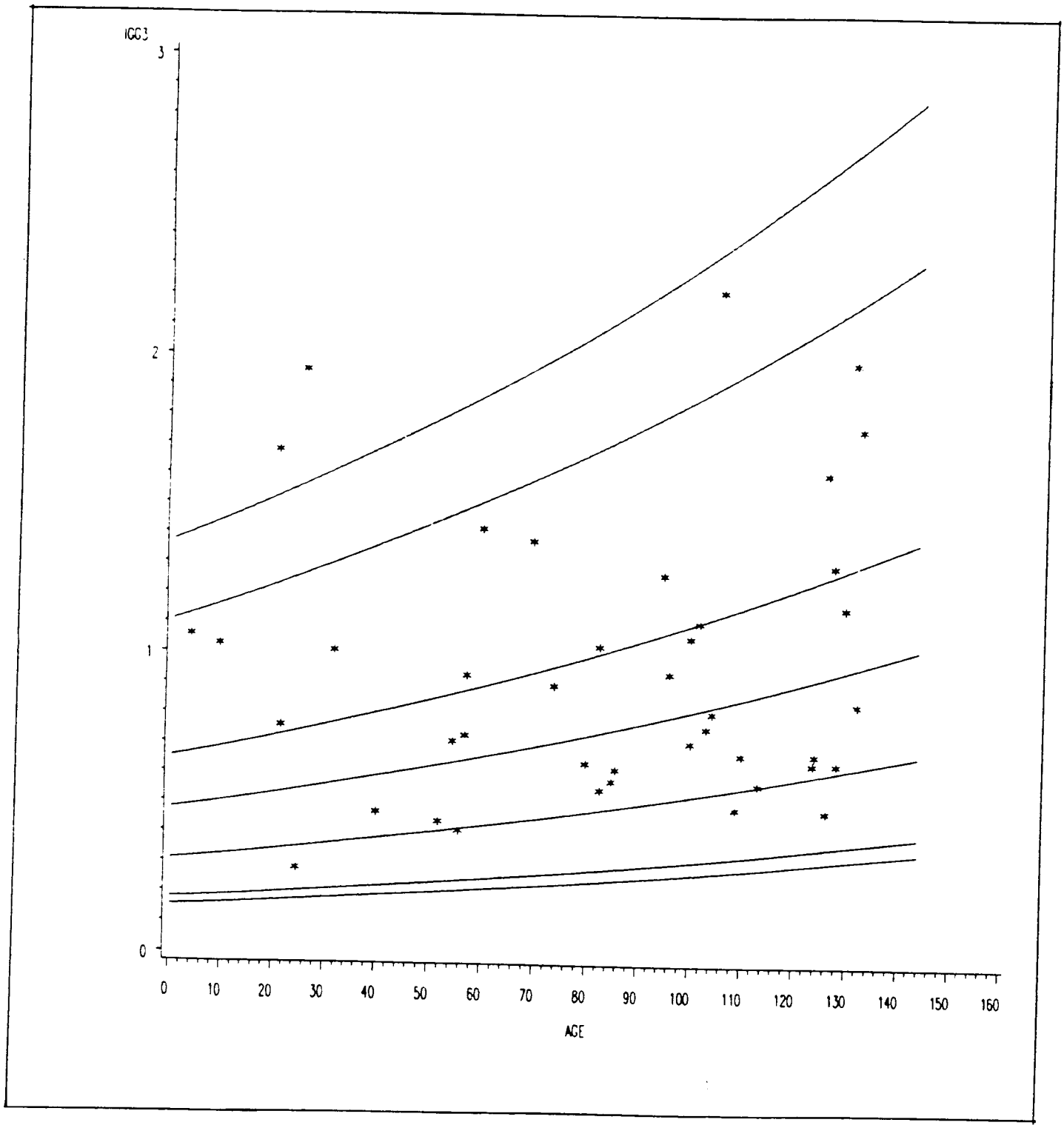

BLACK PATIENTS WITH *S.AUREUS* OM/SA

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