Qualitative and Quantitative aspects of Human IgG subclass responses with special reference to a common microorganism, *Moraxella (Branhamella) catarrhalis.*

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ABSTRACT

Although much is known of the physico-chemical characteristics of the IgG subclasses, their specific biological function remains poorly understood. The consequence of either absence or low levels of one or more subclasses is still unclear. In an effort to document the clinical manifestation of IgG subclass deficiency in a paediatric population, a clinical study of children presenting with IgG subclass deficiency was undertaken. 42% of the 232 children reviewed presented with frequent infections, 17% presented with allergy and 13% presented with a combination of infection and allergy. No clear trends emerged relating the deficiency of a particular subclass with any given clinical manifestation.

Since healthy individuals may have low levels or a complete absence of certain IgG subclasses the functional study of IgG subclasses needs to go beyond their quantitative estimation. It is known that individuals with normal IgG subclass levels may nevertheless suffer from frequent infection due to an inability to respond in an isotype appropriate way to carbohydrate antigens. This phenomenon suggests that an appropriate qualitative assessment is needed to further elucidate IgG subclass function. To this end a study of *Moraxella (Branhamella) catarrhalis* was undertaken. This bacterium is a ubiquitous gram negative organism which is emerging as an important paediatric pathogen. While healthy children may acquire infection with this organism, in adults it infects predominantly those with compromised immunity or chronic lung disease. To investigate whether this difference might have an immunological basis, a study of the antigens of *M.catarrhalis* and the age related IgG subclass response to them was undertaken. The surface antigens were defined by the purification of outer membrane proteins (OMPs) followed by SDS-PAGE and immunoblotting. This revealed that the OMPs were targets of human antibody and, furthermore, this recognition differed between

the various isotypes with IgG3 recognising an extended range of proteins. By modification of a newly developed enzyme-linked immunosorbent assay (ELISA) the binding affinities of the *M.catarrhalis* specific IgG subclasses were measured and IgG3 antibodies were found to be of higher affinity than IgG1 and IgG2 antibodies, further emphasising the importance of this isotype. An ELISA procedure was also used in an analysis of the age related appearance of IgG subclasses specific for the organism and this revealed that IgG3 antibody was undetectable in children under the age of four yet present in healthy adults and older children. Age related delay in IgG subclass antigen specific responses has previously only been described for carbohydrate antigens and the IgG2 response. Extending these findings to IgG3 regulation and function emphasises the need to acquire antibody response data on both an organism and subclass specific basis.

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Publications

The following publications form part of the work presented in this thesis:

Goldblatt D, Morgan G, Seymour N, Strobel S, Turner MW, Levinsky RJ. The clinical manifestations of IgG subclass deficiencies. International Congress and Symposium Series. Royal Society of Medicine. 1989;143:19-26

Goldblatt D, Seymour N, Levinsky RJ, Turner MW. An enzyme-linked immunosorbent assay for the determination of human IgG subclass antibodies directed against *Branhamella catarrhalis*. J Immunol Methods 1990;128: 219-225

Goldblatt D, Turner MW, Levinsky RJ. *Branhamella catarrhalis:* Antigenic determinants and the development of the IgG subclass response in childhood. J Inf Dis 1990;162:1128-1135

Goldblatt D, Turner MW, Levinsky RJ. Delayed maturation of IgG3: A variant of paediatric immunodeficiency? International Congress and Symposium Series, Royal Society of Medicine. 1991;173:109-114

Declaration

All the work presented in this thesis is that of the candidate with the following exceptions which are found in Chapter 5 and the appendix:

(a) Total serum IgG3 levels (Figure 5.10 and Appendix C) were measured by Nigel Seymour in the Department of Immunology at the Institute of Child Health

(b) Assessment of the specificity of the monoclonal antibodies using myeloma proteins (Appendix A) was performed by Nigel Seymour in the Department of Immunology at the Institute of Child Health

(c) Gm allotyping (Table 5.2) was performed as part of a collaborative project by Professor JP Pandey in the Department of Microbiology and Immunology at the Medical University of South Carolina, Charleston, South Carolina, USA.

Abbreviations

ADCC	antibody-dependent cell-mediated cytotoxicity
APS	ammonium persulphate
C_H	Constant heavy chain domain
C_L	Constant light chain domain
DEA	Diethylamine
EBV	Epstein-Barr virus
E. coli	*Escherichiae coli*
ELISA	enzyme linked immunosorbent assay
Fab	Fragment antigen binding
Fc	Fragment crystalline
$Fc\gamma R$	Fc gamma receptor
Hib	*Haemophilus influenzae* type b
$IFN-\gamma$	Interferon gamma
kDa	kilodalton
HUVEC	human umibilical vein endothelial cell
Ig	immunoglobulin
LOS	lipooligosaccharide
LPS	lipopolysaccharide
LRTI	lower respiratory tract infection
M. catarrhalis	*Moraxella (Branhamella) catarrhalis*
MHC	major histocompatibility complex
MW	molecular weight
N. meningitidis	*Neisseria meningitidis*
OMP	outer membrane protein
OMV	outer membrane vesicles
OPD	o-phenylenediamine
P. aeruginosa	*Pseudomonas aeruginosa*
PBMC	peripheral blood mononuclear cell
PHA	phytohaemagglutinin
PWM	pokeweed mitogen
RAST	radioallergosorbent test
S. pneumoniae	*Streptococcus pneumoniae*
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SpA	Staphylococcal protein A
URTI	upper respiratory tract infection
V_H	Variable heavy chain domain
V_L	Variable light chain domain

CHAPTER 1

IgG subclasses: General Introduction

(a) Immunoglobulins and humoral immunity

The recognition of a foreign cell or substance triggers a complex set of events that results in the acquisition of specific immunity against the corresponding antigen(s). The contribution of antibodies to this specific immunity is termed *humoral immunity* because antibodies, by virtue of their solubility, were initially noted to be present in all body fluids ("humors"). Great progress has been made in the last 30-40 years in our knowledge of the nature of antibodies and since the 1950's the existence of several major classes of antibodies has been recognised. Some of the physicochemical, metabolic and biological properties of the five major immunoglobulin classes are summarised in table 1.1(a) and (b)

Table 1.1(a): Physical, chemical and metabolic properties of the human immunoglobulins

	IgG	IgM	IgA	IgD	IgE
Basic structural form	monomer	pentamer	mon/dimer	monomer	monomer
Heavy chain	γ1-4	μ	α1-2	δ	ϵ
Subclasses	4	-	2	-	-
Molecular weight	150 000	950 000	160-380 000	175 000	190 000
Sedimentation constant	6.6S	19S	7S/11S	7S	8S
Electrophoretic mobility (pH 8.6)	γ	Fast γ to β	Fast γ to β	Fast γ	Fast γ
Carbohydrate content (%)	2-3	12	7-11	9-14	12
Valency for antigen	2	5(10*)	2/4	2	2
Distribution (% intravascular)	50	80	50	75	50
Mean adult serum level (mg/ml)	13.5	1.5	3.5	0.03	5×10^{-5}
Half-life (days)	23	5.8	5.1	2.8	2.5

* IgM interactions with haptenic antigens

Table 1.1(b): Biological properties of the human immunoglobulins

	IgG	IgM	IgA	IgD	IgE
Major biological properties	2° Ab response	1° Ab response	Secretory form on mucosal surfaces and in secretions	B cell surface marker	Anaphylaxis, allergy
Placental transfer	+	-	-	-	-
Complement fixation	+	+	-	-	-
Interactions with Fc receptors on:					
Neutrophils	+	-	-	-	-
Eosinophils	+	-	-	-	+
Basophils	-	-	-	-	+
Lymphocytes	+	+	+	+	+
Mast cells	-	-	-	-	+
Platelets	+	-	-	-	?
Macrophages	+	-	-	-	+

IgG is the major immunoglobulin in normal human serum and accounts for 70-75% of the total immunoglobulin pool. IgG production is dominant during the secondary immune response, where it is produced early on in the response and demonstrates an increase in affinity with time. The inability to produce IgG is associated with life long susceptibility to infection. The importance of this immunoglobulin isotype in protection from infection has concentrated research on the structure and function of this molecule.

(b) Immunoglobulin G: General Structure

All IgG molecules are glycoproteins composed of two heavy and two light chains linked together by interchain disulphide bonds as well as other non-covalent forces (eg hydrogen bonding). It is convenient to regard the IgG molecule as comprising three structural units; two are identical and are involved in antigen binding (the fragment antigen binding or Fab portion of the molecule) and the third is the fragment crystalline or Fc portion which is involved in interactions related to

antigen elimination. The structure thus reflects the dual role of IgG in recognising that which is foreign and then attempting to eliminate it.

The Fab arms are linked to the Fc via a region of the polypeptide chain known as the hinge which is sensitive to proteolytic attack. The Fab arms are able to swing out to an angle of greater than 100° because there are numerous proline residues in the hinge region and the loose folding of this region provides flexibility. This flexibility accounts for the ability of the molecule to bind to two sites on a single particle (eg bacterium or virus) or to link two particles. An IgG molecule has a molecular weight of approximately 150 000 and is made up of two identical heavy chains (MW ~50 000) and two light chains (MW ~25 000). The light chains are solely associated with the Fab arms of the molecule whereas the heavy chain spans the Fab and Fc portion. A single disulphide bond connects each light chain to a heavy chain and a variable number of disulphide bonds, depending on IgG subclass, connect the two heavy chains in the hinge region. Papain cleaves heavy chains to the N-terminal side of these hinge disulphides producing two Fab and an Fc fragment. Pepsin cleaves to the C terminal side producing a divalent $F(ab')_2$ fragment and smaller fragments of Fc including a C-terminal pFc' fragment. The heavy chains of the human IgG subclasses are designated γ1, γ2, γ3, and γ4 to correspond to the four IgG subclasses IgG1, IgG2, IgG3 and IgG4. The light chains exist in two forms known as kappa (κ) and lambda (λ).The general features and domain structure of a representative IgG molecule are shown in Figure 1.1 (overleaf).
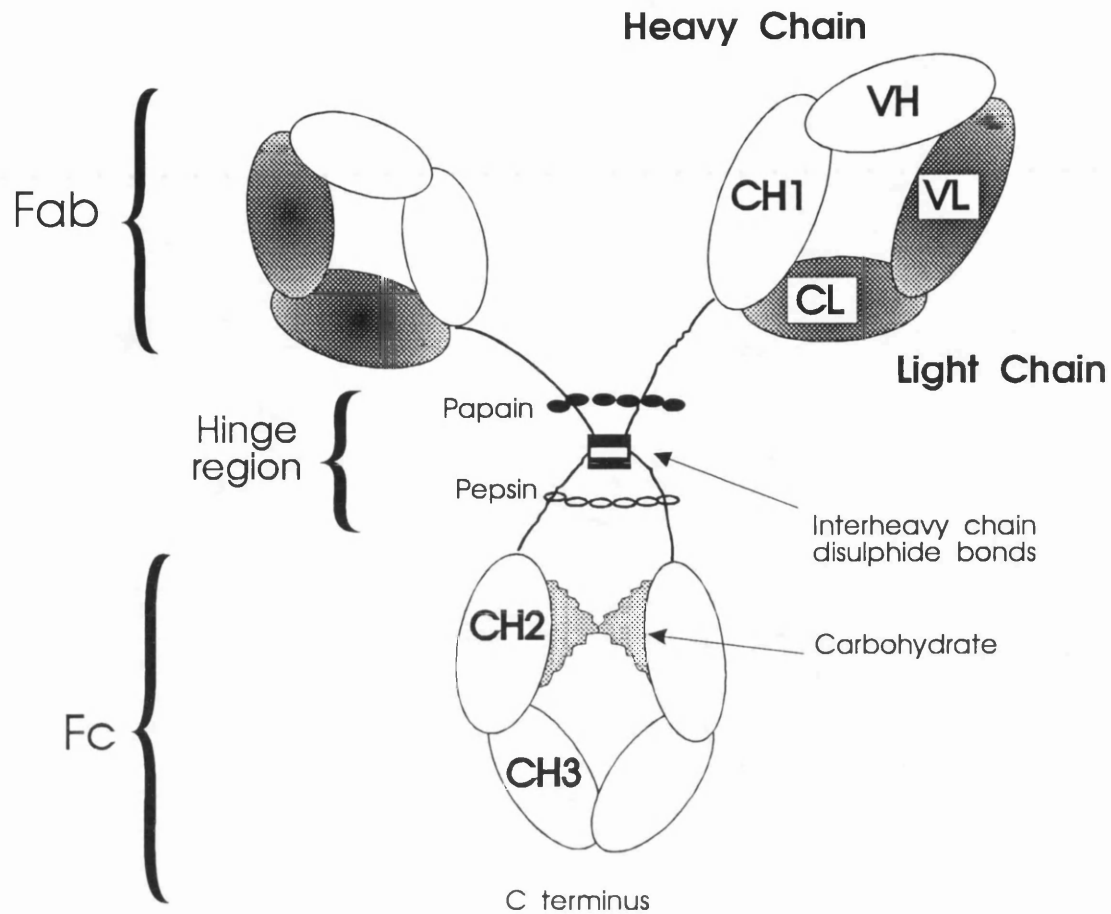
Figure 1.1: General features of a representative IgG molecule indicating the domain structure (after Jefferis, 1990).

Crystallographic studies of whole IgG and fragments of IgG have revealed that each domain has a common pattern of polypeptide chain folding. This folded structure is maintained by internal disulphide bridges between cysteine residues which are separated by 50-60 residues in the linear sequence of the variable or constant region. The characteristic tertiary structure, the immunoglobulin fold, is described as two surfaces of anti-parallel β-pleated sheets between which the disulphide bridge is formed. This characteristic folded structure is one of the features shared by proteins which are identified as members of the immunoglobulin supergene family.

The four individual domains making up the Fab region (V_H, V_L, C_H, C_L) are paired, and the interacting faces of the domains are predominantly hydrophobic, hence the driving force for domain pairing is the removal of these residues from the aqueous environment. The Fab arrangement is further stabilised by a disulphide bond between the C_H and C_L domains. Contact between V_H and V_L is also made by loops from each domain, the hypervariable loops, which come together in space to constitute the antigen binding sites. It is the extreme variability of these loops on the common framework of the immunoglobulin fold which provides for the enormous diversity of antigen recognition by antibodies while retaining the same basic structure. In the Fc region the two C_H3 domains are paired in a pattern similar to that found for the C_H-C_L interaction. The two C_H2 domains show no close interaction but have interposed between them two branched N-linked carbohydrate chains which do make some contact between one another (see Figure 1.1). The Fc carbohydrate chains appear to be important in resisting proteolysis and have also been suggested to have functional roles which will be addressed later.

The four subclasses of IgG were originally defined by the antigenic uniqueness of their heavy chains (Dray, 1960, Terry and Fahey, 1964) which is now known to be determined by the primary amino acid sequence of the constant region of the heavy chains. Each IgG heavy chain is composed of peptide sequences that are encoded by distinct gene segments. The genes for the immunoglobulin heavy-chain family are located in the human on the long arm of chromosome 14.

The N-terminal 110 amino acids constitute the variable region that, in combination with the variable region of the light chain generates the antigen specific binding domain. The remainder of the heavy chain is of constant amino acid sequence for molecules of the same class or allotype. Examination of the primary structure of the heavy chain reveals repeating units of approximately 110 amino acid residues that exhibit very significant sequence homology. Comparison

within a heavy chain defines the V_H (variable heavy) domain and the C_H1, C_H2 and

C_H3 homology regions. The variable domain is the product of genetic

recombination events (see later) while the C_H1, C_H2 , C_H3 and hinge domains are

each encoded within separate exons at the DNA level. Comparisons between like

homology regions of each of the four IgG subclasses reveals >90% sequence

homology supporting the concept that the four human $C\gamma$ genes arose late in

evolution, probably by gene duplication and after the emergence of the primates

[Flanagan and Rabbitts, 1982]. A hypothetical scheme for the early duplication of

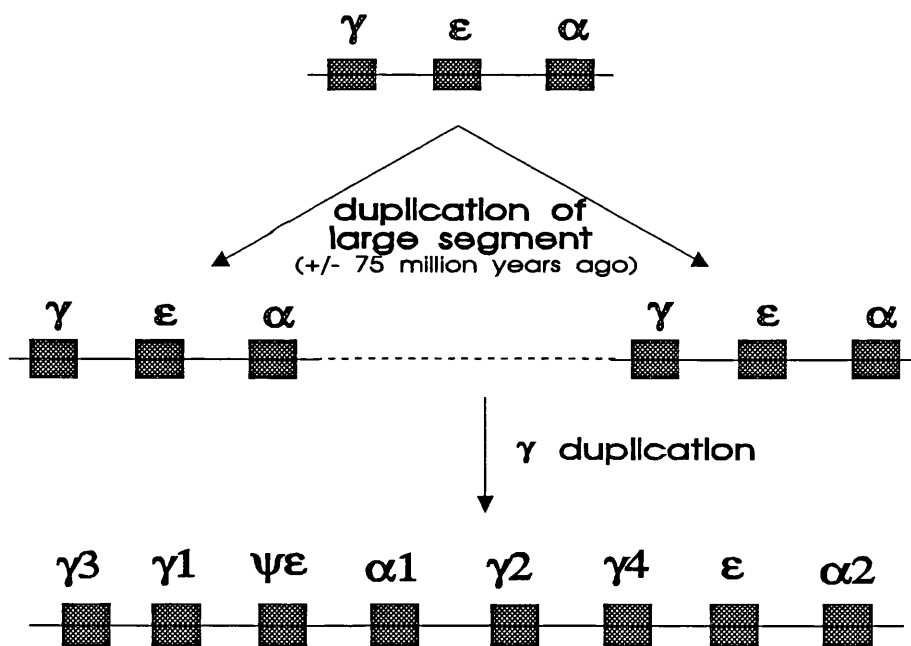the human immunoglobulin heavy chain gene is shown in Figure 1.2.

Figure 1.2: Hypothetical scheme for the evolution of the human immunoglobulin C_H locus. γ duplication could be followed by gene conversion between γ genes allowing for the observed differences between the structure of the four isotypes of IgG [after Flanagan and Rabbitts, 1980]. $\psi\varepsilon$ represents a non-expressed pseudogene for IgE.

In contrast to the four human IgG subclasses, sequence homology between the C_H1 and C_H2 domains in the mouse is much less (60-70%) indicating much earlier divergence, probably shortly after speciation of the mouse [Callard and Turner, 1990]. While all vertebrate species have Ig molecules, not all have IgG subclasses. The IgG subclasses found in mammalian species differ in number from species to species, and appear to be the product of recent evolutionary events and hence are not evolved directly from the lower vertebrates. Species in whom IgG subclasses have been identified include the guinea pig, hamster and oxen (2 subclasses), donkey, mule and zebra (3 subclasses), mouse and rat (4 subclasses) and the horse (5 subclasses) [Mota, 1986]. Some species such as the rabbit have no subclasses.

(c) Molecular control of immunoglobulin production, B cell differentiation and isotype switching

B cell precursors are generated from haemopoietic stem cells which arise in the fetal liver and adult bone marrow. B cell development is accompanied by discrete molecular rearrangements in the immunoglobulin (Ig) genes. During B cell differentiation an antigen specific B cell clone may switch the class of immunoglobulin it secretes although the antigen specificity of the Ig molecule essentially remains unchanged. The molecular events which lead to this phenomenon (isotype switching) are not fully delineated.

As previously discussed, antibody molecules are composed of two identical heavy and light chains. The heavy chain protein is encoded by a cluster of four closely linked families of genes (variable, V_H; diversity D, joining, J_H; and constant, C_H genes) located on the long arm of chromosome 14. A series of rearrangements results in the appropriate juxtaposition of single V, D and J segments to form a functional VDJ exon. The VDJ exon encodes the variable

region of the heavy chain peptide which, together with the L chain variable region provides the molecule with it's antigen specificity. The C_H gene encodes the part of the molecule which defines the isotype and hence the effector functions. The genes for κ and λ-light chains are located on chromosome 2 and 22 respectively and are rearranged in an analogous manner except that they do not possess D region genes.