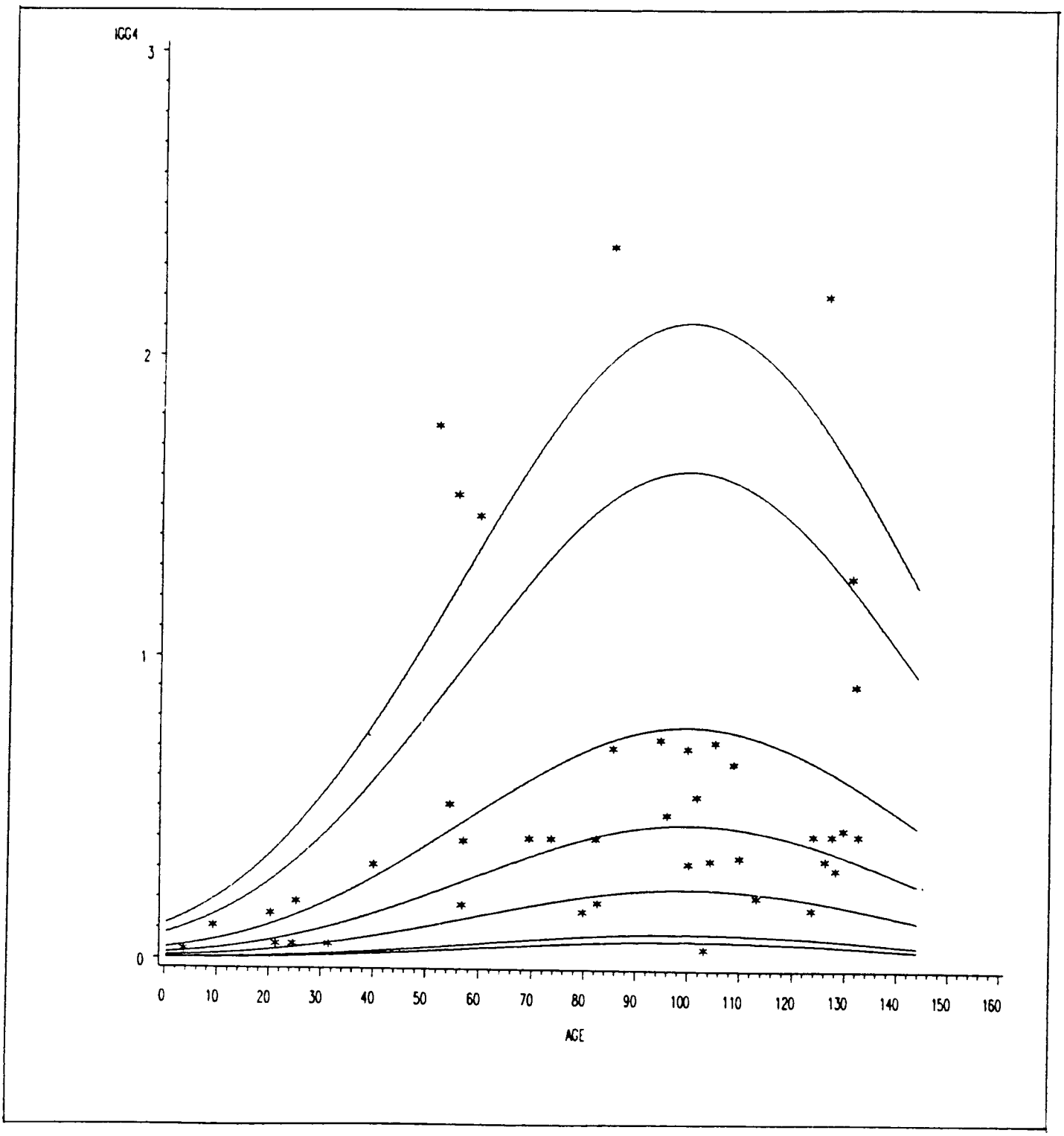
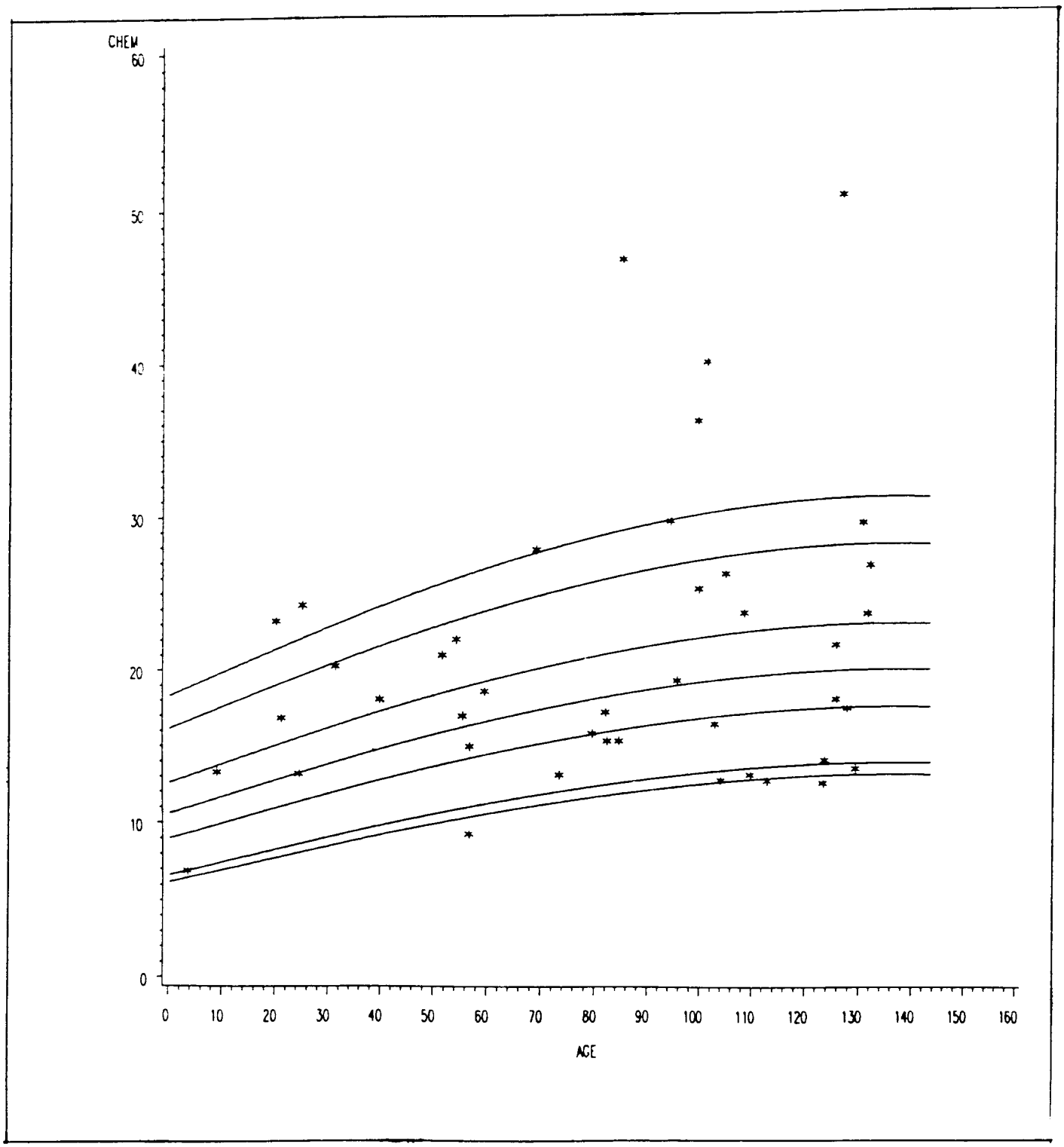


BLACK PATIENTS WITH *S.AUREUS* OM/SA

IgG



COLOURED MALE PATIENTS WITH *S.AUREUS* OM/SA

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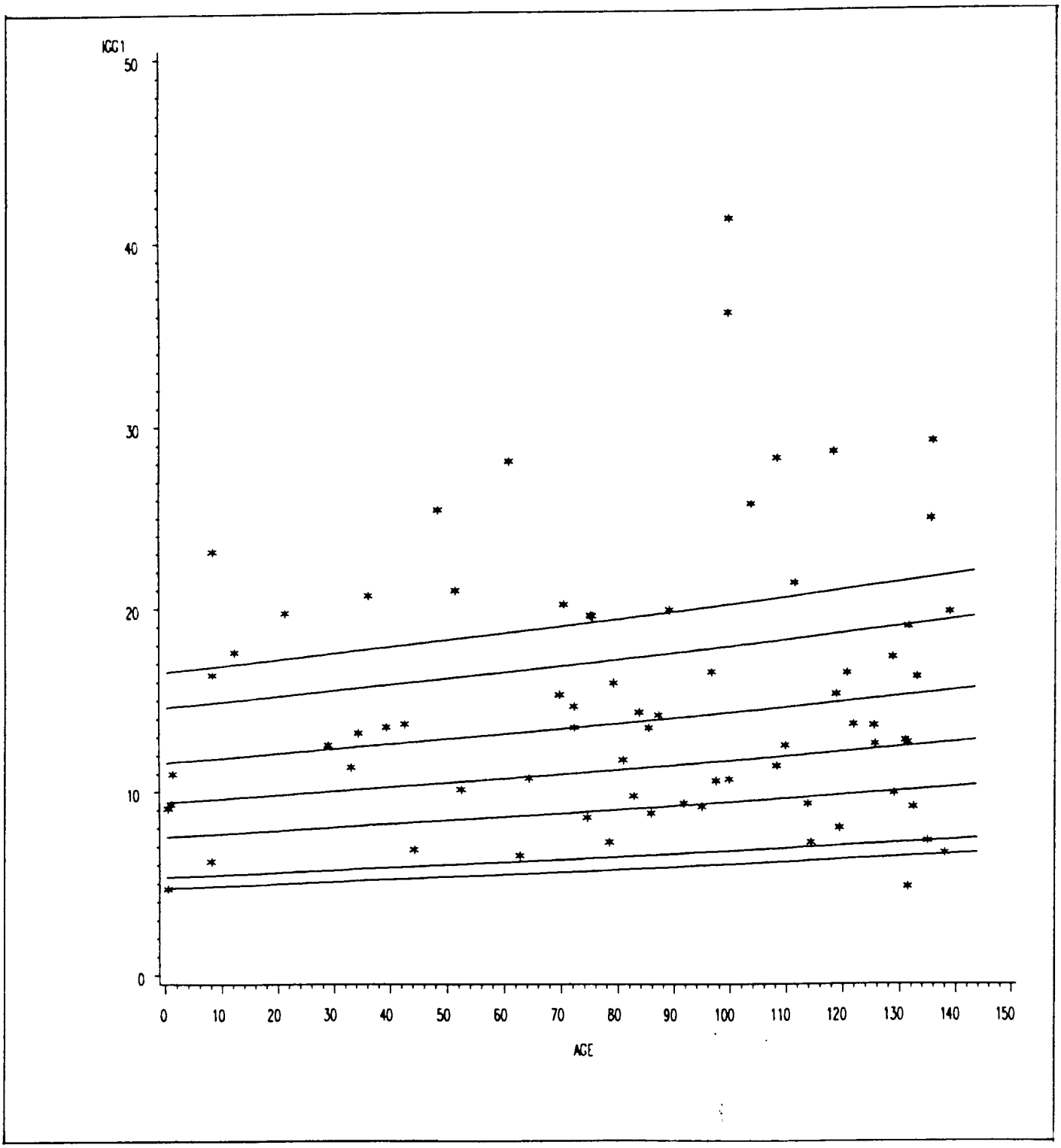
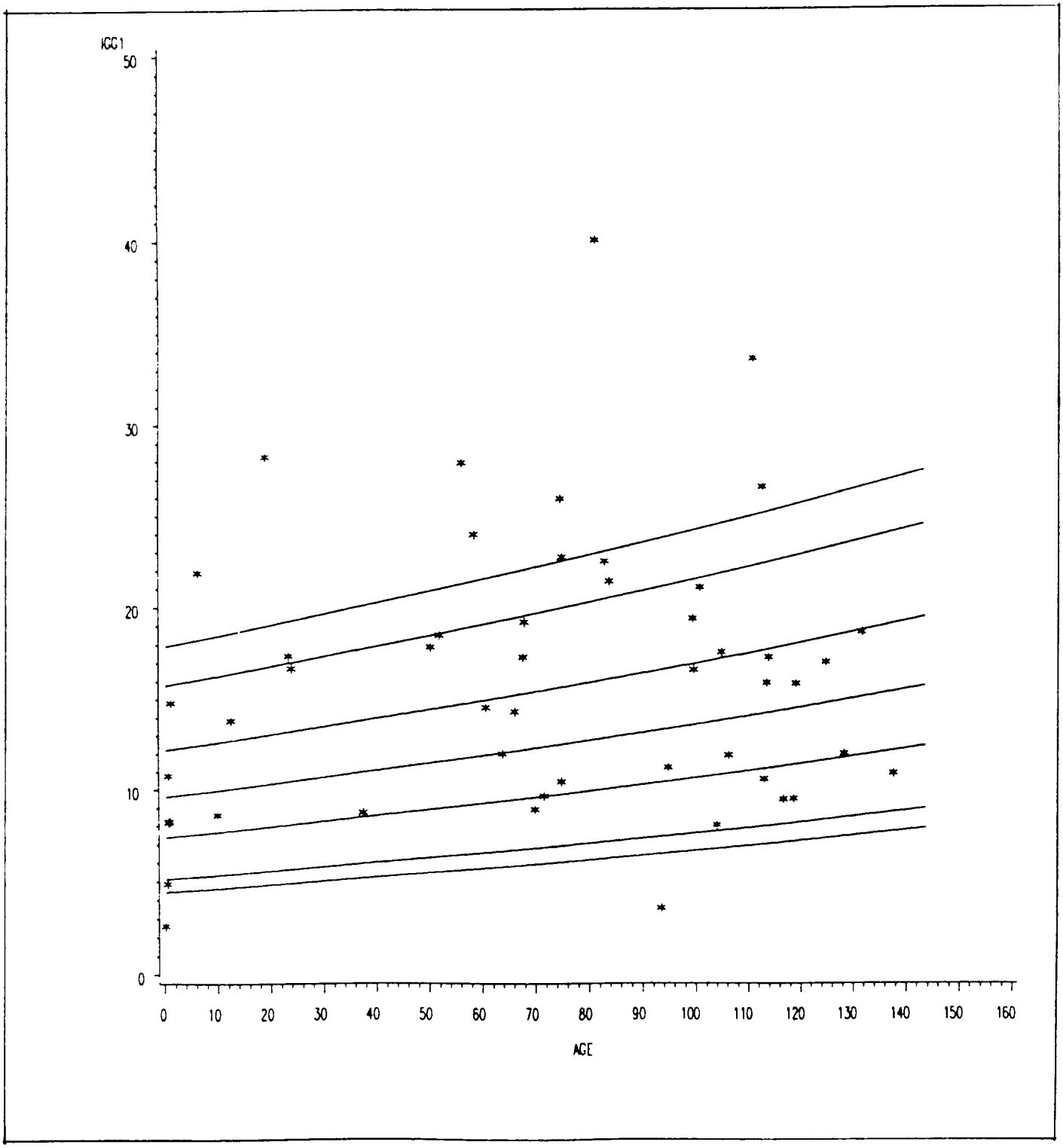