The earliest Ig gene rearrangement is the juxtaposition of one of the D genes with one of the J_H genes. Following successful DJ rearrangement, selection and juxtaposition of a V_H gene adjacent to the rearranged DJ gene segment takes place. This allows a $C\mu$ gene to be transcribed and expressed in the cytoplasm of the first easily identifiable Ig^+ B lineage cell, the pre-B cell. Pre-B cells produce only μ-heavy chains with no light chains and express no surface Ig. Progression to the next stage of B cell differentiation requires the functional rearrangement of either κ or λ light chains in a manner similar to that of which occurs for the heavy chain genes. Rearrangements on the λ chromosome occur only if those on the κ have failed. The production of functional κ or λ light chains allow complete antibody molecules to be produced and expressed on the cell surface, giving rise to the immature B lymphocyte which in it's early stages expresses IgM on it's surface. As these B cells mature they may express IgD on their surface although even before the expression of IgD they are able to undergo isotype switching and express surface IgG, IgA or IgE. The end result of isotype switching of an IgM^+ B cell appears to be that the variable/diversity/joining (VDJ) gene segment, which encodes the hypervariable region and confers antigen specificity upon the Ig molecule, is expressed with a new heavy chain constant (C_H) Ig region gene downstream from the $C\mu$ gene. The C_H genes in man are located just 3' to the J_H genes in the order 5' $C\mu$-$C\delta$-$C\gamma3$-$C\gamma1$-$\psi\epsilon$-$C\alpha1$-$C\gamma2$-$C\gamma4$-ϵ-$\alpha2$ 3'. A schematic view of these events is shown in Figure 1.3 (overleaf).
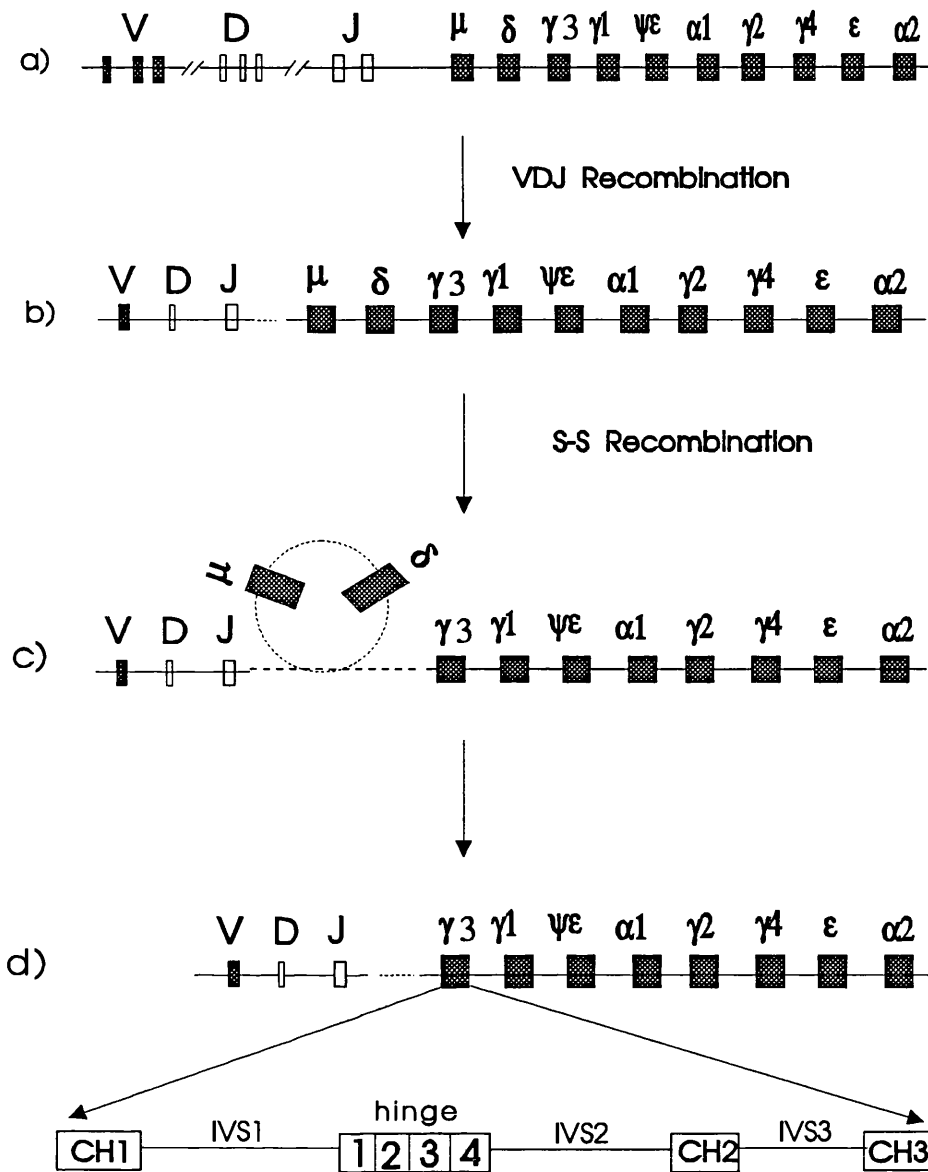
Figure 1.3: Recombination events which occur to form a complete human IgG3 gene during differentiation of a B lymphocyte are schematically represented. VDJ rearrangement (a) occurs in the absence of antigen stimulation at the pre-B cell stage during which μ heavy chains may be produced (b). Following antigen contact, isotype switching may occur (c). C_H genes 5' to the selected C_H gene are deleted and recombination takes place in an intervening sequence that lies between the V_H and C_H genes (S region hence the term S-S recombination). The C_H1, C_H2 , C_H3 and hinge domains of the heavy chain are each encoded within separate exons and an expanded representation of the IgG3 heavy chain locus showing the exon-intron (IVS intervening) sequence is shown in (d). See text for further details and references.

Somatic amplification of both types of immunoglobulin diversity (ie V and C) is mediated by DNA rearrangements of the immunoglobulin genes. Because multiple germ line DNA segments are combined to form a complete V gene, the antigen specificity of each lymphocyte is determined by random association of two or three germ line DNA segments. The progeny of a single lymphocyte retain essentially the same V_H region sequences except for base replacements due to somatic mutations which occur at a high rate.

While the mechanisms underlying isotype switching have not been fully elucidated much progress has been made in this area in the last 13 years. The initial molecular genetic analysis of myeloma DNA's producing different immunoglobulin classes revealed that specific C_H genes are deleted depending on the C_H genes expressed [Honjo T and Kataok T, 1978]. These authors proposed a model that explained class switching by supposing that the expressed V_H gene recombines with a different C_H gene, and the intervening DNA segment is deleted. This model was confirmed in 1980 when several groups [Davis M et al., 1980, Kataoka T, 1980] showed that after switching a complete V_H gene is directly linked to the $C\gamma$ (or $C\alpha$) gene. Subsequently it was shown that the recombination takes place in an intervening sequence that lies between the V_H and C_H genes [Kataoka T et al., 1981]. The nucleotide sequences of the regions containing frequent recombinations are comprised of tandem repeats of short unit sequences that share the following dispersed base sequences, TGAGC and TGGGG. These stretches are termed the switch regions. Questions regarding the regulation of isotype switching and the precise molecular mechanisms by which switching occurs remain unresolved. Early suggestions that class specific regulatory mechanisms controlled switching have been replaced by the more widely accepted view that a general switch recombinase or switch inducing protein is more likely. Alt's model of switch region accessibility [Alt F et al., 1986] suggests that similar to the regulation of V_H gene assembly,

directed class switch events (ie the predisposition of a pre-B cell line to switch to a specific isotype), could also be mediated at the level of the accessibility of the different heavy chain switch regions to a common switch recombinase, rather than by a switch region specific recombinase. Support for this comes from several murine pre-B cell lines which switch to γ2b expression in culture and accumulate significant levels of Cγ2b transcripts which are shorter than normal and not associated with V_H region sequences. Transcripts of other C_H genes (except the normal μRNA) are not found. It has been suggested that there is an intermediate step in these switching events where the C_H gene is fully intact and surface Ig of a new isotype arises from the post-transcriptional processing of long RNA transcripts which incorporate Cμ downstream to the newly synthesised mRNA immunoglobulin C_H region. The observation that switching may take place without deletion explains the ability of B cells to co-express IgM and IgG on their surface.

The regulatory elements that are involved at the mRNA stage of isotype-switching remain elusive. It appears however, that at a cellular level there are at least four important determinants in isotype regulation. These are: (a) the antigen stimulating the immune response, (b) the maturity of the host, (c) the site of antigen presentation and (d) T cell lymphokines produced during the immune response. There is a large body of evidence in the human system demonstrating that protein and carbohydrate antigens generate different immunoglobulin isotypes (see section f, this chapter). This antigen restriction also appears to be age related with infants being unable to mount an IgG2 response to polysaccharide antigens. The route of administration of the antigen is also important in determining the isotype response, since an antigen that elicits a predominantly IgA response when presented to gut-associated lymphoid tissue elicits an IgG response when administered systemically [Svennerholm AM, et al., 1980].

Studies of human IgG subclass expression contrast with the animal models described. The single lineage model of switching (ie all B cells from a single lineage are able to express all isotypes utilising exogenous factors such as T cell help) suggests that T cell independent antigens such as carbohydrates, induce a preferential IgG3 response in the mouse because they do not recruit T cell help and therefore utilise the C_H region closest to the μ gene (Cγ3 in the mouse). In humans however it is IgG2 that is preferentially induced in response to CHO antigens and not IgG3 which is the C_H gene closest to the μ gene. Furthermore, the extensive literature describing murine T cell factors involved in switching (eg. IL4 enhances IgG1 and IgE [Snapper et al., 1988], IL5 enhances IgM and IgA [Takatsu et al., 1988], TGF-β enhances IgA [Coffman et al., 1989] and the stimulatory effect of IFN-γ on IgG2a production [Snapper et al., 1989]), has on the whole, not been reproduced in experiments involving human B cell populations free of T cell contamination. The exception is that of IL4 which has been shown to stimulate the production of IgE in Epstein Barr virus-infected purified human B lymphocytes [Thyphronitis et al., 1989]. While many will extrapolate freely from the murine system to the human system, certain facts (see section b, this Chapter) suggest that the IgG subclass systems in the two species are not analogous [Callard and Turner, 1990].

(d) The Four Human IgG subclasses: Structural Determinants.

Differences in the amino acid content of the heavy chains and the κ:λ ratio of the light chains are characteristic of the different subclasses of IgG. The kappa:lambda ratio varies for the four subclasses and is 2.4, 1.1, 1.4, and 8 respectively for IgG1-IgG4.

While the primary amino acid sequences of the constant regions of the IgG subclass heavy chains are greater than 90% homologous, major structural

differences are found in the hinge region in terms of the number of residues and the

number of interchain disulphide bonds. The hinge region is the site of maximum

structural difference not only between the IgG subclasses but also between all

immunoglobulins. Thus IgM and IgE molecules do not have a hinge region

sequence separately encoded at the DNA level, and the hinge regions of IgA1 and

IgA2 differ widely. Schematic diagrams of IgG1, IgG2, IgG3 and IgG4

emphasising these differences in the hinge regions are shown in Figure 1.4.
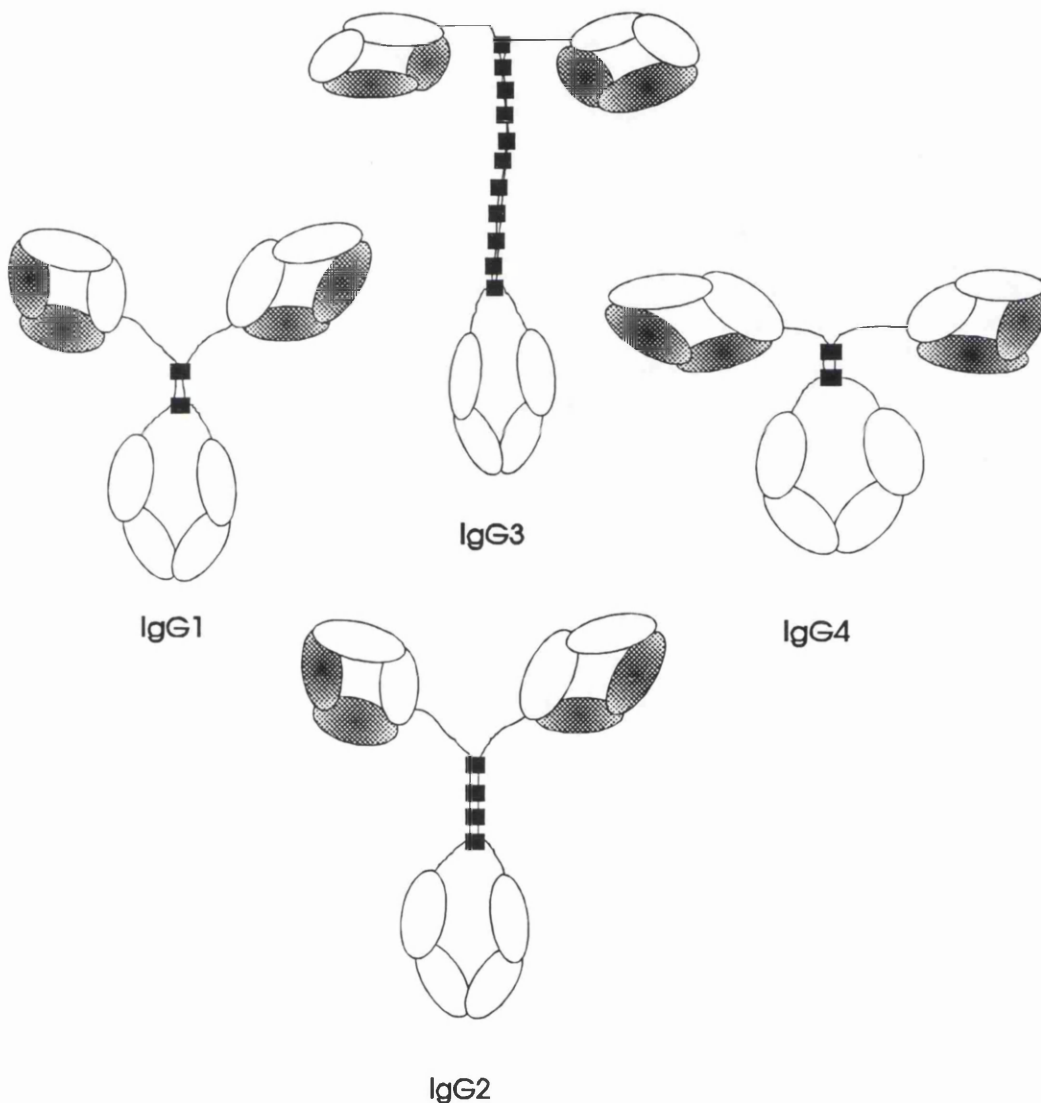

IgG3

IgG1

IgG4

IgG2

Figure 1.4: Schematic representation of the four IgG subclasses. Note the different number of inter-heavy chain disulphide bonds (closed squares) and the elongated hinge region of IgG3 [after Pumphrey RSH, 1990]. See text for details.

The IgG1 hinge is 15 amino acid residues long and freely flexible so that in the intact immunoglobulin molecule the Fab regions can rotate about their axes of symmetry and move within a sphere centred at the first of the two interchain disulphide bridges. IgG2 has a shorter hinge than IgG1 with 12 amino acid residues and 4 disulphide bridges. The hinge region of IgG2 also lacks glycine residues, which together with the shorter length, almost completely prevents rotation and restricts lateral movement of the Fab regions. IgG3 has a unique elongated hinge region containing 62 amino acids (21 prolines and 11 cysteines) that is probably the result of two separate genetic duplications. Structurally it has been described as an inflexible polyproline double helix. The IgG3 Fab regions appear to rotate and wave at a rate similar to that of IgG1; however, the remoteness of the Fc fragment from the Fab regions causes the latter to be less frequently near the Fc over time. This facilitates the binding of the complement component C1q to the Fc region of IgG3 relative to its binding to IgG1 Fc. The extended hinge region of IgG3 is also thought to render the molecule more vulnerable to proteolytic degradation. The rapid catabolism noted for this subclass probably accounts for the apparent shorter serum half life of IgG3. Finally, the hinge region of IgG4 is shorter than that of IgG1, it's flexibility is intermediate between IgG1 and IgG2 and some rotation may occur around the single glycine residue of the hinge region. Access of C1q to the IgG4 Fc is hindered by the shortness of the IgG4 hinge which leads to the Fabs spending more time close to the Fc and hence the inability of this subclass to bind complement.

The point of light chain attachment to the heavy chain also differs among the subclasses. IgG1 light chains are bound near the midpoint of the heavy chain whereas in the case of IgG2, IgG3 and IgG4 the light chains join the heavy chains at the V_H-C_H1 junction. Both the heavy and the light chains are characterised by a

regular pattern of repeating intrachain disulphide bonds that fold the peptide chains into the compact globular domains described above.

(e) Structure-Function relationships of the IgG subclasses

Various effector functions are mediated via the Fc portion of the immunoglobulin molecule and these differ somewhat between the four subclasses (See Table 2.2).

Table 2.2: Some physicochemical and biological properties of the IgG subclasses

	IgG1	IgG2	IgG3	IgG4
Heavy chain	$\gamma 1$	$\gamma 2$	$\gamma 3$	$\gamma 4$
Hinge amino acid number	15	12	62	12
Molecular weight (kDa)	146 000	146 000	170 000	146 000
Light chain $\kappa:\lambda$ ratio	2.4	1.1	1.4	8
Inter-heavy chain disulphide bonds	2	4	11	2
% total IgG (adult serum)	60-70	20-30	5-8	0.7-4
Range in normal serum (mg/ml)	5-12	2-6	0.5-1	0.2-1
half life (days)	21-23	20-23	7	21
complement fixation	+ +	+	+ +	-
Specific anti-CHO responses	+	+ + +	-	-
Specific anti-protein responses	+ +	-	+ +	-
SpA binding	+	+	-	+

The classical pathway of complement is triggered by the interaction of component C1 with IgG in an associated state. Only IgG1 and IgG3 are thought to be able to activate complement via the classical pathway. Many studies do however describe a weak ability of IgG2 to bind complement and it has been suggested that it is the ability of IgG2 to bind rabbit complement [Dangl et al., 1988] that accounts

for this [Jefferis R, 1990]. The subcomponent C1q interacts with the C_H2 domain of IgG although the exact location of the binding site remains unclear. Utilising mouse IgG2b molecules engineered with single site mutations, it has recently been shown that the C1q binding is dependent on a motif found within the $F\gamma2$ strand of the C_H2 domain. However, this motif is present in all human IgG subclasses and therefore does not explain the inability of IgG2 and IgG4 to bind complement. Other studies have shown that the lack of glycosylation results in changes in the structure of the lower hinge region distant from the identified motif [Lund J et al., 1990], and that aglycosylation produces a three-fold lower association constant for C1q. It is possible therefore that C1q interacts with the Fc through a composite site straddling the lower hinge region and the $F\gamma2$ region of the C_H2 domain.

Many important biological roles fulfilled by IgG antibodies are due to their interaction with various cell types. The basis of this interaction is the specific binding of IgG Fc regions to membrane bound Fc receptors. Three classes of human Fc γ receptors (FcγR) have thus far been identified, huFcγRI, huFcγRII and huFcγRIII. They are distinguished by their presence on various cell types, by their molecular weights, and by their differing abilities to bind IgG molecules of the four subclasses. Binding of IgG in complexed form to FcγRs may trigger a variety of responses depending on cell type, FcγR type and the nature of the IgG complex. These responses include processes concerned directly with the elimination of antigens eg phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), generation of reactive oxygen intermediates, release of lysosomal enzymes and the uptake of soluble immune complexes.