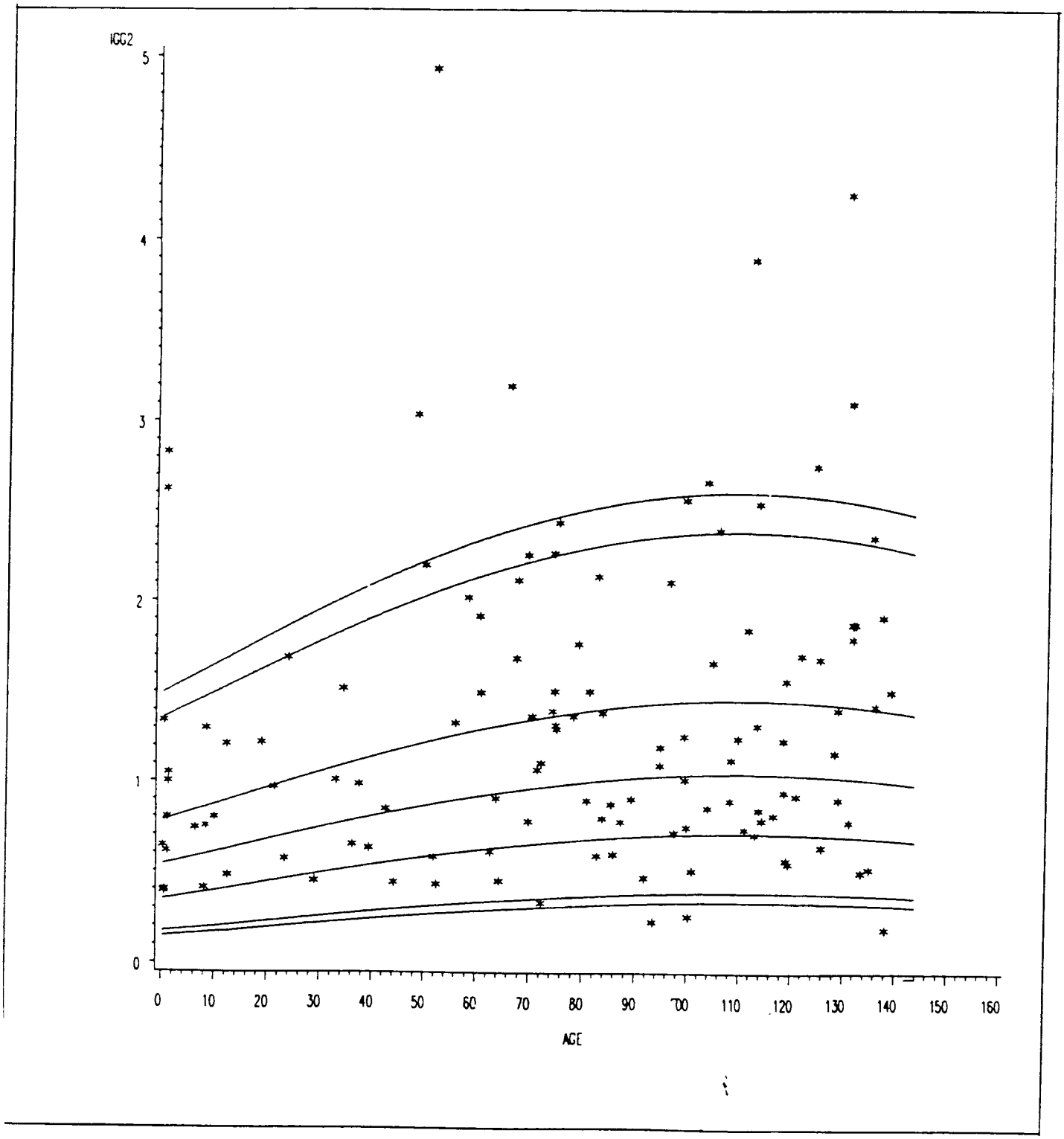
COLOURED FEMALE PATIENTS WITH *S.AUREUS* OM/SA

IgG1



COLOURED MALE AND FEMALE PATIENTS WITH *S.AUREUS* OM/SA

IgG2



COLOURED MALE PATIENTS WITH *S.AUREUS* OM/SA

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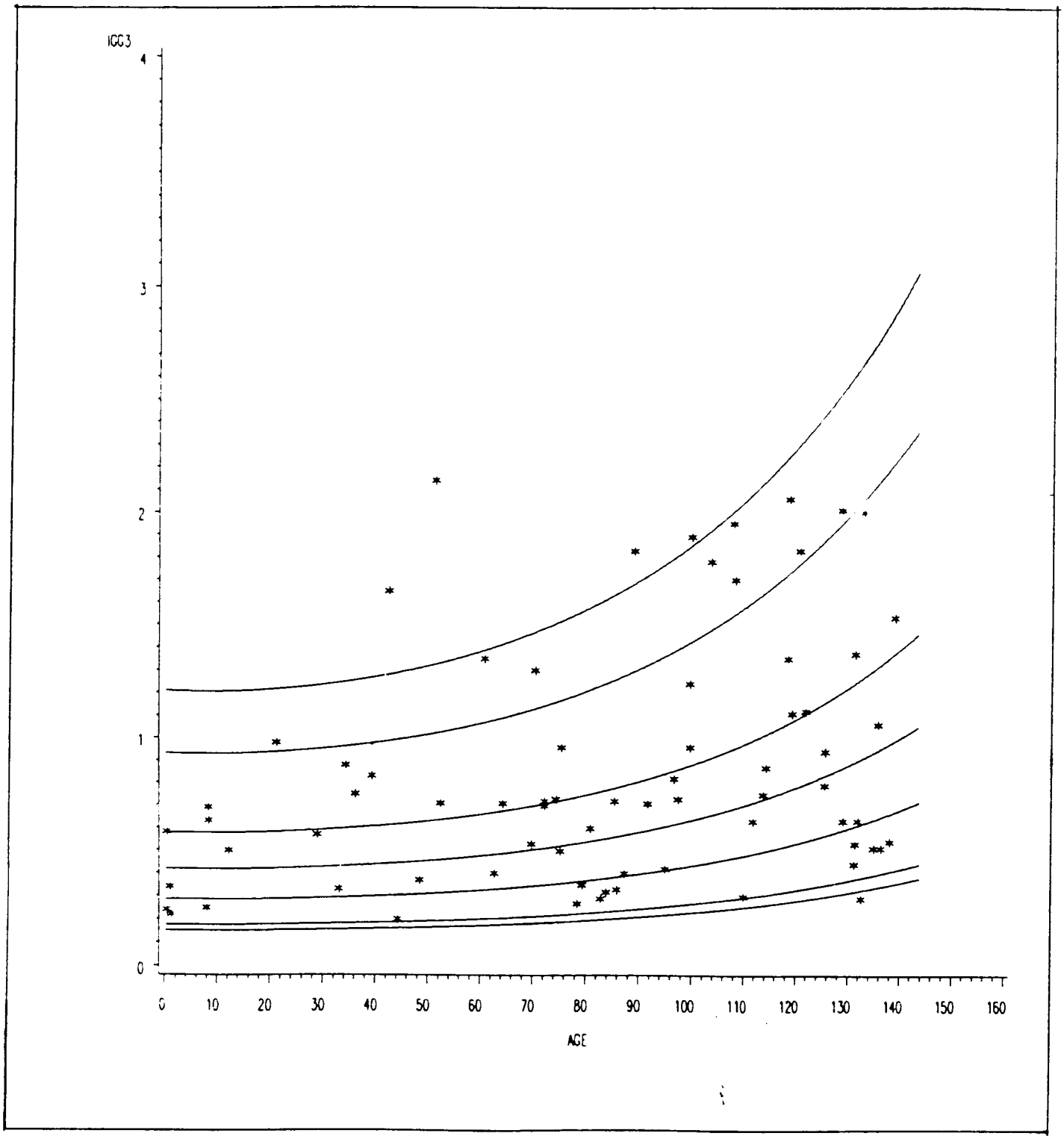

COLOURED FEMALE PATIENTS WITH *S.AUREUS* OM/SA

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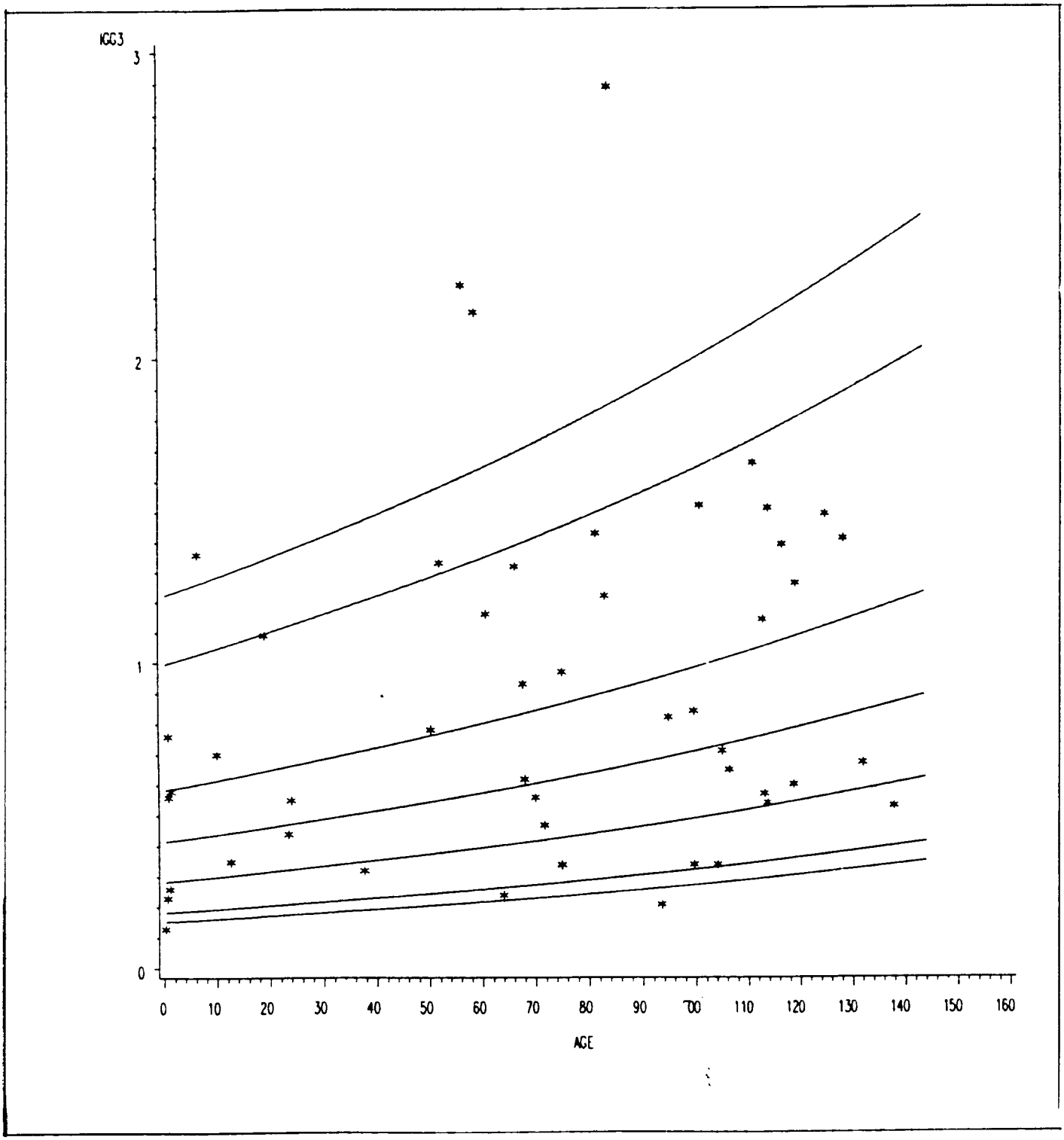