HuFcγRI (CD64) is expressed constitutively only on mononuclear phagocytes (monocytes and macrophages) although it's expression may be induced on neutrophils *in-vitro* by interferon gamma (IFNγ) [Perussia B et al., 1983]. IgG1 and IgG3 bind with an affinity 5-10 fold greater than that of IgG4 [Burton DR,

1985]. It was thought that IgG2 bound with an affinity 100 fold less than IgG1 and IgG3 although recent evidence suggests that IgG2 does not bind at all and that the original finding of IgG2 binding was due to contamination of the IgG2 paraprotein with IgG1 [Walker et al., 1989]. This receptor is thought to mediate the oxidative burst, phagocytosis and ADCC [Anderson et al., 1986] although much of the evidence is indirect and derived from *in-vitro* studies on peripheral blood monocytes or monocytic cell lines. Studies using monoclonal anti-rhesus D antibodies have shown IgG3 to be more efficient than IgG1 at mediating red cell attachment to cells expressing huFcγRI [Wiener et al., 1988] although there is disagreement on their relative abilities to opsonise for phagocytosis [Hadley and Kumpel, 1989].

HuFcγRII (CD32) is expressed by a wide range of cells including mononuclear phagocytes, neutrophils, B lymphocytes, eosinophils, platelets and placental trophoblasts. There is some disagreement in the literature as to the IgG subclass specificity of binding for this receptor. Early studies on the platelet huFcγRII receptor suggested that IgG1 and IgG3 bound with equal affinity and IgG2 and IgG4 less well [Karas et al., 1982]. Subsequent studies with COS cells transfected with recombinant huFcγRII have suggested an order of binding IgG1 > IgG2 = IgG4 > IgG3 [Simmons and Seed, 1988]. Finally some studies have suggested that only IgG1 and IgG3 are bound by this receptor [Walker MR et al., 1989]. The reason for these inconsistencies is unclear but may reflect heterogeneity in the huFcγRII structures expressed by different cells. Binding to this receptor produces a wide range of functions depending on the cell type. On myeloid cells binding may induce the oxidative burst, phagocytosis and ADCC. Binding to platelet huFcγRII mediates aggregation and the release of granule contents and binding to the neutrophil receptor stimulates the oxidative burst as well as phagocytosis [reviewed by Pound and Walker, 1990].

HuFcγRIII (CD16) is found on macrophages (but not monocytes), neutrophils, K/NK cells and a small subset of T cells denoted Tγ cells. Important differences exist between huFcγRIII expressed on different cell types. For instance, neutrophil huFcγRIII is linked in the membrane to phosphatidyl inositol glycan (PIG) whereas on macrophages and NK cells the receptor is an integral membrane protein with a trans membrane portion and an intra-cytoplasmic tail [Ravetch and Perussia, 1989]. The specificity of huFcγRIII appears to be exclusively for IgG1 and IgG3 [Simmons D and Seed B, 1988]. The consequences of binding to huFcγRIII are dependent on the cell type involved for reasons discussed above. Hence binding to the neutrophil PIG linked receptor apparently triggers no effector functions but rather has the effect of enhancing the avidity of binding to huFcγRII [Huizinga et al., 1989]. In the conventionally anchored huFcγRIII on NK cells and lymphocytes, ADCC is mediated by receptor binding and, in macrophages, binding promotes phagocytosis [reviewed by Pound and Walker, 1990].

The ability to bind staphylococcal protein A (SpA) has been extensively studied and shown to be limited to IgG1, IgG2, IgG4 and to IgG3 proteins which bear an allotypic marker characteristic of Oriental populations, G3m(s,t) [Matsumoto et al., 1982]. The molecular specificity of SpA is known and the binding site has been shown to involve residues on both the C_H2 and C_H3 domains [Diesenhofer, 1981]. Hisitidine residue 435 has been shown to be a critical contact residue and this explains the inability of certain IgG3 molecules to bind to SpA. Most IgG3 molecules of Caucasians have the G3m(u) allotypic marker characterised by an arginine residue at position 435.

(f) Selective IgG subclass expression

The first publication to describe the IgG subclass restriction of specific antigen responses was that of Yount et al. in 1967. These authors examined serum

from adults who were immunised with a variety of preparations and found that

IgG2 dominated the response to dextran, levan and teichoic acid despite the fact that

IgG2 only makes up 25% of total IgG. They also showed that antibodies to tetanus

toxoid, diphtheria toxoid and blood group antigens were restricted to IgG1. Their

important findings have been subsequently confirmed and extended in many

laboratories yet the underlying mechanisms responsible for this isotype restriction

have remained elusive to this day.

It is now well recognised that antibody responses in the mouse differ for T

dependent and T independent antigens. IgG1 is the antibody mounted in response to

proteins (T dependent antigens) such as dinitrophenyl-protein conjugates [Scott and

Fleishman, 1982], IgG3 is the antibody mounted in response to T independent

antigens such as dextrans and pneumococcal polysaccharide [Perlmutter et al.,

1978], and IgG2a antibodies are induced by certain viruses [Coutelier et al., 1988].

Similar antigen restricted responses are seen in the human IgG subclass system.

Anti-carbohydrate responses are traditionally thought to be restricted to the IgG2

subclass, while anti-protein (T dependent) antibody responses appear to be

preferentially mounted within the IgG1 or IgG3 subclasses. In support of this many

studies have shown that adult responses to polysaccharide vaccines (eg *Haemophilus*

influenzae type b polysaccharide, pneumococcal polysaccharide), or to natural

infections where the dominant antigen is thought to be a polysaccharide (eg

Neisseria meningitidis group A and C and *E.coli*), are largely restricted to IgG2

[reviewed by Hammarstrom and Edvard Smith, 1986, Barrett and Ayoub, 1986]. In

contrast, responses to protein antigens in vaccines (eg diphtheria and tetanus toxoid)

or protein antigens encountered naturally through viral infections, are

predominantly of the IgG1 and IgG3 subclass [Mäkelä et al., 1987, Seppala et al.,

1984, reviewed by Skvaril, 1986]. However, the antigen restriction of isotype

production is complex and it is becoming clear that a proportion of the adult

response to carbohydrate antigens may be found in the IgG1 subclass [Mäkelä et al., 1987] and that this subclass may be equally effective in protecting against Hib infection [Weinberg et al., 1986].

In children the selective production of IgG subclasses is different to that encountered in adults. Children under the age of two are generally poor responders to polysaccharide antigens and when an antibody response is mounted it tends to be predominantly IgG1. This pattern is seen both for naturally acquired antibodies such as those mounted against teichoic acids (a constituent of the cell wall of gram positive organisms such as *Staphylococcus aureus*) [Hammarstrom et al., 1985] and for antibodies mounted in response to polysaccharide vaccines such as the pneumococcal vaccine [Schatz and Barrett, 1987]. In contrast, the response to protein antigens does not appear to be age restricted and qualitatively the IgG subclass profile of anti-tetanus and anti-diphtheria antibodies appears to be similar in adults and children ie predominantly IgG1 [Seppala et al., 1987]. The ability to mount an effective antibody response to protein antigens in young children makes it possible to administer vaccines such as those against tetanus, diphtheria and pertussis to young infants, whereas the use of unconjugated carbohydrate vaccines is limited to older children. The inability of young children to mount an IgG2 response to polysaccharide antigens has been linked to the delayed ontogeny of this subclass. In contrast to IgG1 and IgG3, adult serum levels of IgG2 are not reached until approximately 8-10 years of age [Lee et al., 1986]. However, the mechanisms underlying both the delayed ontogeny of IgG2 and the isotype restricted response to antigens are still unclear.

The role of IgG4 is even less clear than that of IgG1, IgG2 and IgG3. Following the initial observation that IgG4 may mediate basophil degranulation [Vijay et al., 1977], it has been shown that IgG4 is produced late in the secondary immune response and usually following repeated stimulation with an injected

protein such as phospholipase A2 (a constituent of bee venom) or the neoantigen keyhole limpet haemocyanin [Aalberse et al., 1983, Bird et al., 1990]. Furthermore, IgG4 produced following immunisation with tetanus toxoid has been shown to be of low affinity [Devey et al., 1985] and the functional significance of the IgG4 response remains to be established.

The selective expression of IgG subclasses may reflect regulation of isotype switching which in turn may be determined by antigen presentation, the quality or quantity of T cell help and/or inherent B cell control mechanisms. Furthermore the effect of factors such as Gm allotype on the serum level and response to antigens within a particular subclass needs to be taken into account.

Isotype responses elicited by the same antigen presented via different routes may differ significantly. The investigation of IgG subclass responses in individuals exposed naturally to or vaccinated against both pertussis [Zachrisson et al., 1989] and cholera [Jetborn et al., 1988] have shown differences in the isotype profile of the immune response. While infection with hepatitis B induces an IgG1 and IgG3 response, responses to the vaccine also include IgG4 responses [Persson et al., 1988]. Bird et al. [1990] have shown that the development of IgG4 responses to KLH take place over a prolonged time course which suggests that the IgG4 response might depend on long-term antigen retention, possibly in the germinal centres of follicular dendritic cells. It is here that antigen driven selection of B cells and B memory cell development have been shown to occur [MacLennan and Gray, 1986]. Since antigen localisation within the germinal centre is dependent on complement fixation, Bird et al. cite the observation that IgG4 is the only subclass to be severely depressed in genetically determined deficiencies of early components of the classical pathway of complement activation [Bird and Lachmann, 1988], in support of their assertion that IgG4 production may depend on long term antigen contact.

The role of T cell help and intrinsic B cell mechanisms that might influence switching are considered in section (c) of this chapter and in the general discussion (Chapter 6).

While the question of IgG subclass deficiency and the relationship between the absence of a subclass (or combination of subclasses) and the susceptibility to infection are dealt with fully in Chapter 3, it is interesting to note here that certain authors have attempted to link antigen specific responses to serum levels of a given subclass prior to vaccination. The well known association between infection with encapsulated organisms in those under the age of two and the inability to mount an IgG2 response to bacterial polysaccharides in this age group led investigators to examine the relationship between pre-vaccination serum IgG2 and the ability to mount anti-bacterial polysaccharide antibodies. Siber et al. [1980] showed that the level of IgG2 prior to immunisation correlated directly with the mean antibody response to both pneumococcal and Hib polysaccharide vaccines although not to the response to a protein vaccine (influenza A virus) in a group of 53 adults previously treated for Hodgkins disease and in 10 healthy controls. The same correlation has been demonstrated in children immunised with Hib vaccine [Ambrosino et al., 1985, Shackleford et al., 1985] although these children represent a highly selected population who have shown an undue susceptibility to natural infection with Hib. Recently, Shackleford and colleagues have reported a study where healthy children with low IgG2 subclass levels were immunised with Hib conjugate vaccines and showed normal vaccine responses while a group of symptomatic children (frequent infections) with low levels of IgG2 analogous to the low IgG2 levels of the healthy children, showed impaired responses [Shackleford et al., 1990a, Shackleford et al., 1990b]. It is clear from these studies that the relationship between IgG2 levels, IgG2 deficiency and bacterial immunity is complex and still requires much investigation. These aspects are further discussed in Chapter 3.

(g) Gm Allotypes

Allotypes or genetic variants of protein molecules are the products of allelic genes (alternative forms of genes at a single locus) inherited in a single Mendelian manner. They differ in individuals of the same species and their expression correlates with differences in the amino acid sequence of the protein. Human immunoglobulin allotypes are carried by the constant region of κ light chains and by $\gamma 1$, $\gamma 2$, $\gamma 3$, $\alpha 2$ and ϵ heavy chains. It has been shown that the γ heavy chain (Gm) markers are located in the CH2 and CH3 regions of the molecule with the exception of the G1m(f) and G1m(z) which are located in the CH1 region of the IgG1 molecule. A list of the currently studied human Gm markers is given in Table 2.3

Table 2.3: Nomenclature and localisation of allotypic determinants to different CH regions of the γ and κ chains [adapted from Lefranc M-P and Lefranc G, 1990].

Localisation	Domains	Alphabetical	Numeric
Heavy chain subclass:			
IgG1	CH3	G1m(a)	G1m(1)
	CH3	G1m(x)	G1m(2)
	CH1	G1m(f)	G1m(3)
	CH1	G1m(z)	G1m(17)
IgG2	CH2	G2m(n)	G2m(23)
IgG3	CH3	G3m(b0)	G3m(11)
	CH2	G3m(b1)	G3m(5)
	CH3	G3m(b3)	G3m(13)
	CH2	G3m(b4)	G3m(14)
	CH3	G3m(b5)	G3m(10)
	CH3	G3m(c3)	G3m(6)
	CH3	G3m(c5)	G3m(24)
	CH2	G3m(g)	G3m(21)
	CH2	G3m(u)	G3m(26)
	CH3	G3m(v)	G3m(27)
	CH3	G3m(s)	G3m(15)
	CH3	G3m(t)	G3m(16)
	CH3	G3m(g5)	G3m(28)
Kappa light chains			
	CL	Km(1)	Km(1)
	CL	Km(2)	Km(2)
	CL	Km(3)	Km(3)

In 1971 Wells et al. investigated the association between Gm allotype and the qualitative antibody response. They showed that individuals with the G1m(a) and G3m(g) allotypes demonstrated high titres of both natural and induced antibodies to the bacterial immunogen flagellin derived from *Salmonella adelaide*. This observation led to many further studies investigating the association between Gm allotypes, antibody levels and the ability to mount an antibody response to a

particular antigen. The influence of the G2m(n) allotype on anti-bacterial antibody production has been particularly thoroughly investigated and many studies have suggested that G2m(n) positive individuals have an advantage in mounting antibody responses to polysaccharide antigens. Ambrosino et al. [1985] showed that G2m(n) positive adults had significantly higher post-immunisation antibody levels to Hib vaccine and 8 out of 11 pneumococcal types than those lacking this antigen. They also showed that a highly selected group of 25 Caucasian children with Hib infections other than epiglottitis (contracted before 18 months of age), were significantly more likely to lack the G2m(n) allotype than controls. Sarvas et al. [1989] have recently shown in an adult Finnish population that following pneumococcal vaccination, G2m(n) homozygotes produced higher levels of anti-pneumococcal IgG2 antibodies than controls. Their methodology permitted the division of the population into positive and negative homozygotes and heterozygotes. Interestingly, Rautonen et al. [1990] have shown that in 47 children with coeliac disease, G2m(n) homozygotes had a higher proportion of IgG2 antibodies to the wheat protein, gliadin.

In contrast, Granoff et al. [1986a] were unable to show G2m(n) related differences of IgG antibody levels to Hib after immunisation of Caucasian children although the same group reported an excess of G2m(n) negatives in a selected group of 37 children previously shown to have impaired antibody responses to natural Hib infection, who had also failed to respond to the Hib polysaccharide vaccine [Granoff DM et al., 1986b]. More recently Granoff's group [Granoff, 1989] and Takala and colleagues from Finland [1991] have again demonstrated no significant association between the G2m(n) haplotype and susceptibility to Hib disease. Takala's study of the G2m(n) allotype in 172 Finnish children with invasive Hib disease revealed no difference in the G2m(n) allotype distribution between the children infected with Hib and that seen in the general population. The reason for

the lack of agreement in the studies described may, perhaps, be due to the size and selected nature of the populations investigated in the earlier studies. With regard to Hib disease, it is children under the age of 2 who are most at risk of invasive disease and as all children in this age group are poor at responding to polysaccharide antigens and unable to mount vigorous IgG2 responses the relevance of the G2m(n) allotype in this group is perhaps questionable. Recent work has focussed on the effect of extended haplotypes and serum levels of the IgG subclasses. Oxelius [1990] has investigated the link between Gm allotype and IgG2 and IgG3 deficiency in a group of adult Caucasian patients in Sweden. She confirmed the association between the absence of G2m(n) and IgG2 deficiency but went on to show that the lack of G2m(n) may also be associated with IgG3 deficiency and further showed the association between IgG2 deficiency and the lack of G1m(a) and G3m(g) and IgG3 deficiency and the lack of G1m(f) and G3m(b). She concluded that whilst earlier studies had focussed mainly on G2m(n) and antibody responses it might be more relevant to study specific antibody response with reference to gene and gene dosage of the accompanying G1m and G3m allotypes. Morrel and colleagues [1989] have investigated the effect of extended haplotypes on total IgG subclass concentrations and on natural antibody concentrations to Group A *Streptococcal* carbohydrate. They showed that individuals with the G1m(f), G2m(n), G3m(b) haplotype had higher concentrations of serum IgG2 and IgG2 anti-carbohydrate antibody while having lower IgG1 and IgM antibodies (total and specific). The advantage of this extended haplotype in influencing the production of IgG2 antibodies to carbohydrate antigens may be of critical importance in young children.

The mechanism by which Gm allotypes exert their control over the serum concentrations of immunoglobulin isotypes and specific antibody responses remains unclear. Regulatory elements linked to certain haplotypes may influence switching

and it has been postulated that B cells positive for G1m(f), G2m(n), G3m(b) may be more responsive to lymphokines that may influence switching [Vitteta et al., 1985]. Alternatively, as has been shown in the mouse, T cells could directly upregulate B cells depending on the Ig allotype [Nutt et al., 1981]. Yet another possibility is that immunoglobulin allotype-linked genes could act at the level of the antigen presenting cell; for example it has been shown that HLA-DR3 and G1m(f), G2m(n), G3m(b) positive individuals are slow degraders of sheep red blood cell antigens and consequently high antibody producers [Legrand et al., 1985]. Sarvas et al., [1989] have suggested that the G2m(n) positive allele may in some way be associated with a V_H gene repertoire that contains "good/advantageous" V genes for several anti-polysaccharide responses thereby explaining their findings of significant anti-pneumococcal responses. However, that would result in similar responses in all the subclasses mounted and a positive correlation was only seen with the IgG2 response; hence these authors favour the switch region efficacy hypothesis as described above.

(h) Ontogeny of the IgG subclasses

Although IgG synthesis occurs during fetal life and can be detected in the fetus as early as eleven weeks gestation, the amount produced by the fetus is negligible. Most of the IgG present in cord serum is of maternal origin since all IgG subclasses transfer across the placenta. In the full term neonate, cord serum levels of IgG1 are consistently higher than in maternal serum which suggests active transport. IgG2, IgG3 and IgG4 are similar in maternal serum and in cord sera [Morell et al., 1972a]. Most of the transplacental transfer of IgG occurs in the last trimester of pregnancy and immature infants born before the 30th week of gestation have very low concentrations of all IgG subclasses. The immature infant does, however, have the capacity to produce immunoglobulins and Oxelius et al. [1984b]

have shown that immature infants have the ability to synthesise IgG1 and IgG3 during the first week after birth.

Serum concentrations of all four IgG subclasses decline rapidly during the first month of life with a nadir between one month (IgG3) and 3-6 months. Subsequently, IgG1 and IgG3 concentrations rise rapidly, reaching two thirds of adult concentrations by 3-4 years of age. IgG2 and IgG4 concentrations increase more slowly, reaching adult levels between 8-10 years of age. These differences in the serum levels of the IgG subclasses are similar to the differences seen in the study of B cells ontogeny. Circulating B cells obtained from cord blood and stimulated with Epstein-Barr virus stain positively for cytoplasmic IgG1 and IgG3 but not IgG2 and IgG4. The latter subclasses are only found in B cells from two years of age onwards [Andersson et al., 1981].