COLOURED MALE PATIENTS WITH *S.AUREUS* OM/SA

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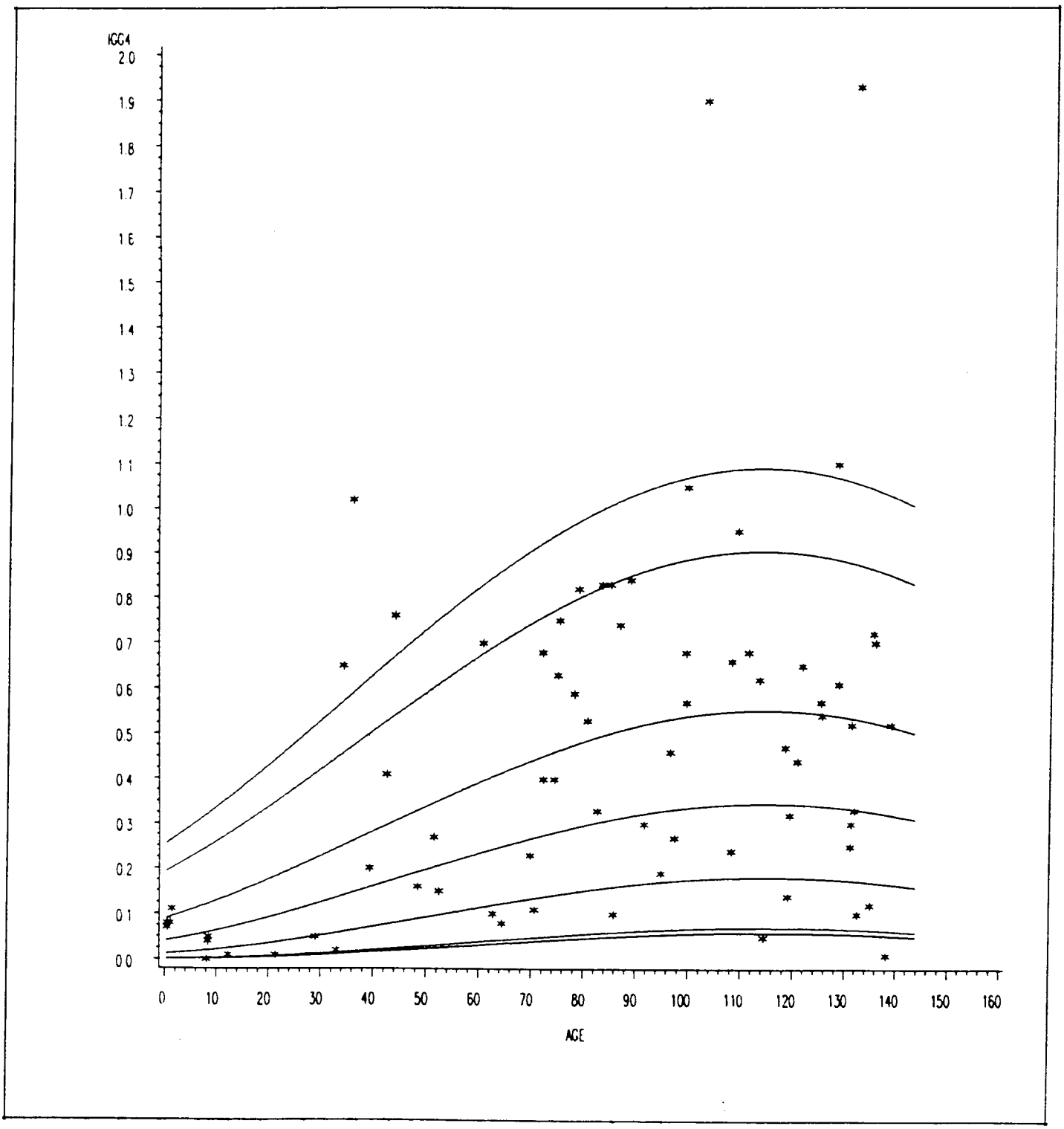
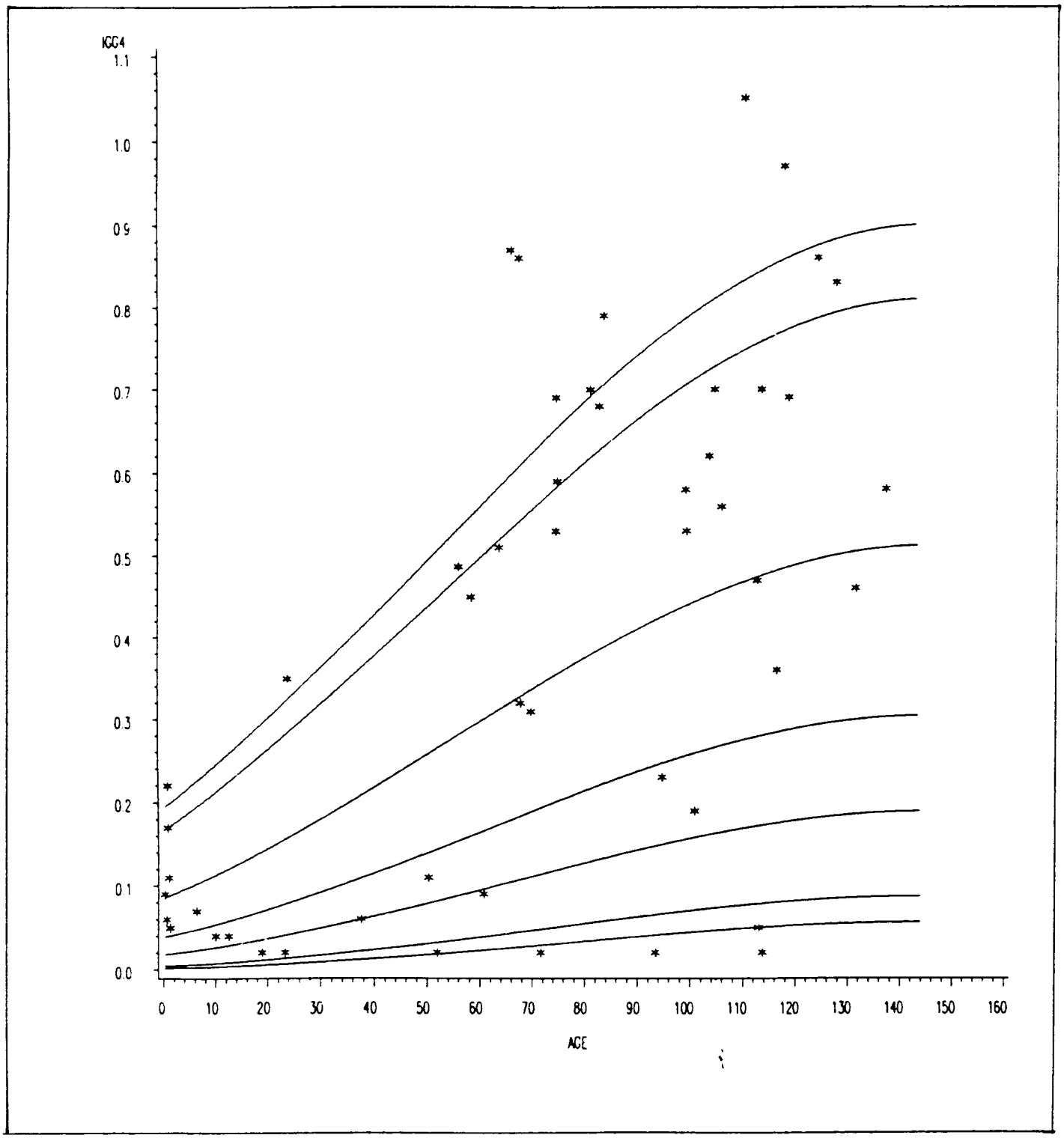


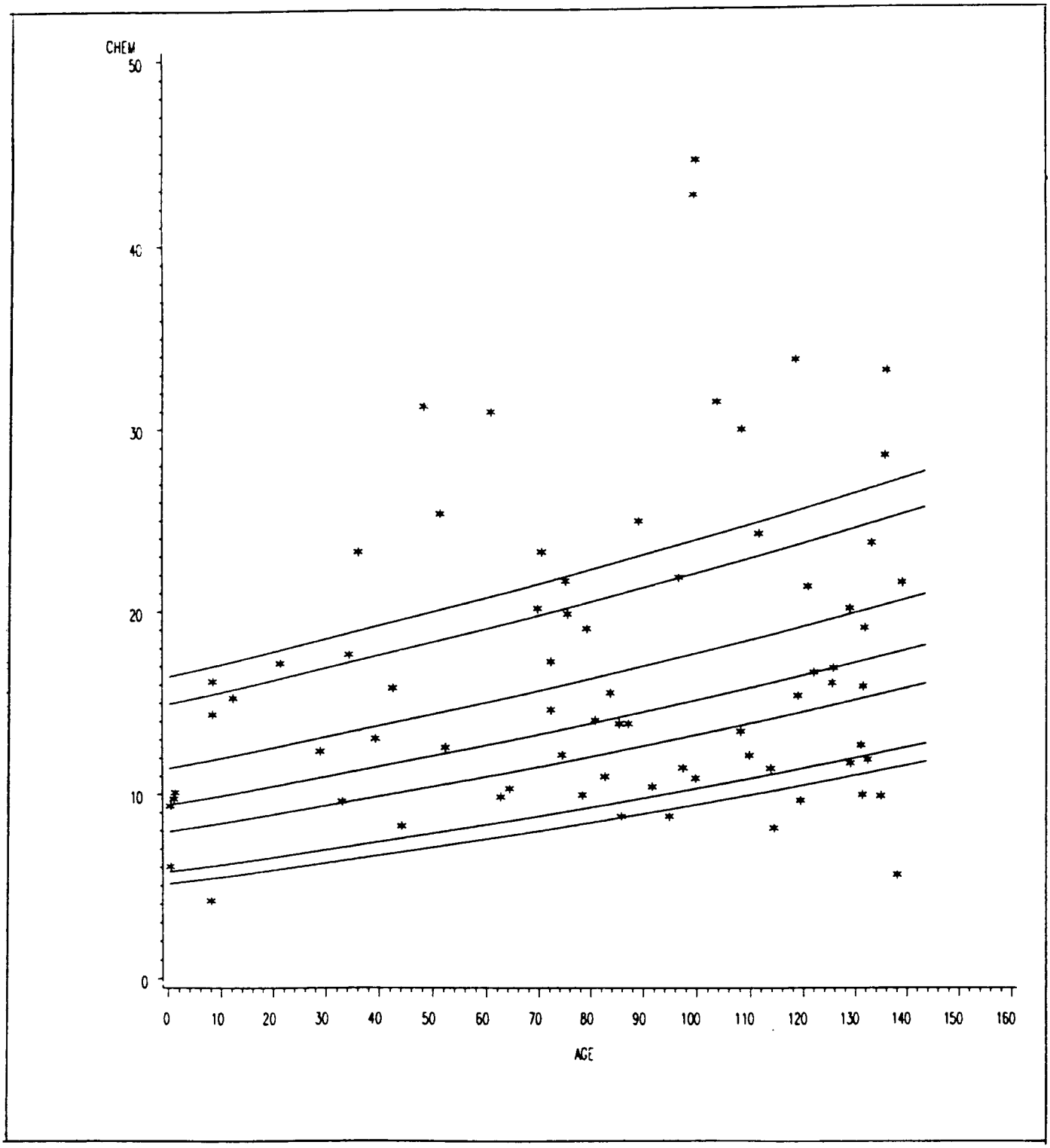
COLOURED FEMALE PATIENTS WITH *S.AUREUS* OM/SA

IgG4



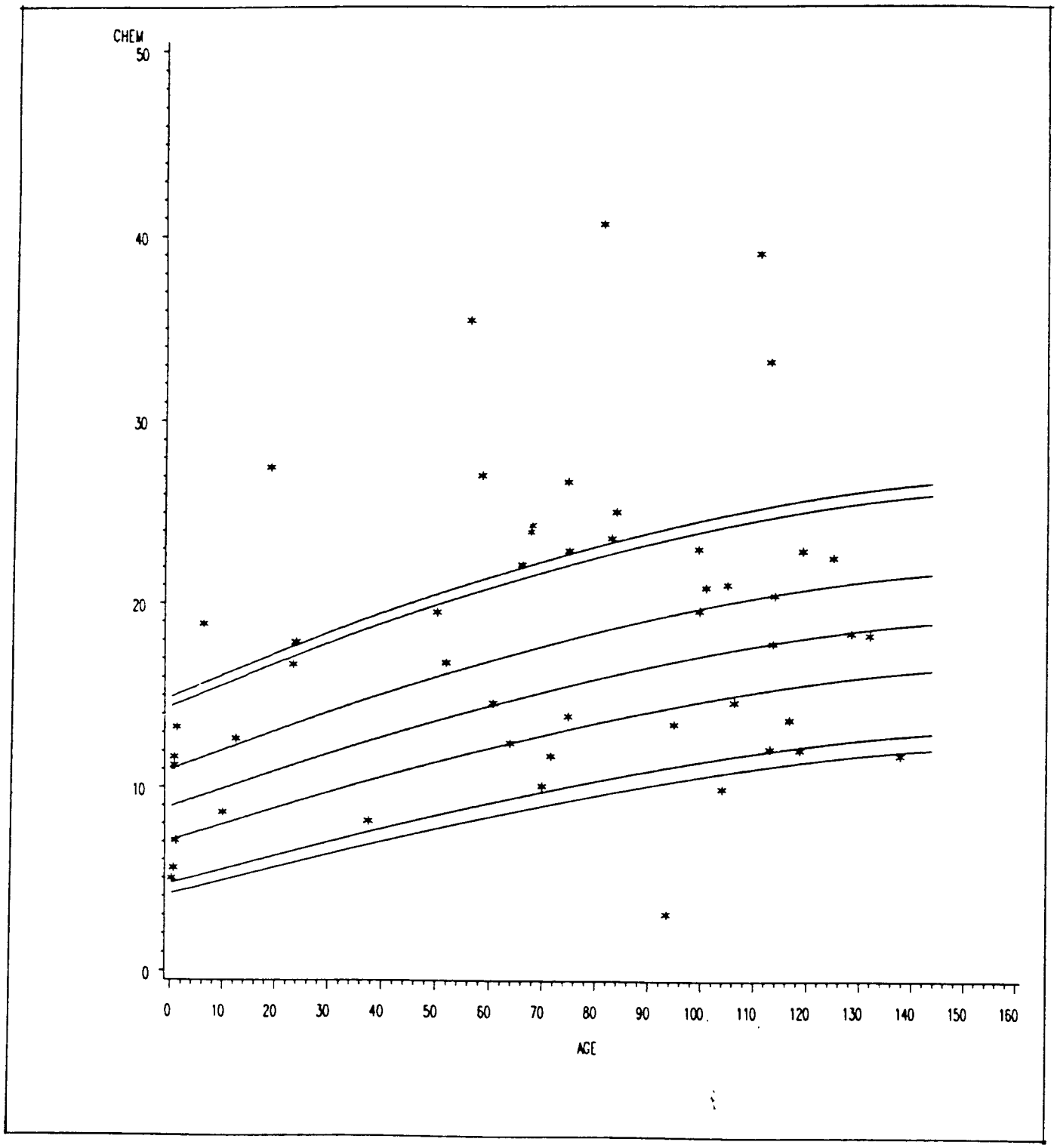
COLOURED MALE PATIENTS WITH *S.AUREUS* OM/SA

IgG



COLOURED FEMALE PATIENTS WITH *S.AUREUS* OM/SA

IgG



BUFFERS AND SOLUTIONS

Buffers and Solutions

1. Glycine/Tris Buffer

Stock Solution

Glycine (0.05M) (Merck)	3.75 g

Dissolve in 900 ml distilled water

Adjust to pH of 2.5 with IN HCl

Make up to 1000 ml with distilled water

Store at 4°C

Working solution

Add solid Tris (Boehringer Mannheim) to stock solution to pH of 7.2

Make up freshly before use

ii. Phosphate Buffered Saline (PBS)

10x PBS

NaCl (137 mM) (Univar)	400 g
KCl (2.68 mM) (NT Lab Supplies)	10 g
Na_2HPO_4 (8.1 mM) anhydrous (BDH Analar)	57.5 g
KH_2PO_4 (1.47 mM) (BDH Analar)	10 g

Make up to 5000 ml with distilled water

Dilute 1:10 before use

pH 7.3

iii. PBST

Add 250 μl (0.005%)* Tween 20- (Sigma) (Catalogue No P1379) to 5 litres PBS

Add 2500 μl (0.05%)** Tween 20 to 5 litres PBS

* 0.005% PBST used in subclass ELISAs

** 0.05% PBST used in tetanus toxoid specific ELISAs

iv. 0.25% BSA in PBST

BSA (Boehringer Mannheim) (Catalogue No 735086)	0.25 g

Dissolve in 100 ml PBST

v. Tris Buffered Saline (TBS)

Tris* (10 mM) (Boehringer Mannheim)	1.21 g
Tris** (20 mM) (Boehringer Mannheim)	2.42 g
NaCl (154 mM)) (Univar)	9 g

Dissolve in 900 ml distilled water

Adjust to pH of 7.5 with 1N HCl

Make up to 1000 ml with distilled water

* 10 mM Tris used in IgG subclass and tetanus toxoid ELISAs

** 20 mM Tris used in Gm and Km allotype ELISAs

vi. TBST

Add * 100 μl (0.01%) Tween 20 (Sigma) (Catalogue No P1379)

Add ** 500 μl (0.05%) Tween 20 (Sigma) (Catalogue No P1379) to 1 litre TBS

* 0.01% TBST used in washing

** 0.05% TBST used in blocking

vii. 1% Casein in 10 mM TBS

Casein (1%) (Hammarsten) (Catalogue No 44020)	10 g

Dissolve in 1000 ml 10 mM TBS

Store at 4°C

pH 7.6

1% casein in PBS

Casein (1%) (Saarchem) (Catalogue No 152813)	10 g

Dissolve in 1000 ml PBS pH 7.6

Store at 4°C

pH 7.6

viii. 2% Bovine serum albumin (BSA) in 0.05% TBST (20 mM)

Gm allotype blocker