In normal adult sera IgG1 is the predominant subclass (58-71% of total IgG), followed by IgG2 (19-31%), IgG3 (5-8%) and IgG4 (1-5%). Reported values vary significantly for both the adult normal ranges and the pediatric normal ranges [Morell et al., 1972b, Shakib et al., 1975, Van der Giessen et al., 1975, Schur et al., 1979, French, 1986, Lee et al., 1986](see Figure 6.2). These variations may be due to technical differences between laboratories in reagents and the assay protocols used, differences in the methods of expressing normal ranges and the variations in the expression of Gm allotypes in different populations. These issues are discussed further in Chapter 6

(i) IgG subclass affinity

Isotype switch is only one of the consequences of antigen dependent B cell maturation. The increase in antibody affinity that occurs following contact between an antigen and a B cell is an important phenomenon with functional consequences. Antibody affinity is defined as the strength of the primary interaction between an

antibody (single combining site) and an antigen. Antibody avidity or functional affinity is the term used to describe the reaction(s) between multivalent antibodies and complex antigens [Steward, 1981]. Affinity has been shown to influence a wide range of biological activities and in general high affinity antibodies are more effective than low affinity antibodies in mediating such activities as neutralisation of toxins, immune elimination of antigen and viral elimination [reviewed in Steward, 1981]. While the classical phenomenon of affinity maturation (ie the increase in antibody affinity with time following antigen exposure) has been recognised for many years it is only recently that a fuller understanding of the mechanisms underlying this maturation have become evident. Previously affinity maturation has been explained by the clonal selection theory ie high affinity antibodies are produced as a consequence of the selection and expansion of B-cell clones that existed before immunisation and are committed to the production of high affinity antibodies. Recently, however, it has become apparent that affinity maturation involves a process of intraclonal diversification through somatic hypermutation. This process appears to be triggered after immunisation and leads to the accumulation of high affinity memory B cells [Griffiths et al., 1984, Kocks and Rajewsky, 1988]. While isotype switch and affinity maturation are both consequences of antigen stimulation of B cells it appears that the two phenomena are independent [Manser T, 1989]. The affinity of different subclasses produced in response to the same antigen may differ significantly.

IgG subclass responses to tetanus toxoid have been shown to be localised mainly to the IgG1 and IgG4 subclasses. Using a solid phase assay, Devey et al. [1988] showed that the functional affinity of the IgG1 antibody fraction was higher than the affinity of the IgG4 fraction. An IgG4 restricted response might therefore not carry the biological advantages of an IgG1 response although the same author had previously been unable to show that a low affinity response to tetanus toxoid

was related to any general susceptibility to recurrent, acute or chronic infection [Devey et al., 1985]. Nevertheless, it is clear that IgG4 responses are not always low affinity since high affinity responses have been described for IgG4 antibodies to antigens such as thyroglobulin [Weetman et al., 1989]. Persson et al., [1988] have investigated the affinity and relative avidity of the IgG subclass antibodies with specificity for various hepatitis B antigens (proteins) and purified pneumococcal polysaccharide type 3. The affinity of the anti-hepatitis B antigens ranked from high to low accordingly: IgG1 > IgG2 > IgG3 > IgG4. Despite the higher affinity of IgG2, IgG3 was more commonly detected. In contrast to the anti-protein responses, the relative avidity of polysaccharide specific IgG2 was higher than that of IgG1. Interestingly individuals in whom the subclass response was restricted to a single subclass (some due to Ig heavy chain deletions) the affinity of the restricted subclass produced was the same as that of the equivalent subclass in an individual producing all subclasses. It is apparent therefore that the subclass restriction of an antigen specific response is not solely related to affinity (IgG2 anti-hepatitis B antibodies would occur more often than IgG3 if that were the rule) and that the affinity of a given subclass may vary between antigens.

(j) Unanswered questions and the aim of the studies described

It is clear from the evidence in this review that much progress has been made in understanding the structural basis for functional differences of the IgG subclasses as well as in our knowledge of the molecular structure of the immunoglobulin molecule. This understanding has not, however, been matched by elucidation of the mechanisms controlling isotype switch and, by implication, the mechanisms underlying antigen specific IgG subclass production. Nor do we have more than a superficial understanding of the clinical/biological significance of an IgG subclass deficiency. In the studies described in this thesis the first question

addressed has been that of IgG subclass deficiency. In an effort to link the absence of a specific subclass with an identifiable clinical presentation, the records of a large number of children defined as IgG subclass deficient have been analysed. It was hoped that the opportunity to analyse a large group of children might provide a clue to the biological consequences of deficiency in one or more subclasses and possibly the identification of distinct deficiency patterns amongst individual IgG subclass deficiency syndromes.

A second approach to the analysis of IgG subclass function, is to investigate antigen specific immunity using reagents that can distinguish the four subclasses and hence provide information as to which subclass is being mounted in response to the antigen. Much of the work to date on antigen specific responses has focussed on IgG2 production in response to carbohydrate antigens. Responses to proteins have always been assumed to be adequate from early on in life. In an attempt to investigate the IgG subclass ontogeny to a specific organism *in-vitro*, an organism causing disease in children but not in adults, yet with the potential to be troublesome in immunosuppressed adults, has been investigated. This organism, *M. catarrhalis*, has been used to illustrate the acquisition of protective humoral immunity following contact in childhood. In parallel studies, the antigens on the surface of the organism have been analysed and used to explore the ontogeny of the IgG subclass response. Furthermore, methods for measuring antibody affinity have been adapted to provide information on antigen specific IgG subclass affinity. This approach has been adopted in an effort to develop a way of viewing antibody function that goes beyond the usual quantitative assays and which hopefully will provide a model for the analysis of humoral immune responses in general.

CHAPTER 2

Materials and General methods

(a) Chemicals and Buffers:

(i) Chemicals

Chemical	Supplier	Code
Acetic acid	BDH	1001
Acrylamide	BDH	44313
Agarose	Sigma	A6877
Ammonium persulphate	Sigma	A9164
Bovine Serum Albumin	Sigma	A4503
Bromophenol Blue	BDH	20015
Calcium Chloride	Sigma	C3881
Citric Acid	BH	10081
Diethylbarbituric acid	Sigma	B0375
Diethylamine	Sigma	D3131
Di-sodium carbonate	BDH	10240
Dithiothreitol (DTT)	Sigma	D9779
EDTA	Sigma	E5134
Formaldehyde	BDH	10113
Glutaraldehyde	Sigma	G6257
Glycine	Sigma	G2879
Hydrogen Peroxide	Sigma	H1009
Magnesium Chloride	Sigma	M0250
2-Mercaptoethanol	Sigma	M6250
Methanol	BDH	10158
Methylglyoxal	Sigma	M0252
o phenylenediamine	Sigma	P-1526
Periodic Acid	Sigma	395-1
Polyethyleneglycol 4000	BDH	44273
Potassium dihydrogen phosphate	BDH	10203
Poly-l-lysine	Sigma	P8920
Schiffs reagent	Sigma	395-2-016
Silver nitrate	BDH	10233
Sodium azide	BDH	10369
Sodium barbitone	BDH	10365
Sodium carbonate	BDH	10240
Sodium Chloride	BDH	10241
Sodium dodecyl sulphate (SDS)	Sigma	L4509
Sodium Hydrogen Carbonate	BDH	10247
Sodium dihydrogen orthophosphate	BDH	10245
di-Sodium hydrogen orthophosphate	BDH	10249
Streptavidin peroxidase	Zymed	434323
TEMED	Sigma	T8133
Tris	BDH	10315
Tween 20	BDH	66368

(ii) Buffers

Barbital buffer for single radial immunodiffusion	Sodium barbital Diethylbarbituric acid	75 mM (15.14g /litre) 15 mM (2.76g /litre)	pH 8.6
Carbonate buffer for ELISA coating	di-Sodium carbonate Sodium hydrogen carbonate	15 mM (1.59g /litre) 35 mM (2.93g /litre)	pH9.6
Citrate buffer for ELISA	Citric acid	0.1 M (21g /litre)	pH 5.2
EDTA buffer	Di-sodium hydrogen phosphate Sodium chloride EDTA	0.05M 0.15M 0.01M	pH 7.4
Phosphate buffered saline (PBS)	Sodium chloride Potassium chloride di-Sodium hydrogen phosphate Potassium di-hydrogen phosphate	140.0 mM (8g /litre) 2.7 mM (0.2g /litre) 8 0 mM (1.15g /litre) 1.5 mM (0.2g /litre)	pH 7.3
Phosphate buffer for ELISA	Sodium hydrogen phosphate	0.2 M (28.5g /litre)	pH 5.2
OPD (ELISA substrate)	o-phenylene diamine Citrate buffer phosphate buffer 30% hydrogen peroxide	10 mg 10 ml 10 ml 10 μl	pH 5.2

Buffers (contd)

PBS-Tween	PBS (as above) with		pH 7.3
	Tween 20	0.05% (v/v)	

(b) Antibodies

Biotinylated monoclonal mouse anti-human IgG subclass and anti-total IgG antibodies were used both in the ELISA and for immunoblotting and were obtained from Zymed Laboratories Inc. (San Francisco, CA) and were of the following clones: IgG1 clone HP 6069, IgG2 clone HP6002, IgG3 clone HP6047, IgG4 clone HP6025 and total IgG clone HP6045. Additional monoclonal mouse anti-human IgG subclass antibodies used for single radial immunodiffusion and immunoblotting were obtained from Unipath, UK and were of the following clones: IgG1 clone JL 512, IgG2 clone GOM1, IgG3 clone ZG4, IgG4 clone RJ4. The specificity of the monoclonal antibodies was assessed in a large multicentre collaborative IUIS/WHO study [Reimer et al 1985] and in addition, the Unipath moncolonals had undergone previous analysis [Lowe et al 1982]. The anti-IgG3 monoclonal obtained from Zymed (HP 6047) was the only anti-subclass monoclonal not included in the IUIS/WHO study and was therefore evaluated as shown in Appendix A. In addition, the same set of Zymed monoclonal anti-human IgG subclass reagents have been used in the development of antigen specific IgG subclass ELISA procedures for other antigens. The standard curves obtained for two of these antigen specific ELISAs (tetanus and meningococcal polysaccharide) utilising the same set of monoclonal antibodies as the *M. catarrhalis* ELISA are shown in Appendix B. Iodinated sheep anti-mouse IgG, sheep anti-human IgG and streptavidin were obtained from Amersham, UK.

Table 2.1: Monoclonal antibodies employed in studies described below

MONOCLONAL ANTIBODIES	CODE	SOURCE
Mouse anti-human IgG1 Fc Biotinylated	HP6069	Zymed
Mouse anti-human IgG1	JL512/ HP6007	Oxoid (Unipath)
Mouse anti-human IgG2 Fc Biotinylated	HP6002	Zymed
Mouse anti-human IgG2	GOM1/ HP6008	Oxoid (Unipath)
Mouse anti-human IgG3 Fc Biotinylated	HP6047	Zymed
Mouse anti-human IgG3	ZG4/ HP6010	Oxoid (Unipath)
Mouse anti-human IgG4 Fc Biotinylated	HP6025	Zymed
Mouse anti-human IgG4	RJ4/ HP6011	Oxoid (Unipath)
Mouse anti-human IgG Fc Biotinylated	HP6045	Zymed

(c) Sources of Sera.

Standard serum: Clotted blood from sixty apparently healthy adults was collected at a single sitting, then centrifuged and the serum pooled in equal amounts, aliquoted and stored at -70°C. This pool, which is subsequently referred to as standard serum, was included in all assays. The levels of antibody in an unknown serum were extrapolated from a standard curve derived from this pooled serum, and expressed as a percentage of the antibody bound using the standard serum. An adult range was obtained by analysis of the serum of forty apparently healthy laboratory and hospital workers. Sources of sera used for the individual studies are described fully in the relevant chapters.

(d) Antigen Preparation

A reference isolate of *M. catarrhalis* (code no. NC 11020) was obtained from the National Collection of Type Cultures, Colindale, London. Two further clinical isolates were obtained from the Microbiology Laboratory at the Hospital for Sick Children, Great Ormond Street, London. The identity of the clinical isolates was confirmed by morphology, gram-staining, catalase production, oxidase reduction, the ability to reduce nitrate, and the inability to produce acid from

glucose, maltose, sucrose or lactose. The organisms were stored on blood agar slopes and prior to use were plated out on horse blood agar and incubated at 37°C. Following an 18 hour incubation, colony purity was checked and than a colony scraping was emulsified in 2 ml of broth and this suspension was inoculated into Bactec aerobic culture vials (Becton Dickinson, Maryland). Following an 18 hour incubation at 37°C with agitation the culture medium was centrifuged at 10,000g for 15 minutes at 4°C. The bacterial pellet and supernatant were processed as outlined below.

The bacterial pellet was recovered and washed twice by resuspending in phosphate-buffered saline pH 7.4 (PBS) and centrifuging at 1000g. After washing, the bacterial pellet was resuspended to an optical density of 0.9 at 540 nm. This suspension was then referred to as whole cell antigen and utilised in both ELISA and immunoblotting.

The supernatant was subjected to two further centrifugation steps at 10,000g for 20 minutes at 4°C. The supernatant was recovered on each occasion and the pellet discarded. The supernatant was then concentrated in an Amicon series 8000 concentrator using a 0.2 micron filter. The concentrated supernatant was then subjected to a final centrifugation at 100,000g for two hours at 4°C following which the supernatant was discarded. The pellet recovered contained vesicles of outer membrane proteins. This method of OMP isolation has been evaluated by Murphy and Loeb [1989] and the OMPs isolated in this way have been shown to be free of any cytoplasmic contamination and compare well with OMPs isolated by a sucrose density gradient method. The pellet was resuspended in 100 μl of PBS and subjected to SDS-PAGE and immunoblotting (see Chapter 4) or used in the ELISA procedure (see Chapter 5).
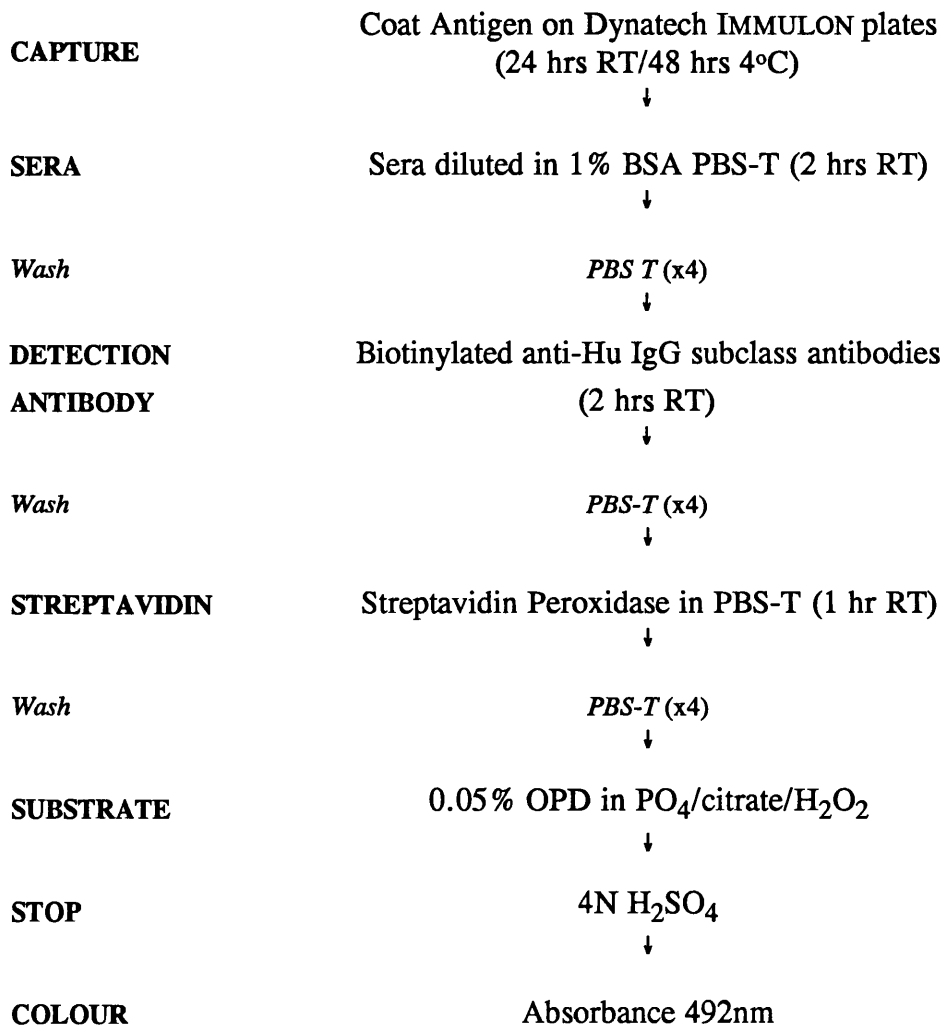
(e) Single Radial Immunodiffusion

Serum IgG subclass levels were determined by a single radial immunodiffusion technique using monoclonal antibodies (Unipath, Bedford, UK): JL512 (anti IgG1), GOM1 (anti IgG2), ZG4 (anti IgG3) and combined RJ44 and GB7B (anti IgG4). The monoclonal antibodies were used as ascitic fluid in 0.1M barbitone buffer pH 8.6 containing 7% PEG 4000 and 1% agarose at the following dilutions: anti-IgG1, 7 μl/ml of buffer; anti-IgG2, 10 μl/ml of buffer; anti-IgG3, 1 μl/ml of buffer and anti-IgG4, 6 μl/ml of buffer. Test samples were applied into 2mm wells and allowed to diffuse at 4°C for 72 hours and the precipitation zones then read. A reference serum calibrated against a commercial standard was used with each assay. The intra-assay variation of a high and low standard serum did not exceed 2.5% and the inter-assay variation did not exceed 6%. Total IgG, IgA and IgM were measured on a centrifugal fast analyser employing monoclonal antibodies (Atlantic, Scarborough, Maine, USA). The normal ranges used for the IgG subclass levels were developed in the Department of Immunology using sera from age matched elective surgical cases and healthy schoolchildren with at least 20 individuals in each 2 year age band (see Figure 3.1). The normal range for total immunoglobulin levels was similarly derived and age matched. For both IgG subclass and total immunoglobulin levels, a deficiency was defined as a level more than two standard deviations below the age matched mean.

(f) General ELISA technique for the detection of antigen specific IgG subclass antibodies

Immulon No. 2 flat bottomed ELISA plates (Dynatech, Virginia, USA) were coated with the antigen of choice (see chapter 5) in carbonate buffer and left for 24 hours at room temperature (or 48 hours at 4°C) before the coating mixture was discarded and the plates allowed to air dry. The plates were then used

immediately or stored wrapped in polythene and aluminium foil at 4°C for a period of up to three months. Aliquots of the test sera and standard serum diluted in 1% BSA in PBS-T were added to the wells (80 μl/well) and the plate incubated for 2 hours at 37°C. The serum was then discarded and the plate washed four times in wash buffer. Biotinylated antibodies were then diluted as follows in 1% BSA PBS-T: IgG1 $^1/_{500}$; IgG2 $^1/_{1000}$; IgG3 $^1/_{5000}$; IgG4 $^1/_{500}$ and IgG total $^1/_{2000}$. The wells were filled with 80 μl of the antibody solution and the plates incubated for a further 2 hours at room temperature (18-25°C). The plates were then washed four times in wash buffer following which 80 vl of a $^1/_{50\ 000}$ solution of peroxidase conjugated streptavidin were added. After standing at room temperature for one hour the plates were washed four times in wash buffer before the addition of 80 μl per well of substrate solution containing 0.5 mg/ml of OPD in a solution of 0.05% H_2O_2/0.1M Citric Acid/0.2M Na_2HPO_4. The colour reaction was stopped after 10 minutes with 4N H_2SO_4 (40 μl/well) following which the optical densities were measured at 492nm using an ELISA reader (Titertek multiskan, Flow). A range of dilutions of the standard serum ($^1/_{40}$ - $^1/_{20480}$ depending on the assay) was included on each plate and the results of the unknown sera expressed as a percentage of this internal standard.

General Scheme For Antigen Specific ELISA procedure for measuring IgG

subclass antibodies

CAPTURE	Coat Antigen on Dynatech IMMULON plates (24 hrs RT/48 hrs 4°C)
	↓
SERA	Sera diluted in 1% BSA PBS-T (2 hrs RT)
	↓
Wash	*PBS T* (x4)
	↓
DETECTION ANTIBODY	Biotinylated anti-Hu IgG subclass antibodies (2 hrs RT)
	↓
Wash	*PBS-T* (x4)
	↓
STREPTAVIDIN	Streptavidin Peroxidase in PBS-T (1 hr RT)
	↓
Wash	*PBS-T* (x4)
	↓
SUBSTRATE	0.05% OPD in PO_4/citrate/H_2O_2
	↓
STOP	4N H_2SO_4
	↓
COLOUR	Absorbance 492nm

(g) SDS-Polyacrylamide Gel Electrophoresis

10 % polyacrylamide gels were prepared using the following stock

solutions:

1) 30% acrylamide with 0.8% NN'methylene bisacrylamide

2) 1M Tris-HCl pH8.8

3) 1M Tris-HCl pH 6.8

4) 10% w/v sodium dodecyl sulphate (SDS)

5) 1.5% ammonium persulphate

6) TEMED N,N,N',N',-tetramethylethylene diamine

0.75mm Thick SDS-PAGE slab gels containing 10% acrylamide solution

with 0.1% SDS in Tris buffer were cast in the Biorad "Protean II" apparatus.

Stacking gels, containing 5% acrylamide and 0.1% SDS in Tris-HCl buffer, were

cast above the main gels. The 10% gels were polymerised with ammonium

persulphate (APS) (final concentration 2.5%) and TEMED (final concentration

0.1%) while the stacking gels contained 5% APS and 0.5% TEMED.

Sample preparation: Protein samples (volume 50μl) were boiled for 5

minutes with 25 μl of a "sample buffer" of 4% w/v SDS, 20% w/v sucrose, 4%

(v/v) 2-mercaptoethanol, 0.001% bromophenol blue and 0.01M Tris pH 6.8.

Coomassie blue stained molecular weight standards (Sigma, UK; subunit molecular

weights in parentheses) were included on each gel. These contained β-galactosidase

(116 kDa), phosphorylase B (97.4 kDa), transferrin (78 kDa), BSA (68 kDa),

ovalbumin (45 kDa), glucose-3-phosphate dehydrogenase (36 kDa), carbonic

anhydrase (29 kDa), trypsinogen (24 kDa) and lysozyme (14.3 kDa).

Electrophoresis: The gels were run in the "Biorad protean II" apparatus

using a Tris-glycine buffer of 0.192M glycine/0.025M Tris/ 0.1% SDS pH 8.3.

Following electrophoresis the gels were stained or blotted

Coomasie staining: Gels were stained for one hour or overnight in a solution of 0.025% Coomassie brilliant blue R (Sigma UK Ltd) in 50% methanol/ 5% glacial acetic acid and were destained in 7.5% glacial acetic acid/ 5% methanol.

Silver staining: Silver staining was performed essentially as described by Morrisey et al., 1981. Coomassie stained gels were fixed in 50% methanol/ 10% glacial acetic acid for 30 minutes, in 5% methanol/7% acetic acid for 30 minutes and in 10% glutaraldehyde for 30 minutes. The gel was washed overnight in deionised water and reduced with 5μg/ml of dithiothreitol (DTT) for 30 minutes and treated with 0.1% silver nitrate for a further 30 minutes. The gel was washed rapidly in distilled water and rinsed twice in a developer of 0.0185% formaldehyde in 3% Na_2CO_3 until all the Coomassie blue stained protein bands changed colour to brown. The reaction was terminated by the addition of 2.3M citric acid until the pH returned to neutral. The gel was rinsed in distilled water and finally in 0.03% sodium carbonate solution to prevent bleaching.

PAS stain for carbohydrates: Following fixation of the gel overnight at room temperature in destain (40% v/v methanol, 7% v/v acetic acid, 53% v/v water), the gel was equilibrated for one hour at 4°C in cold 7% acetic acid. All subsequent steps were then performed in the dark at 4°C. The gel was incubated for one hour in 1% w/v periodic acid in cold 7% acetic acid and then washed for 24 hours in several changes of 7% acetic acid. Following this, the gel was incubated for one hour in cold Schiffs reagent at which point red bands of glycoproteins, if present, should become visible. The sensitivity of this method was: heavily glycosylated proteins 5-10 μg, less glycosylated proteins, 20-100 μg.

(h) Immunoblotting (in the Bio-rad tank blotting system)

The SDS-PAGE gel, membranes , filter papers and scotchbrite pads were soaked in 0.192M glycine/ 0.025M Tris pH 8.3 with 20% methanol. A "sandwich"

was made from the cathode side of the cassette consisiting of the scotchbrite pads, 2-3 layers of Whatman 3MM filter paper, the gel, a "Hybond C Extra" (Amersham) membrane, 2-3 more layers of filter paper and pads. The cassette was closed and submerged in the tank with the running buffer pre-cooled to 4°C. The gel was blotted at 0.35 mAmps for two hours with stirring. Following the blotting step the membrane was marked to indicate the position of the wells and blocked with 3% skimmed milk (Marvel) in PBS/0.02% sodium azide for a minimum of 1 hour. The antibodies were diluted in the skimmed milk/PBS/azide solution and washes were carried in the same solution. When whole serum was used as the detection antibody it was employed at a concentration of $1/50$, secondary detector antibodies (class/subclass specific) were diluted to approximately $1/1000$ and the secondary antibody was ^{125}I labelled anti-species immunoglobulin or ^{125}I labelled streptavidin peroxidase used at 1×10^5 cpm/ml. The blot was washed for 6 x 5 minute periods after incubation with the antibodies. Following the final wash the blot was enclosed in "Saran wrap" and autoradiographed at -80°C between fast intensifying screens (Cronex, Dupont) with Kodak XAR 5 film for 2-7 days depending on the intensity of the signal.

CHAPTER 3

IgG subclass deficiencies: Clinical presentation in Paediatrics

(a) Introduction

Immunoglobulin G (IgG) subclasses were discovered [Dray, 1960] and further characterised [Terry and Fahey 1964] in the early 1960's. While much of the early research on IgG subclasses focussed on questions of their metabolism, Gm allotype distribution, antibody responses and their interactions with cellular Fc receptors, observations on deficiencies of one or more of the IgG subclasses in individual sera were reported. Some of these reports were of patients with an increased susceptibility to infection but others were apparently healthy normal blood donors [Terry, 1968]. The first definitive report of patients with IgG subclass deficiency was published by Schur et al. from Boston in 1970. They described recurrent bacterial infection in three children with reduced IgG subclasses. While they suggested this was a new and isolated phenomenon, subsequent interpretation has suggested that they were simply describing IgG subclass imbalances in patients with common variable immunodeficiency, a view now endorsed by one of the authors [Knutsen, 1989]. Nevertheless, their report and the increasing availability of reagents permitting the measurement of IgG subclass levels, spurred many researchers to evaluate the potential clinical role of the IgG subclasses by analysing the biological consequences of their deficiency. Despite the twenty one years that have elapsed since Schur's report, the definition of subclass deficiency and the clinical relevance of low levels of IgG subclasses are still poorly understood.

The first clear-cut evidence that IgG subclass deficiency could represent a new entity surfaced with Oxelius's 1974 paper on hereditary deficiency of IgG2 and IgG4. She described a family which suffered from recurrent infection (ear and lung) due to *H. influenzae*. Although total serum levels of IgG, IgA and IgM were normal, IgG2 and IgG4 were barely detectable. In addition their serum contained virtually no antibody specific for *H. influenzae* polysaccharide or teichoic acids suggesting that they had an inability to mount antibody responses to carbohydrate

antigens. The authors suggested that the lack of polysaccharide specific antibody

could be attributed to the IgG2 deficiency, since IgG2 had already been shown by

Yount et al. [1968] to be particularly important in responses to polysaccharide

antigens in humans. However, the family described by Oxelius also demonstrated

low levels of isohaemagglutinins (IgM) and one of them had a low PHA response

suggesting that their immunological defect was not simply isolated IgG subclass

deficiency. In 1981 when Oxelius and colleagues reported their findings on IgG

subclass levels in patients with IgA deficiency, IgG subclass deficiency was again

drawn to the attention of clinical immunologists. As a result, there has been a

profusion of literature describing the levels of IgG subclasses in the blood of

patients with a variety of different clinical syndromes. IgG subclass deficiencies

have been described in association with cystic fibrosis [Fick, et al., 1986], AIDS

[Parkin, 1989], asthma [Page et al., 1988], eczema [Merrett et al., 1984], epilepsy

[Duse et al., 1986], diabetes and ataxia telangiectasia [Rivat-Peran et al., 1981] to

name but a few. In addition to the measurement of IgG subclass levels in patients

with recognised clinical syndromes or diagnoses, much attention has been focussed

on attempting to identify clinical syndromes that might be characterised by the

absence of one or more of the IgG subclasses.

IgG1 Deficiency

IgG1 is the predominant isotype in the serum and hence a deficiency in this

subclass is usually reflected in a reduction of total IgG serum. Clinically, IgG1

deficiency has been associated with recurrent pyogenic infections but because of the

association with a reduced total IgG it is thought that this clinical presentation is

synonymous with common variable immunodeficiency. Low levels of IgG1 have

been described in association with a variety of primary and secondary antibody

deficiencies including Common variable immunodeficiency with hypogamm-

aglobulinaemia, Ataxia telangiectasia and X-linked agammaglobulinaemia [Aucouturier et al., 1986, Wedgewood et al., 1986]. An inability to respond appropriately with an IgG1 subclass response to a hepatitis B vaccine despite normal levels of serum IgG1 has also been described in patients with Down's syndrome although in this group of patients an increased risk of frequent infection points to a more generalised immunodeficiency [Avanzini et al., 1988]

IgG2 Deficiency

Selective IgG2 deficiency and susceptibility to infection with organisms containing a polysaccharide capsule is perhaps the most accepted clinical association of all the IgG subclass abnormalities. IgG2 deficiency is the most commonly described deficiency in certain selected paediatric populations such as those with frequent chronic chest symptoms [Smith et al., 1986], asthma [Loftus et al., 1988] and epilepsy [Duse et al., 1986], as well as being described more commonly than the other subclasses in association with IgA deficiency [Oxelius et al., 1981, Roberton et al., 1990]. The frequency with which a low IgG2 level is reported in childhood might simply relate to the slower maturation of this subclass, while a non age-matched interpretation of the level of IgG2 may erroneously lead to an assessment of deficiency. However, IgG2 has been shown to be an important constituent of the immune response to, and provide protection from, organisms with a polysaccharide capsule such as *S. pneumoniae*, *H. influenzae* and *N. meningitidis*. Susceptibility to infection with these organisms has been linked by many authors to both an absolute deficiency of IgG2 [Bass et al., 1983, Gottsegen, 1987] and to an inability to mount an effective IgG2 response to the polysaccharide capsule either during a natural infection or following vaccination [Siber et al., 1980, Käyhty et al., 1981, Umetsu et al., 1985]. Furthermore, some children with frequent upper and lower respiratory tract infections who have normal levels of IgG2 have been

shown to have an impaired response to polysaccharide vaccines [Smith et al., 1990, Sanders et al., 1991], an observation which has also been noted in adults with IgA deficiency [Lane and MaClennan, 1986]. The evidence suggests that while a low level of IgG2 in the serum may be a marker of an immunodeficiency, the ability to mount an isotype appropriate response to an antigen is independent of the level of the subclass in the serum. Important work from Shackleford and colleagues [Shackleford PG et al., 1990] has also highlighted the fact that children may have low levels of serum IgG2 (levels analogous to those found in children with frequent infection), yet remain entirely healthy and respond normally to polysaccharide vaccines. Furthermore patients with C_H deletions who lack the $C\gamma_2$ gene and therefore have no serum IgG2, may be entirely healthy and may make anti-polysaccharide antibodies of the IgG1 and IgG3 subclasses [Hammarström et al., 1987].

IgG3 Deficiency

IgG3 deficiency has been described as the commonest IgG subclass deficiency detected in adults and accounted for 54% of the deficiencies noted in a group of 151 Swedish adults with recurrent respiratory tract infection and IgG subclass deficiencies [Söderström et al., 1986]. Oxelius and colleagues [1986] analysed 6580 consecutive samples from patients mainly suffering from recurrent infections. A total of 1864 were adults with recurrent or chronic infection and 4716 were children with acute recurrent, severe or chronic infections. 313 (4.75%) were found to have a serum IgG3 more than 2SD below the normal range and in 186 patients it was the sole abnormality. Recurrent respiratory tract infection, pharyngitis, rhinitis and/or sinusitis were the most common diagnoses amongst those patients with an isolated IgG3 deficiency. The commonest associated subclass deficiency was that of IgG1 (113 patients) with only 14 having an associated IgG2

deficiency. Ten of the IgG3 deficient patients were given pneumococcal vaccination and all responded to the vaccine, a finding which is not unexpected since most anti-pneumococcal antibody would be of the IgG2 subclass. IgG3 antibodies are known to be important in immune responses to a number of viruses including herpes simplex, varicella zoster and Epstein Barr [reviewed by Skvaril, 1986] and appear to predominate early in the response to primary infection. This same group [Björkander et al., 1985] have previously reported an association between impaired lung function, IgA deficiency and low levels of IgG3 in two patients and IgG2 in four patients. There was no obvious difference in the pattern of, or the pathogens involved in, the infections of the IgG3 and IgG2 deficient patients. The basis for the apparent preferential production of IgG3 following an antigenic stimulus is unclear.

IgG4 Deficiency

Selective IgG4 deficiency has been described but the technical difficulties associated with the measurement of IgG4 in the serum makes interpretation of the data difficult. Between 20-30% of the general population will have IgG4 levels that are undetectable using immunodiffusion methods and hence only studies using more sensitive methods (eg radioimmunoassay or ELISA) identify unequivocally, patients with selective IgG4 deficiency [Heiner et al., 1983]. While Heiner and colleagues [1986] have argued that low levels of IgG4 (ie < 10mg/l) may be associated with clinically recognisable syndromes (most commonly recurrent infection), 75% of the patients they described had other associated IgG subclass abnormalities. Needless to say some argument still exists as to the clinical relevance of the relatively common finding of low levels of IgG4 [Jefferis and Kumararatne, 1990].

(b) Aims of the Study

The Hospital for Sick Children, Great Ormond Street is a tertiary paediatric referral centre with an established Immunology Department. The Department

consults on both inpatients and outpatients where approximately 200 new patients

are seen per year, the majority being referred because of suspected

immunodeficiency or allergy. The patients attending the Immunology Department

are a highly selected population since most will have been referred from and

evaluated by another medical practitioner, usually a paediatrician. This pattern of

referral has led to the collection of many patients with the same diagnoses attending

a single institution and thus providing an opportunity for the study of large numbers

of patients with the same, yet relatively uncommon, conditions.

The Department of Immunology has been measuring IgG subclass levels

since 1985 and these records therefore provided a large resource of information.

The majority of studies published to date analysing IgG subclass deficiency in

childhood have described relatively small numbers of children. In an effort

therefore to document the clinical associations of IgG subclass deficiency in a large

group of children, the case notes of patients who had been investigated and found to

have an IgG subclass deficiency over a three year period, were analysed

retrospectively. In particular an attempt was made to link an individual subclass

deficiency with a particular clinical association so as to better understand the

biological consequences of a deficient IgG subclass.

(c) Methods

Patient selection: Patients included in this study were all those over the age

of six months whose IgG subclasses were found to be low at presentation during the

retrospective three years of the study period. Patients with a known

immunodeficiency syndrome eg Wiskott Aldrich, X-linked agammaglobulinaemia

and severe combined immunodeficiency were excluded from the study. Deficiency

of an IgG subclass was defined as a level more than two standard deviations below

the age matched mean derived from a normal range that had been developed in the

Department using the same methodology as that used for analysing the clinical material described in this study. Clinical diagnosis was based on a detailed review of the clinical case notes and not based on the diagnostic code given on discharge. The diagnosis of allergy was made on the clinical history together with positive laboratory tests including IgE levels and specific IgE antibodies measured by RAST where these were available. Frequent/chronic/recurrent infection was interpreted as such when it interfered with lifestyle, caused end organ damage and/or necessitated referral and investigation.

Immunochemistry: Serum IgG subclasses were determined by Mr N Seymour in the Department of Immunology, Institute of Child Health, London. A single radial immunodiffusion technique utilising monoclonal antibodies was employed and is described in Chapter 2. The inter- and intra-assay variation for the IgG subclass assay were a maximum of 2.5% and 6% respectively and a reference serum calibrated against a commercial standard was used with each assay. Lower limits of detection for the assays were IgG1 1.5mg/dl, IgG2 and IgG3 1mg/dl, and IgG4 0.6 mg/dl. The age matched normal range was developed in the Department using sera from age-matched elective surgical cases and healthy schoolchildren with at least 20 individuals in each group. Normal ranges for our laboratory are shown in Figure 3.1 (overleaf). All results were expressed with reference to this normal range. Total IgG, IgA and IgM were measured by the Department of Biochemistry at The Hospital for Sick Children, Great Ormond Street, on a centrifugal fast analyser employing monoclonal antibodies (Atlantic, Scarborough, Maine, USA).
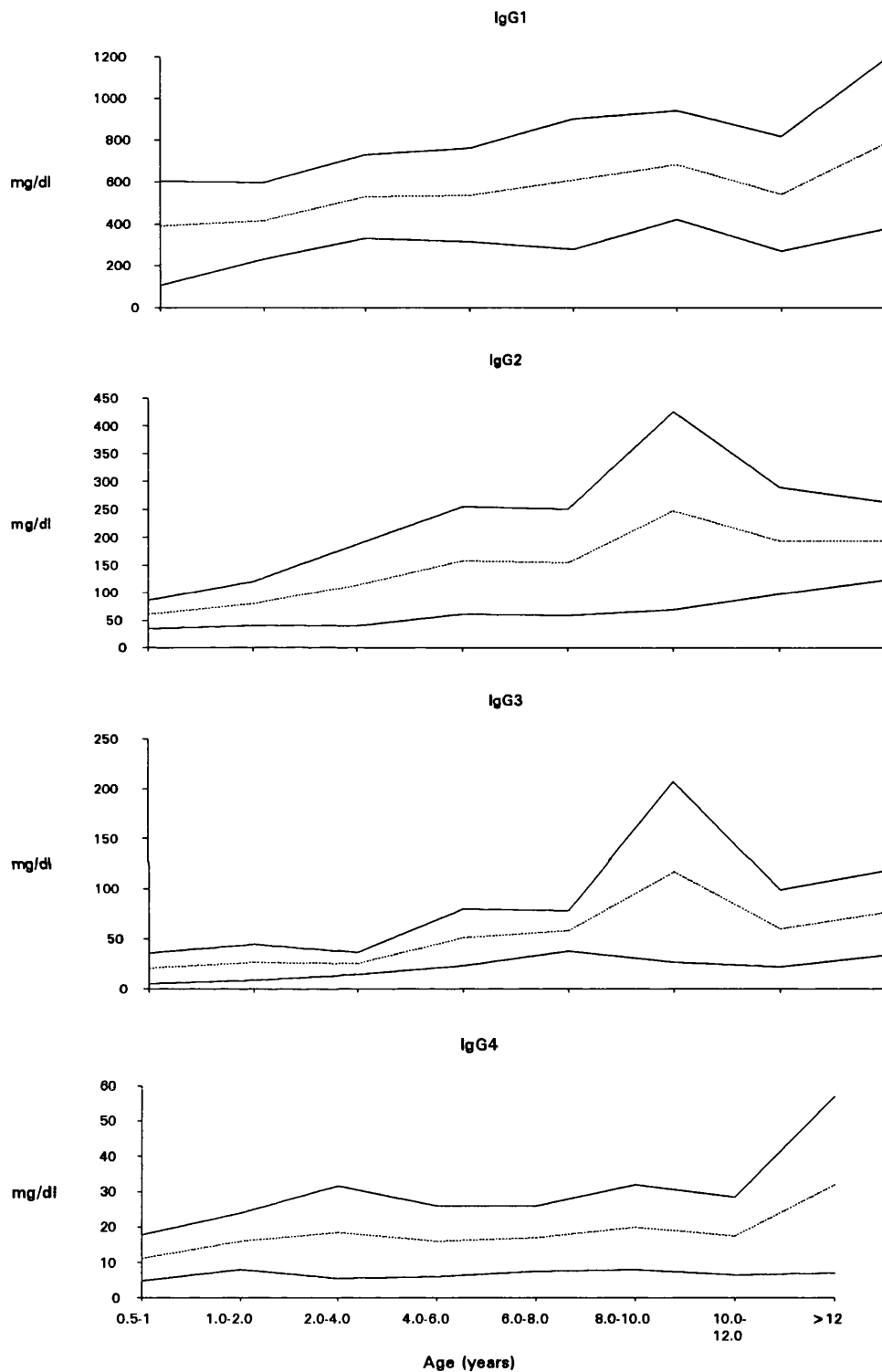
Figure 3.1: Age related IgG subclass normal ranges established in the laboratories of the Department of Immunology, Institute of Child Health. Mean (dotted line) and + and - 2 standard deviations (solid lines) are indicated on the graphs.