BSA fraction V (2%) (Boehringer Mannheim) (Catalogue No 735086)	20 g

Dissolve in 1000 ml 20 mM TBS

Add 500 μl (0.05%) Tween20

Store at 4°C

1:10 blocker solution

Gm allotype diluent

0.2% BSA/TBS/0.005% Tween20

ix. Carbonate buffer

Na_2CO_3 (15 mM) anhydrous (BDH Analar)	1.59 g
$NaHCO_3$ (35 mM) (BDH Analar)	2.93 g
Thymol (0.67 mM) (BDH Analar)	0.1 g

Dissolve in 1000 ml distilled water

Note that the pH is 9.6

x. O.1 M Citrate Buffer

Solution A

Citric Acid (0.1 M) (BDH Analar)	21 g

Dissolve in 1000 ml distilled water

Solution B

Tri Sodium Citrate (0.1 M) (BDH Analar) 29.4 g

Dissolve in 1000 ml distilled water

Solution A *20.5 ml*

Solution B *29.5 ml*

Make up to 100 ml with distilled water

Adjust to pH of 5.0 with 4 M NaOH

Note that the pH is critical

xi. o-Phenylenediamine dichloride (OPD) solution

Note that OPD (Sigma) (Catalogue No P1526) may be weighed in small aliquots and stored at -20°C in the dark

Add 2.5 ml citrate buffer per mg of OPD

Add 1 μl of 30% H_2O_2 per ml of citrate buffer

Make up freshly before use

xii. 2N H_2SO_4

Formula: N required = $\frac{\text{Equivalent weight}}{\text{Specific Gravity}} \times \frac{100}{\%\ \text{Impurities}}$

H_2SO_4 (NT Lab Supplies) 53.3 ml

Add to 500 ml distilled water and make up to 1000 ml when cool

xiii. 1% Agarose/3.8% PEG

Agarose A (1%) (Pharmacia) (Catalogue No 17-0422-01) 1 gm

0.05 M Phosphate buffer, pH 7.4 ± 80 ml

Dissolve agarose in buffer, by heating and stirring,

in boiling waterbath.

Add: PEG 6000 (BDH) 3.8 gm

Dissolve as above, with swirling

Add:	NaCl	0.9 gm
	Na Azide	0.2 gm

Make up to 100 ml with 0.05 M Phoshpate buffer

Aliquot and store in 10 ml amounts

For use: Dissolve in boiling waterbath

Pour onto glass slide or Gelbond

xiv. 0.9% NaCl

NaCl	9 gm
Distilled water	1 litre

xv. Amido Black Stain

Amido Black	0.5 gms
Methanol	90 ml
Glacial Acetic Acid	10 ml

Dissolve Amido Black in Methanol/Acetic acid

xvi. Destainer

Methanol	900 ml
Glacial Acetic Acid	100 ml

xvii. 2% acetic acid

Glacial acetic acid	2 ml
Distilled water	98 ml

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