(d) Results

Patterns of IgG subclass deficiency: During the period of analysis 583

patients between the ages of 6 months and 16 years had their IgG subclass levels

estimated. Of these, 267 were found to have one or more subclasses >2 SD below

the age matched mean. Of these 267, the records of 232 patients were eligible for

analysis and are included in the results below. The 139 males and 93 females in the

study group gave an overall male to female ratio of 1.5:1. This ratio held true for

males and females within each individual subclass deficient group as well as for the

group as a whole, irrespective of age.

Overall the most common deficiency encountered, either isolated or in

combination, was that of IgG4 (n=125), followed by IgG2 (n=88), IgG1 (n=78)

and IgG3 (n=64). Figure 3.2 illustrates the distribution of deficiency by subclass.
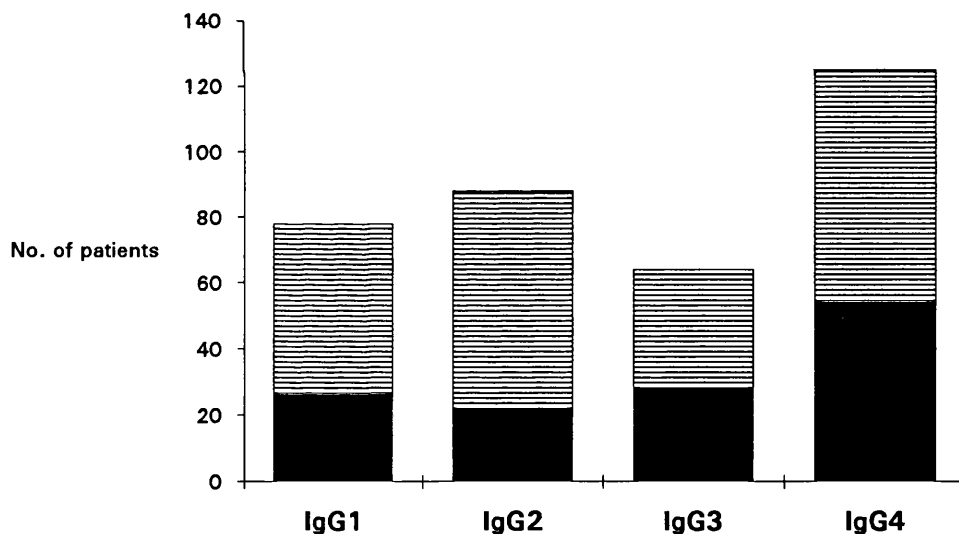
Figure 3.2: Distribution of IgG subclass deficiencies in 232 selected paediatric patients. Patients presented with either isolated deficiencies (solid) or combined deficiencies (lines).

The most common isolated deficiency encountered was that of an isolated

IgG4 deficiency (n=55) followed by isolated IgG3 (n=27) and isolated IgG1

(n=26). The most common combination of deficiencies was the association between

IgG2 and IgG4 seen in 44 patients. A full list of all the deficiencies encountered in

the study group is shown below in Table 3.1. Levels of subclasses were shown to

vary with time quite significantly for some patients in whom levels were measured

on several occasions, although many were persistently low.

Table 3.1: IgG subclass deficiency in 232 paediatric patients. Distribution of deficiencies by subclass in decreasing order of frequency.

IgG Subclass deficiency	Number of patients
Isolated IgG4	55
Isolated IgG3	27
Isolated IgG1	26
Combined G2 & G4	26
Isolated IgG2	23
Combined G1 & G2	16
Combined G3 & G4	14
Combined G1 & G4	12
Combined G1,G2 & G4	10
Combined G1 & G3	8
Combined G2 & G3	4
Combined G2, G3 & G4	4
Combined G1, G2, G3,& G4	3
Combined G1, G2 & G3	2
Combined G1, G3 & G4	2

The average number of patients in each age group was 28.9 (SEM = 3.0)

and the percentage of the total number was similar for all the age groups. However,

differences were found in the frequency of subclass deficiency encountered in the

different age groups. The proportion of patients presenting under the age of six

years with an IgG3 deficiency was significantly less (p < 0.05) than those in the

same age group with an IgG4 deficiency and also less than those with an IgG2 or

IgG1 deficiency (Figure 3.3). While seldom detected under the age of 6 years,

IgG3 deficiency was found more commonly than IgG1 and IgG2 deficiency in

children over the age of six. This pattern tended to last until adolescence (> 12

years) at which point the various IgG subclass deficiencies tended to be equally

represented.

Figure 3.3: Age at presentation of paediatric patients (n = 232) with IgG subclass deficiencies.

Of 209 patients in whom total IgG, IgA and IgM were measured, 49 (24%)

had an associated IgA deficiency (more than 2 SD below the mean). Of these 49

patients 21(43 %) had an associated IgG2/IgG4 deficiency (either alone or in

combination). The commonest association was with an isolated IgG4 deficiency

(n=9) followed by isolated IgG3 (n=7), combined IgG2/IgG4 (n=7) and isolated

IgG2 (*n*=5). Total serum IgG deficiency was identified in less than 10% of the 209

patients.

Clinical characteristics: The most common clinical presentation of the group

as a whole was that of recurrent or severe infection (Figure 3.4). Forty two percent

of all the patients had such infections as their presenting problem. A further 17%

had allergy as a presenting complaint and a further 13% presented with a

combination of allergy and recurrent infection. The remaining group of patients

(26%) was heterogeneous with a wide range of diagnoses. The two largest groups

of patients with a diagnosis other than infection or allergy, were those with a

seizure disorder (n=22) and patients with autoimmune disease (n=8).
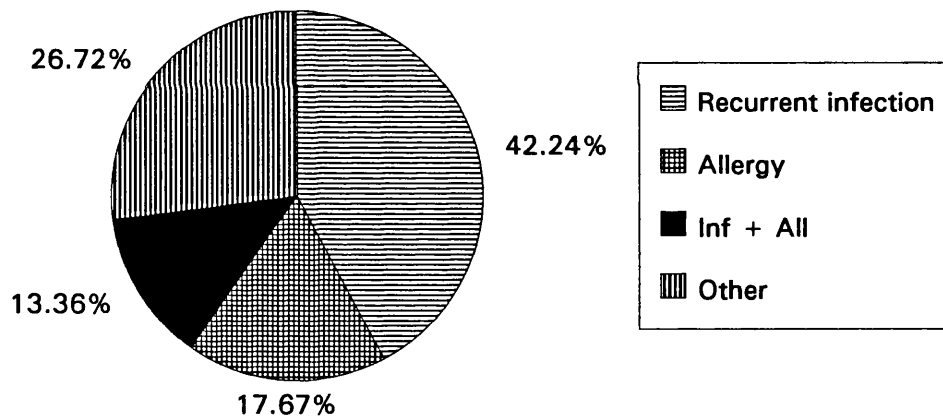
Figure 3.4: Clinical presentation of 232 patients with IgG subclass deficiencies, illustrating the preponderance of cases presenting with infection and/or allergy.

Analysis of each individual subclass deficient group revealed no particular

deficiency associated with any diagnosis, and the proportion of patients with

recurrent infection or allergy in each subclass group reflected the overall trend and is illustrated in Figure 3.5.
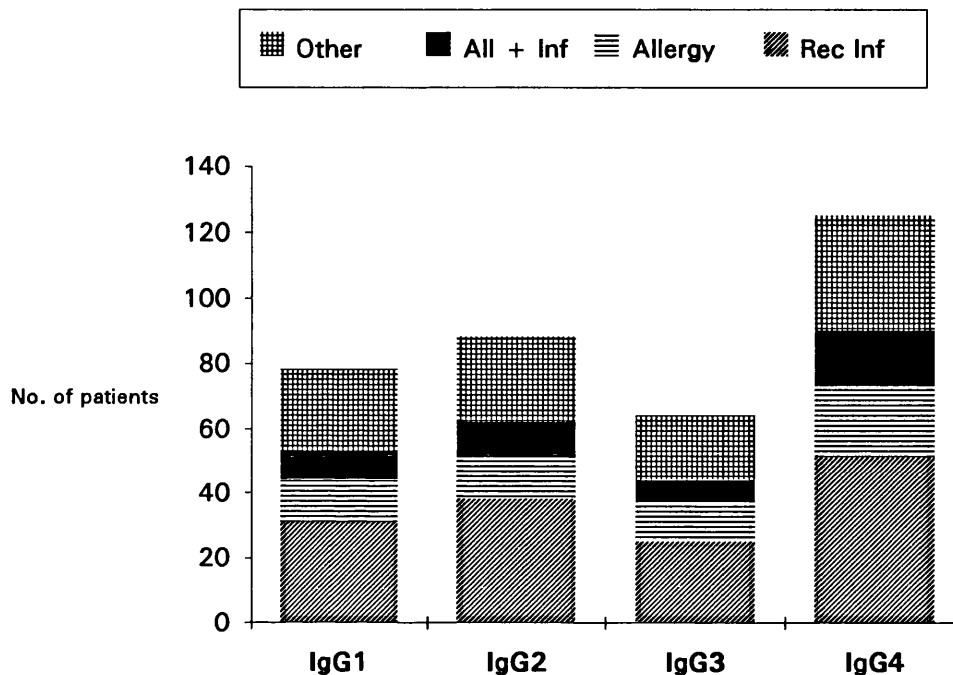

Figure 3.5: Breakdown of clinical presentation of 232 subclass deficient patients by subclass, illustrating similar proportions of patients with infection and/or allergy within each subclass deficient group. Clinical categories shown are; recurrent infection (diagonal stripes), allergy (horizontal stripes), combined allergy and infection (solid) and other miscellaneous diagnoses (hatched).

Of all those patients presenting with recurrent infection the spectrum of disease was broad and is illustrated in Table 3.2. The majority of patients in the group with recurrent upper and lower respiratory tract infection were children with an average of one infection per month who required frequent courses of antibiotics. The patients with recurrent severe chest infection usually had significant sputum production and abnormal chest X rays. The patients with septicaemia or severe infection included a group with meningitis or overwhelming sepsis. No specific subclass deficiency was associated with any particular form of recurrent infection,

although in the group with overwhelming sepsis, a low level of IgG1 was found more commonly than in the group with recurrent infection.

Table 3.2: Diagnoses in patients presenting with recurrent or severe infection (n = 128)

Clinical diagnosis	No. of patients
URTI/LRTI	63
Severe chest infection	17
Septicaemia/severe infection	15
Skin infection	11
Otitis Media	10
Mouth ulcers	7
Sinopulmonary disease	5

A total of 22 children had seizures as a major clinical problem. Fourteen of these had an associated IgG2/IgG4 deficiency (alone or in combination) and seven had an associated IgA deficiency.

(e) Discussion

A low level of IgG4 was the most frequent abnormality detected and the previously described association between IgG2 and IgG4 deficiency was confirmed in this study. The ratio of males to females presenting with a subclass deficiency remained consistently 1.5:1 and there was no tendency for this to reverse in adolescence. There were similar numbers of patients in each age group although the distribution of subclass deficiencies did not remain constant. IgG4 deficiency presented more commonly below the age of six and the incidence fell in the older age groups. This finding would be consistent with what we understand of the maturation of the immune system, and in particular, the maturation of IgG subclass production (Van der Giessen et al., 1975). However, IgG3 deficiency was rarely

found below the age of six years yet appeared more commonly in older children. The maturation of IgG3 is thought to occur earlier than IgG4 and hence a different explanation must be sought for the later presentation of this deficiency. It is possible that this group of children represents those who, when entering school at the age of 5 or 6, encounter for the first time a spectrum of infectious agents which unmasks their pre-existing immunological defect.

It has been established that the genes encoding the heavy chains of human IgG and IgA are closely linked on chromosome 14 (Flanagan and Rabbitts, 1982), and deficiencies involving both IgA and IgG subclasses have been reported. Our study confirms this association but unlike previous reports selectively linking IgG2/IgG4 deficiency with IgA deficiency (Oxelius et al., 1981), we have found that all four subclasses may be associated .

In common with others (Heiner, 1984, Oxelius 1984), we have observed the association between IgG subclass deficiency and frequent or severe infection. However, because of the highly selected nature of our patient group we can conclude little about the frequency of IgG subclass deficiency in association with recurrent infection. Others (Flanagan and Rabbitts, 1982) have described patients with undetectable or deficient subclasses who were apparently healthy. Shackleford et al., [1990] have recently published a survey of IgG2 levels in healthy children recruited from well-child care clinics and those undergoing elective surgical procedures. 11 out of 575 (1.9%) screened were noted to have low levels of IgG2. However, as children with frequent infection were excluded, the true incidence of IgG2 subclass deficiency in this population cannot be identified and levels of IgG1, IgG3 and IgG4 were not measured in the original screening procedure. It will require large population based studies to define the frequency of IgG subclass deficiency in the healthy paediatric community and the frequency of patients with recurrent infection having normal levels of IgG subclasses. Furthermore, as levels

of subclasses in this study were shown to vary for any given patient with time, the diagnosis of an IgG subclass deficiency should only be made if a persistent abnormality is noted.

The group of patients in the recurrent or severe infection category represented a group with a broad spectrum of clinical severity. No quantifiable difference in IgG subclasses between those having severe infection and those with mild though frequent infection could be identified. Individuals in whom a subclass was undetectable were not more severely affected than those who were simply low in a subclass. Further research is required in the area of subclass specific antibody responses in order to determine why patients with a comparable subclass deficiency and no other abnormality should be so differently affected and what influences compensation for any one subclass deficiency.

Many of our patients with recurrent infection were treated with prophylactic antibiotics (cotrimoxazole once daily) as a first line treatment and appeared to improve. A minority of patients were identified who did not respond to antibiotic prophylaxis and who had in addition evidence of end organ, especially lung, damage. This group required intravenous immunoglobulin replacement and in general showed a good response to treatment with a reduced frequency of infection.

The association between epilepsy and subclass deficiency has been previously described (Duse et al., 1986) and intravenous immunoglobulin has been suggested as a form of therapy. Ten percent of our total group had seizures although three of these had recurrent infection and a further seven had a diagnosis of food allergy. The role of IgG subclass deficiency in epilepsy is unclear and needs further investigation.

Part of the difficulty in interpreting IgG subclass deficiency data is the definition of deficiency and the inadequacy of the normal range used. Central to the interpretation of IgG subclass data is the whole question of when a low level of a

subclass is considered a deficiency. We have accepted moderately reduced levels (ie more than 2 standard deviations below the age related mean) as deficiency although we accept that this will include 2.5% of normal individuals. Furthermore, it has been argued that IgG subclass levels do not follow a normal distribution, which suggests that geometric means and standard deviations of the means would not adequately describe the distribution. The definition of deficiency in relation to an age related mean is of course critical but may further confound the issue since normal ranges (particularly for IgG2, IgG3 and IgG4) vary significantly from laboratory to laboratory [Beard et al., 1990] (see Chapter 6). The reason for this variation is multifactorial but includes differences in methods for measuring the IgG subclasses, different antisera, different reference standards as well as the inherent difference in the populations studied and the possible influence of Gm allotype. Any study therefore, that relates levels of IgG subclasses measured "in house" to a normal range derived from a different laboratory runs the risks of incorrectly assessing the "normality" or otherwise of the levels measured. An alternative approach to defining IgG subclass deficiency is to include only those who have a trace or undetectable amount of subclass in their sera. While easier to define, this definition may well only represent the "tip of the iceberg" of IgG subclass deficiency and exclude a potentially affected population. The sensitivity of the radial immunodiffusion technique most commonly used is inadequate when measuring IgG4 and hence an undetectable level of IgG4 by this technique may well represent an overinterpretation of those who are truly deficient.

IgG subclass levels are affected by various factors which need to be considered. Clearly one of the most important is age. In their ontogeny IgG subclasses are known to differ from each other: IgG3 and IgG1 antibodies are produced in early infancy whereas IgG2 and IgG4 appear later [Lee et al., 1986]. Thus, low IgG2 and IgG4 levels in children may indicate delayed maturation.

Factors such as infections, surgery and immunosuppressive drugs may all influence the level of IgG subclass levels in the serum and need to be taken into account. Furthermore, the influence of Gm allotype is important and may influence serum levels of the IgG subclasses. A normal range derived from a homogeneous local population may compensate for any Gm allotype influence, especially if deficiency is defined in terms of standard deviations below the mean.

The clinical relevance of IgG subclass deficiency has also been called into question because of the existence of patients who lack subclasses due to heavy chain deletions yet are completely healthy [Lefranc et al., 1982]. This apparent contradiction suggests that there are compensatory mechanisms that may be relevant although little is understood of these. While there is little adequate epidemiological data, approximately 10% of sera in some studies have shown decreased concentrations of one or (more commonly) combinations, of the IgG subclasses [Ochs et al., 1987]. In Swedish and Swiss national registries, IgG subclass deficiency was present in 14 and 10% respectively, of children with primary immunodeficiencies [Fasth, 1984; Ryser et al., 1988] representing one of the commonest immunodeficiencies. Clinically the common denominator amongst such patients was frequent infection (especially respiratory tract infection) although this is not surprising since the majority of patients were selected on this basis.

In conclusion, for the data presented here to be viewed in proper perspective, more information will be required on the incidence of IgG subclass deficiencies in the paediatric population as a whole. What proportion of healthy children have low levels of IgG subclasses and what proportion of children presenting with the clinical manifestations we have described have normal levels of IgG subclasses? The variation of subclass levels in an individual with time has been noted and hence for a meaningful assessment of a subclass deficiency no single measurement should suffice, and a subclass should be documented to be low on at

least two occasions three months apart. Furthermore, since in 90% of cases an IgG

subclass deficiency is associated with a normal total level of IgG, total IgG levels

are inadequate as a screening procedure for subclass deficiencies. In an effort to

explain why patients with a similar quantitative defect of single or combined

subclasses are so differently affected, assessment of subclass specific antibody

produced in response to protein or carbohydrate antigens needs to be explored.

Finally, it is noted that prophylactic antibiotics are an important form of therapy for

patients with recurrent infection and can be used profitably. However a small group

of patients do not respond to this form of therapy and need intravenous

immunoglobulin. As yet the characteristics of the latter group have not been fully

evaluated.

CHAPTER 4

IgG subclass recognition of the antigens of a common microorganism

(a) *Moraxella (Branhamella) catarrhalis*: Rationale for it's use as a model of IgG subclass ontogeny

Despite the recognition of *M. catarrhalis* as a pathogen by Sir William Osler in 1919 [Cushing, 1927] and it's implication in otitis media in 1927 [Hart, 1927], the pathogenic potential of *M. catarrhalis* has not been widely recognised in modern clinical practice. It has in the past been regarded as one of the commonest inhabitants of the pharynx [Mackowiak, 1982] and the long held assertion that it is a harmless commensal has led medical technologists to refrain from individual identification of *M. catarrhalis* and a group of upper respiratory tract organisms referred to as non-gonococcal non-meningococcal *neisseria* or "normal" flora. The 1980's have, however, witnessed an increasing awareness of the clinical relevance of this organism, mainly due to numerous reports describing *M. catarrhalis* as the sole pathogen in a variety of clinical settings. The reason for this increased reporting is unclear but it has highlighted the important differences between adult and paediatric *M. catarrhalis* infection. The possibility that this difference might have an immunological basis has led to the studies described in the next two chapters.

(i) Microbiology

M. catarrhalis is an aerobic, oxidase positive, non-motile, gram negative diplococcus. During the early 1900's it was known as *Micrococcus catarrhalis* but because of it's similarity to *neisserial* species and it's cytochrome oxidase activity it was classified as *Neisseria catarrhalis* in the 1920's. In 1970, on the basis of Catlin's nucleic acid hybridisation studies, analysis of guanine plus cytosine content and genetic transformation experiments, it was transferred to the new genus *Branhamella*, named after Sara E. Branham (1888-1962), an important contributor to the development of meningococcal group classification based on capsular

reactions. However, there has been a further taxonomic change and the most recent

edition of *Bergey's Manual of Systematic Bacteriology* has assigned this organism as

a subgenus of *Moraxella*, based on studies by Bore [1984]. The full name is

therefore *Moraxella (Branhamella) catarrhalis* although many continue to call the

organism *Branhamella catarrhalis*.

On gram stain of sputum this organism is identified as a gram- negative,

intra- or extra-cellular, kidney bean-shaped diplococcus. The organism grows after

$+/-$ 18 hours on 5% sheep blood or chocolate agar at 37°C in 10% humidified CO_2.

Colonies are smooth, small, mucoid, 1-3 mm in diameter and white to grey in

colour. Oxidase is produced and nitrate reduction accomplished, but the organism

fails to metabolise glucose, maltose, sucrose, lactose or fructose. Polysaccharides

are not produced from sucrose while DNase is produced [Doern et al., 1980].

There is no cross reaction with the rapid diagnostic assays currently used for the

detection of polysaccharide capsules of *Neisseria meningitidis* (A-D and X-Z) or

with the direct fluorescent antibody test for *Neisseria gonorrhea*.

The presence of this organism as a common inhabitant of the upper

respiratory tract has contributed to the confusion surrounding it's role as a

pathogen. While the heavy growth of the organism in the sputum together with a

large amount of polymorphs is suggestive of infection, sputum cultures may still be

difficult to interpret. Thornley et al. [1982] compared sputum and transtracheal

aspirates to investigate the reliability of sputum examination in the diagnosis of

lower respiratory tract infection with *M. catarrhalis*. Their method of direct

examination of sputum together with growth in direct and quantitative culture

identified *M. catarrhalis* as a pathogen in 11 patients and in 10 of these *M.*

catarrhalis was subsequently isolated from transtracheal aspirates. The authors also

stressed the significance of finding the organism within leucocytes in the sputum. A

Lancet editorial [1982] on this subject concluded that in adults with underlying lung

disease or compromised immunity, the isolation of *M. catarrhalis* from purulent sputum needed to be taken seriously. In contrast, it's isolation from the sputum of healthy adults could probably be disregarded. The question of *M. catarrhalis* isolation in childhood was not addressed.

Isolation rates of *M. catarrhalis* from the upper respiratory tract of healthy children vary from study to study depending on various factors including the choice of culture media. Overall trends however show that carriage is higher in the winter than the summer months and that carriage appears more common amongst the younger age groups. Nasopharyngeal colonisation of healthy children in Cleveland, Ohio, was 46% in autumn and winter but only 9% in spring and summer [Van Hare et al., 1987]. Higher percentages of *M. catarrhalis* were isolated from children younger than 24 months than from older children. Further evidence comes from three studies in the 1980's of nasopharyngeal specimens from Swedish children who showed no symptoms of respiratory infection. *M. catarrhalis* was isolated from 45% of children 2 years or younger, but from only 17% aged 6-7 years [Lundgren and Ingvarrson, 1986] and similarly, from 36% of children 2 years or younger but from only 13% who were 6 to 9 years old [Brorson and Malmvall, 1981]. Molstad et al., [1988] studied 190 children aged 1-7 years who attended day-care nurseries and isolated *M. catarrhalis* from 58%. In contrast to children, *M. catarrhalis* was recovered from only 3% of blood agar cultures of posterior nasal specimens from 286 healthy men who were military service recruits in Finland [Jousimies-Somer et al., 1989]. Part of the problem in comparing studies describing isolation rates of *M. catarrhalis* is the great variability of culture media used. While selective media designed to improve the detection of meningococci are inhibitory for *M. catarrhalis* (due to the action of colistin) [Christensen et al., 1986], semi-selective media containing vancomycin and trimethoprin enhance the detection of this organism [Van Hare et al., 1987].

(ii) Clinical Review

Despite being considered a harmless commensal, reports in the literature describe this organism as being pathogenic in a variety of clinical settings including septicaemia [Baron et al., 1985], endocarditis [Douer et al., 1977], meningitis [Pfister et al., 1965, Feigin et al., 1969], urethritis [Smith, 1987], mastoiditis [Marcinak et al., 1987], conjunctivitis [Righter et al., 1983] and tracheitis [Wong et al., 1987]. It's role as a pathogen in immunocompromised patients has also been stressed [McNeelly et al., 1976, Srinivasan et al., 1981, Diamond et al., 1984, Wong et al., 1988] and *M. catarrhalis* may cause either pneumonia or septicaemia in patients with malignancy, those undergoing chemotherapy or patients with acquired immunodeficiency syndrome (AIDS). An increasing number of papers have discussed the role of *M. catarrhalis* as a lower respiratory tract pathogen in adults with underlying lung disease [Ninane et al., 1978, Mcleod et al., 1983, Slevin et al., 1984] while the organism's emergence as an important pathogen in otitis media and sinusitis in childhood [Kovatch et al., 1983, Van Hare et al., 1987] has more recently brought it to the attention of both clinicians and research workers.

Hager et al. [1982] reviewed the features of 429 previously reported cases of *M. catarrhalis* bronchitis and pneumonia and showed that 84% of the patients had pre-existing cardiopulmonary disease. Chest X-Ray findings, where present, were described as patchy and predominantly affected the lower lobes. The question of when the presence of *M. catarrhalis* in the sputum is clinically significant still remains controversial. Coffey was one of the first to recognise the importance of *M. catarrhalis* in the aetiology of acute otitis media. His study published in 1966 [Coffey, 1966] reported a pure growth of *M. catarrhalis* in 8% of 267 children with acute otitis media. More recent reports have suggested that there has been a marked increase in the occurrence of *M. catarrhalis* in middle ear exudates of children with

acute otitis media. Bluestone [1988] quotes an isolation rate for *M. catarrhalis* of

11.7% in acute otitis media and 8.5% in chronic otitis media, making it the third

most common organism (after *S. Pneumoniae* and *H. influenzae*) isolated in otitis

media. The increase in frequency of otitis media due to *M. catarrhalis* has been

associated with the appearance of β lactamase producing strains of the organism.

Prior to 1971 all middle ear isolates of *M. catarrhalis* were β lactamase negative

[Kamme et al., 1971]. The first reports of β lactamase production by *M. catarrhalis*

were published in 1977 [Malmvall et al.,] while a mere ten years later β lactamase

positive strains represented 75% of all isolates described by Van Hare et al. [1987].

Table 4.1 (overleaf) lists chronologically studies that have addressed the aetiology

of acute otitis media showing the emergence/recognition of *M. catarrhalis* as a

pathogen. The table also includes, where available, the percentage of isolates that

were β lactamase positive.

Table 4.1: *M. catarrhalis* in acute otitis media. Emergence as a pathogen and the increase in β-lactamase positive isolations (NR = not recorded).

Location [reference]	Year	*M. catarrhalis* isolates	% β lact-amase +ve
Literature Review [in Shurin et al., 1983]	1945-1980	$^{170}/_{3263}$ (4.5%)	NR
Natchez, MS [Coffey et al., 1987]	1964-1966	$^{52}/_{698}$ (7.4 %)	0%
Atlanta, GA [Aronovitz GH, 1974]	1973	$^{8}/_{67}$ (8.3%)	0%
Boston, MA [Shurin et al., 1980]	1974	$^{9}/_{132}$ (6%)	NR
Asahikawa, Japan [Fujita et al., 1983)	1976-1977	$^{2}/_{100}$ (2%)	NR
Cleveland, OH [Shurin et al., 1983]	1979-1980 1980-1982	$^{3}/_{47}$ (6%) $^{26}/_{98}$ (26.5%)	76.9%
Cleveland, Ohio [Van Hare et al., 1987]	1979-1984	$^{60}/_{355}$ (16.9%)	75%
Pittsburgh, PA [Kovatch et al., 1983]	1981-1982	$^{38}/_{200}$ (19%)	76%
Cleveland OH [Marchant et al., 1984]	1983	$^{17}/_{129}$ (13%)	85%
Galveston, TX [Chonmaitre et al., 1986]	1982-1985	$^{11}/_{84}$ (13%)	NR
Turku, Finland [Ruuskanen et al., 1989]	1980-1986	$^{22}/_{3332}$ (6.8%)	30-71%
Pittsburgh, PA [Krenke et al., 1988]	1983-1986	$^{199}/_{1087}$ (18.3%)	68-83%

(iii) Immunology

Circumstantial evidence for the importance of immunoglobulins as mediators of defence against *M. catarrhalis* is found in the strong clinical association between various immunodeficiency states and systemic or bronchopulmonary infection with *M. catarrhalis* (see above). Furthermore the age related distribution of infection

with this organism provides a further clue to both the possible pathogenesis and the role of the immune system. Of the 89 cases of *M. catarrhalis* infection reported to the Public Health Laboratory Services of the United Kingdom Communicable Disease Surveillance Centre between 1975 and 1987 (voluntary reporting, not incidence) the majority of patients were either very young or elderly. 26 (29%) were in patients over the age of 65 and 35 (39%) were in patients under the age of 4 years [CDR, 1988]. This age related distribution of infection together with the observation that an immunocompromised host may be susceptible to *M. catarrhalis* infection, suggests that immunity to *M. catarrhalis* may be acquired during childhood and may protect healthy adults from infection. Prior to the development of such immunity or following the loss of immunity (eg immunocompromised adults or the elderly) the individual may be susceptible to infection.

The development of antibodies reactive with *M. catarrhalis* has been investigated in patients suffering from maxillary sinusitis [Brorson et al., 1986], acute bronchopulmonary infections [Chapman et al., 1985, Black and Wilson 1988, Chi et al., 1990], and acute otitis media [Leinonen et al., 1981] . The serological methods have included immunodiffusion and complement fixation [Boroson et al., 1986], enzyme-linked immunoassay [Leinonen et al., 1981, Chi et al., 1990], a bactericidal assay [Chapman et al., 1985] and an immunofluorescent antibody test [Black and Wilson, 1988] (for further discussion of these methods see Chapter 5). It is apparent from these previous studies that infection with *M. catarrhalis* induces an antibody response, predominantly an IgG response, although sera from healthy controls may have antibodies and the ability to kill the organism in a bactericidal assay.

In summary, *M. catarrhalis* has been increasingly recognised as an important pathogen in a variety of clinical settings. It is the third most common organism causing otitis media in childhood and appears to affect predominantly

younger children. The organism does not appear to be an important pathogen in healthy adults although will be found in a pathogenic role in the lower respiratory tract of adults with underlying chronic lung disease including chronic obstructive pulmonary disease and occupation related lung disease. Immunocompromised patients, such as those with malignancy or on immunosuppressive therapy will likewise be at risk of invasive disease and it is within this clinical setting that patients may develop septicaemia. Occasionally *M. catarrhalis* has been isolated from the cerebrospinal fluid and has been reported to produce a purpura fulminans type of clinical picture. The host immunity to this organism remains unclear. Most studies have shown a level of specific IgG in their control groups although this is usually below the level of that found in infected patients. Despite these findings, one study was unable to show any bactericidal activity in pooled normal human serum. The relationship between carriage of the organism in the nasopharynx and serology clearly needs to be studied. Prior to the present investigations there was no information about the levels of specific antibody in children of different ages and no data on the role of the outer membrane proteins and their potential immunogenicity.

(b) The surface of *M. catarrhalis* and host defence

In common with other gram negative organisms the surface of *M. catarrhalis* is composed of outer membrane proteins (OMPs), lipooligosaccharide (LOS)/lipopolysaccharide (LPS) and fimbriae. A poorly characterised fibrillar coat has also been described and is thought to be composed of polysaccharide. Fimbriae, which play a role in adherence and colonisation of mucosal surfaces by some bacteria, have been described on the surface of a proportion, but not all isolates of *M. catarrhalis* [Murphy, 1989c] and recently confirmed by Marrs and Weir [1990] as similar to type 4 pili found on a wide variety of organisms including other *Moraxella* species, *N. menigitidis*, *N gonorrhoeae*, *P.aeruginosa* and *Vibrio*

cholera. The role of these pili and their contribution to the adherence properties and development of *M. catarrhalis* have not been studied.

A large body of evidence indicates that LOS is an important virulence factor that contributes to the pathogenicity of non-enteric Gram-negative bacteria. Johnson et al., [1975] showed that the structure of the *M. catarrhalis* LPS was unique and different in particular from that found in both other so-called non-pathogenic *neisseria* and *N. menigitidis* and *N.gonorrhoeae*. Studies of the antigenic characteristics of the LOS from *M. catarrhalis* [Murphy, 1989c] have shown that there is some variation in the antigenic characteristics of the LOS although much less than in other Gram-negative organisms such as *H. influenzae* [Campagnari et al., 1987].

In contrast to Gram-positive organisms, Gram-negative organisms have an outer membrane which contains proteins that may be important targets for bactericidal antibody. The immunogenicity of outer membrane proteins and their importance as targets of bactericidal antibody have been shown for several Gram negative organisms including nontypable *H. influenzae* [Gnehm et al., 1985], *H. influenzae* type b [Hetherington, 1989] and Group B *N. meningitidis* [Mandrell and Zollinger, 1989]. The outer membrane proteins of organisms such as nontypable *H. influenzae* vary considerably between isolates and this permits the typing of organisms on the basis of OMP variability. In an effort to type *M. catarrhalis* by OMP analysis, initial attempts to isolate the outer membrane by detergent fractionation were unsuccessful (see below) as the preparations were consistently contaminated by cytoplasmic membranes. However, in common with other organisms, *M. catarrhalis* releases endotoxin (LOS) and OMPs in the form of outer membrane vesicles (OMV) without cell lysis. This phenomenon has been shown to occur *in-vitro* in a variety of gram negative bacteria incubated under specific growth conditions including *P. aeruginosa* [Ingram et al., 1973], *S. typhimurium* [Lindsay

et al., 1973], and *N. meningitidis* [Devoe and Gilchrist 1973]. *In-vivo*, outer membrane vesicles derived from *Hib* have been shown to induce meningeal inflammation in rabbits [Mustafa et al., 1989], and increase blood-brain permeability during experimental meningitis in rats [Wispelwey B et al., 1989]. Furthermore, OMVs, pili and other surface appendages have been recovered from the cerebrospinal fluid of a neonate with meningococcal meningitis [Stephen DS et al., 1982]. Collection and purification of OMVs from broth culture supernatants of *M. catarrhalis* and comparison with more sophisticated methods of OMP isolation (sucrose density gradient centrifugation of a whole cell lysate), has shown that vesicle collection from broth culture supernatants yields OMP preparations free of cytoplasmic contamination and identical to those isolated by more sophisticated methodology [Murphy and Loeb, 1989a].

Bartos and Murphy's [1988] attempt to type *M. catarrhalis* by OMP analysis revealed that the outer membrane proteins of fifty different clinical isolates were virtually identical thereby precluding their usefulness as a method of typing. They demonstrated eight major outer membrane proteins ranging in molecular weight from 21,000 to 98,000 daltons. To date no information is available on the immunogenicity or antigenic conservation of these outer membrane proteins. In 1980, Eliasson identified by precipitation of sonicated, acid extracted *M. catarrhalis,* a unique protein antigen that was recognised by 69% of normal human sera. This protein, designated P-antigen, may well represent one of the outer membrane proteins identified in the study of Bartos and Murphy.

In an effort to characterise the immune response to *M. catarrhalis* the OMPs of this organism have been isolated and studied immunochemically. As described earlier the nature of the IgG subclass response may be determined by the type of antigen (ie protein or carbohydrate) and hence it was important to establish the target antigen(s) of antibody directed against *M. catarrhalis*. Using SDS PAGE and
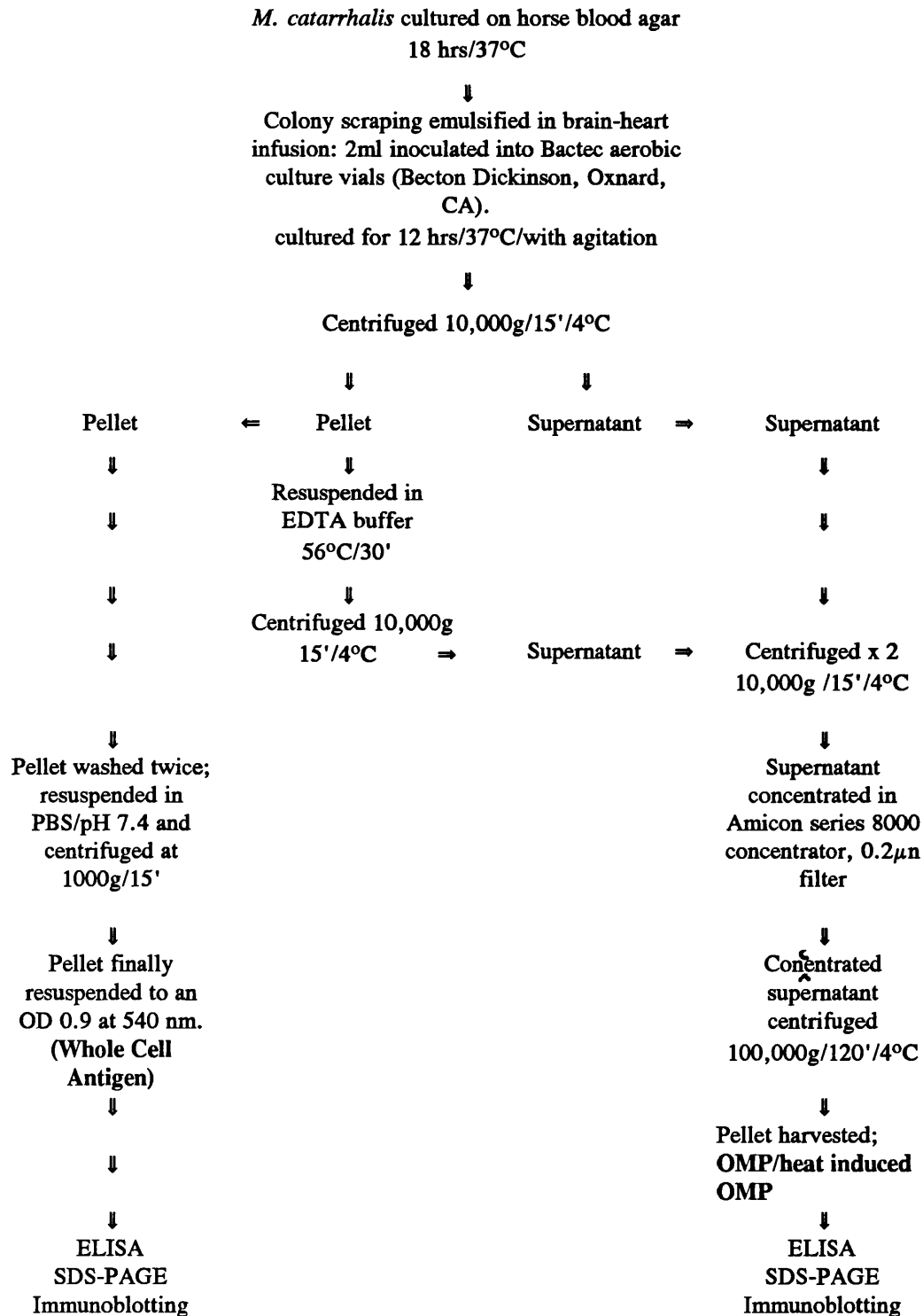
immunoblotting it has been possible to examine the interaction between the outer

membrane proteins of *M. catarrhalis* and naturally occurring human IgG subclass

antibodies.

(c) Studies on the Outer Membrane Proteins of *M. catarrhalis*

Antigen preparation and SDS-PAGE

The isolation of outer membrane proteins from gram-negative organisms can

usually be accomplished by detergent fractionation of the cell envelopes. This

method when applied to *M. catarrhalis* by Murphy and Loeb [1989], proved to be

unsuccessful due to the contamination of the preparations with cytoplasmic

membranes. These authors therefore went on to compare various methods of OMP

preparation including sucrose density centrifugation and methods based on the

differential solubility of cytoplasmic and outer membrane antigens. OMPs of other

gram negative organisms are relatively insoluble to various detergents including

sarcosyl [Filip et al., 1973], Triton-X-100 [Schnaitman et al., 1971] and 0.01M

EDTA [Gnehm et al., 1985], a property which facilitates their purification.

Applying similar methods to *M. catarrhalis* yielded large amounts of protein with

cytoplasmic contamination. In contrast, the collection of OMP vesicles secreted by

M. catarrhalis spontaneously into the culture supernatant yielded uncontaminated

fractions of pure OMPs. The preparation of OMPs for SDS-PAGE and Western

blotting was performed essentially as described by Murphy and Loeb (with some

minor modification), is described fully in Chapter 2 and briefly in schematic form

below. The added step of concentrating the initial supernatant obtained following

the second centrifugation step significantly speeded up the process of OMP

purification and permitted the use of much smaller volumes in the final

centrifugation step.

Scheme used for *M. catarrhalis* OMP preparation

M. catarrhalis cultured on horse blood agar
18 hrs/37°C

⇓

Colony scraping emulsified in brain-heart
infusion: 2ml inoculated into Bactec aerobic
culture vials (Becton Dickinson, Oxnard,
CA).
cultured for 12 hrs/37°C/with agitation

⇓

Centrifuged 10,000g/15'/4°C

⇓		⇓	

Pellet	⇐ Pellet	Supernatant ⇒	Supernatant
⇓	⇓		⇓
	Resuspended in		
⇓	EDTA buffer		⇓
	56°C/30'		
⇓	⇓		⇓
	Centrifuged 10,000g		
⇓	15'/4°C ⇒	Supernatant ⇒	Centrifuged x 2
			10,000g /15'/4°C
⇓			⇓
Pellet washed twice; resuspended in PBS/pH 7.4 and centrifuged at 1000g/15'			Supernatant concentrated in Amicon series 8000 concentrator, 0.2μn filter
⇓			⇓
Pellet finally resuspended to an OD 0.9 at 540 nm. (Whole Cell Antigen)			Conçentrated supernatant centrifuged 100,000g/120'/4°C
⇓			⇓
			Pellet harvested; OMP/heat induced OMP
⇓			⇓
ELISA SDS-PAGE Immunoblotting			ELISA SDS-PAGE Immunoblotting

SDS-PAGE and Immunoblotting.

SDS-PAGE was performed essentially as described by Laemmli [1970] and is described fully in Chapter 2. Western blotting on nitrocellulose after electro-transfer was performed as described by Towbin et al. [1979] and is also described fully in Chapter 2.

(d) Results

Initial experiments were designed to evaluate the method of OMP isolation and to compare the difference in yield between the heated and unheated bacterial preparations. Heating the bacterial preparation was designed to increase the amount of outer membrane protein harvested by stimulating vesicle formation. Figure 4.1 shows the results obtained from an initial experiment where OMPs were isolated from the *M. catarrhalis* culture supernatant as described on the previous page. The yield of OMP following bacterial heating in EDTA buffer prior to vesicle collection was compared to conventional methods and the results of Coomassie staining of these preparations of OMPs can be seen in lanes d and e (heat induced) compared to j (no heating) of Figure 4.1. The added step of heating appeared to offer no advantage in terms of yield and simply added time to the purification procedure and was hence subsequently omitted. Figure 4.1 also includes some important control material. Lane a shows the low OMP yield from supernatant concentrated but not yet centrifuged at 100,000g. Lane b shows the absence of OMPs from the Amicon filtrate, suggesting that the proteins of interest were retained in the concentrated supernatant. Lane f shows that there were no contaminating proteins in a control culture medium preparation that apart from not being inoculated was treated in the same way as the inoculated culture medium.
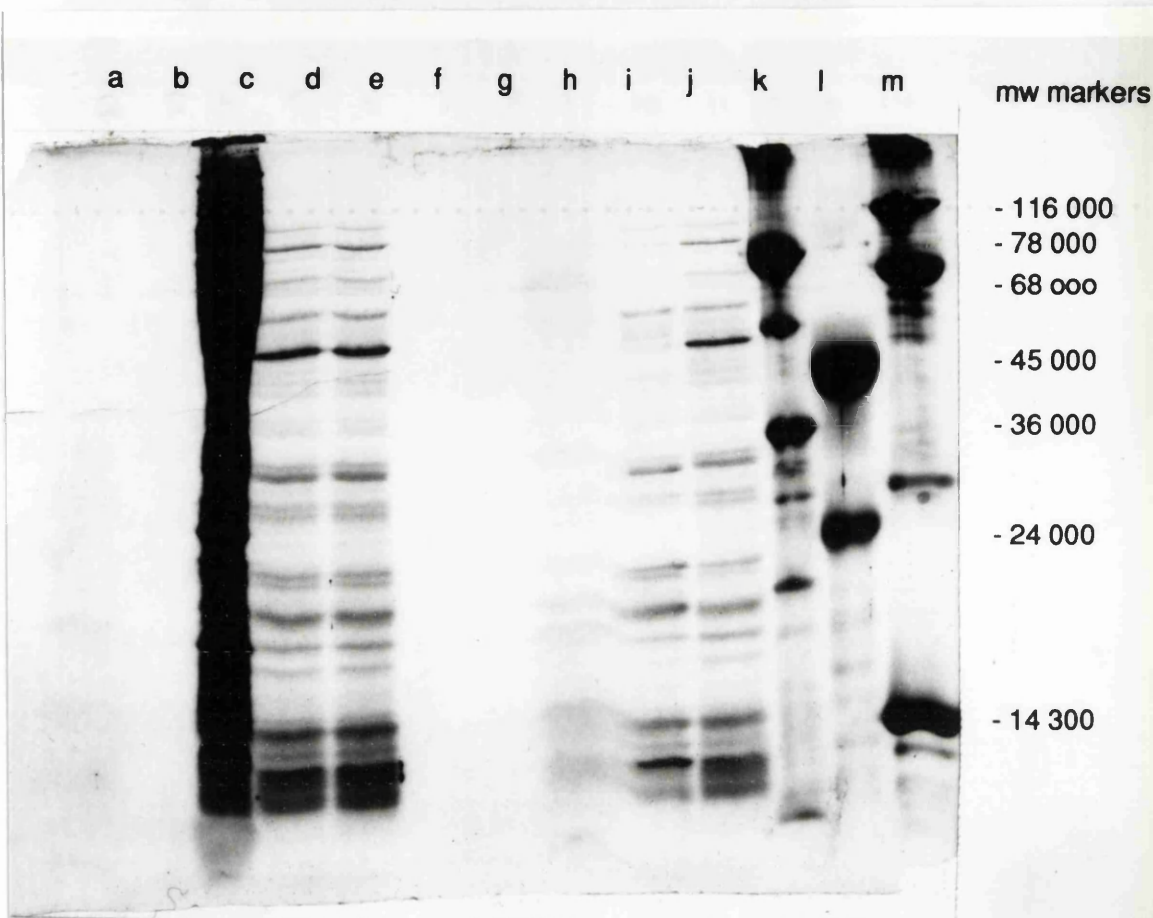
Figure 4.1:Coomassie brilliant blue-stained 10%SDS-polyacrylamide gel electrophoresis of outer membrane protein preparations from *M. catarrhalis*, whole cell antigen and control medium. Lane a) Culture supernatant prior to the final centrifugation step; b) Amicon stirrer filtrate; c) whole cell antigen ie whole bacteria; d and e) heat induced vesicles; f and g) culture medium alone (no bacterial inoculation) following 1st and 2nd centrifugation steps (f) and Amicon concentration (g); h) lane contaminated by overspill from i; i) vesicle preparation stored at -20°C; j) vesicle preparation obtained without bacterial heating; k, l, m) molecular weight markers.

The successful isolation of the OMPs of *M. catarrhalis* permitted the patterns of OMPs obtained to be compared with the results that had been published by Murphy and Loeb [1989]. These authors had identified 6 to 8 major proteins visible on Coomassie staining, whilst noting the presence overall of between 10 and 20 OMP bands. This pattern of major and minor protein bands is typical for gram negative bacteria. Following personal communication and a visit to Dr Murphy's

laboratories in Buffalo agreement has been reached that the OMP patterns obtained independently in the two laboratories are essentially the same.

Following the above experiment silver staining of the OMPs and various control material was undertaken. The increased sensitivity of the silver stain allows visualisation of proteins undetectable by Coomassie staining. Figure 4.2 shows the results obtained from the silver staining of OMPs and control material from bacteria that had not been heated prior to vesicle collection. Once again the Amicon filtrate and control culture medium contained no proteins (lanes A and F respectively). Silver staining revealed bands that were invisible or only poorly seen on Coomassie staining and which later proved to be important when analysing the immunoblots. PAS staining of the gels for the presence of carbohydrates (see Chapter 2) were negative.
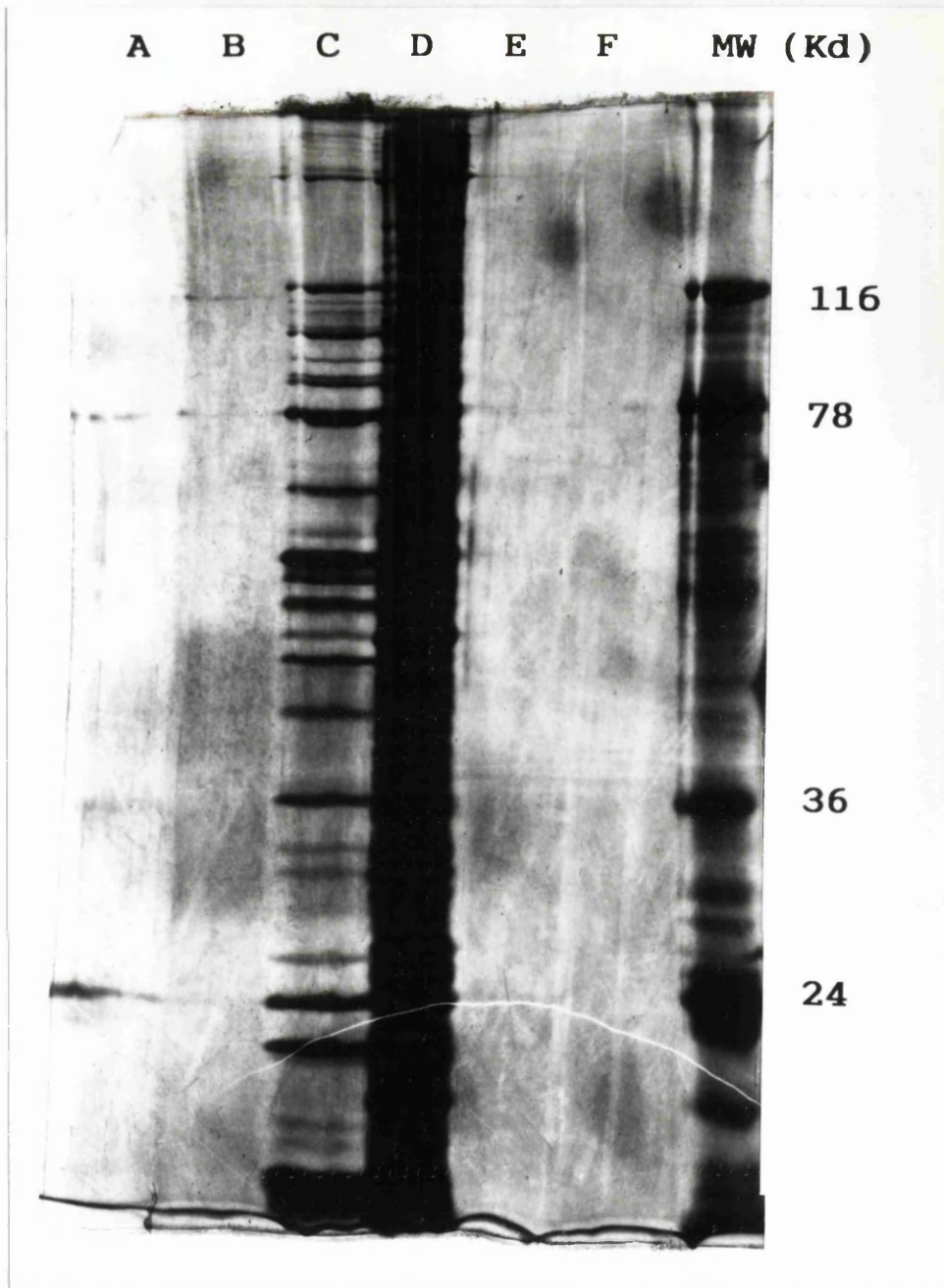
Figure 4.2: Silver-stained 10% SDS polyacrylamide gel showing protein bands from various preparations of *M. catarrhalis*; Lane **A**, Amicon filtrate; **B** Supernatant following final centrifugation step; **C** pellet obtained after final centrifugation step; **D** whole cell antigen; **E** control culture medium (no bacteria added), supernatant following final centrifugation; **F** control culture medium "pellet" following final centrifugation (since no pellet was seen, appropriate site on the tube wall was washed for adherent protein). Molecular mass standards are indicated on the right and were run in the lane marked MW.

Following the successful isolation of the OMPs of the reference strain of *M. catarrhalis* obtained from the NCTC, a comparison was made with the OMPs from clinical isolates of *M. catarrhalis*. These isolates were obtained from the clinical microbiology laboratories of the Hospital for Sick Children, Great Ormond Street and were isolated from a middle ear specimen and a tracheal aspirate of different patients at different times of the year. The ability to analyse the immune response of this organism within various groups of patients relied on the similarity of OMPs between isolates. Figure 4.3 shows the Coomassie stained OMPs isolated from the reference organism and two clinical isolates respectively as well as the stained whole cell antigen. The similarity observed between the patterns of OMPs was striking and suggests that the OMPs are well conserved between the isolates of *M. catarrhalis*.
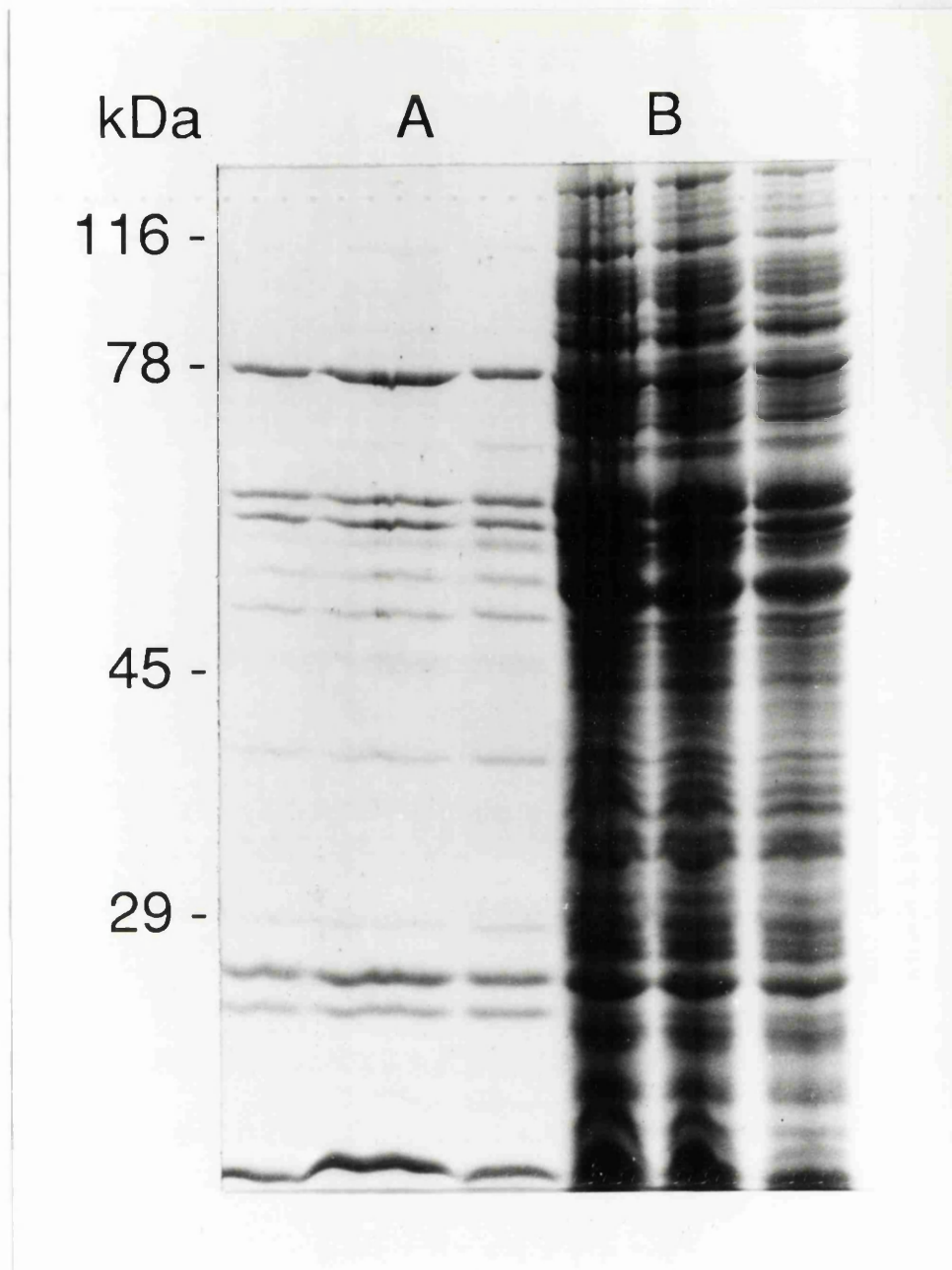
Figure 4.3: Coomassie brilliant blue-stained 10% SDS-polyacrylamide gel after electrophoresis of outer membrane proteins (A) and whole bacterial cell antigen (B) prepared from a reference isolate and two clinical isolates of *M. catarrhalis*. Molecular mass standards are indicated at left.

Having established that the OMPs differed little between clinical isolates the next step was to discover whether these OMPs were targets for human serum

antibody. The methods for immunoblotting and the serum used for this series of experiments have been described fully in Chapter 2.

Initially it was necessary to evaluate the specificity of any binding seen on immunoblotting. Figure 4.4 shows the results from an experiment designed to show the specificity of such binding. OMPs from *M. catarrhalis* and the proteins from a species of pseudomonas were transferred onto nitrocellulose and immunoblotted utilising the pooled standard adult serum as the detector antibody. In addition the binding of the pooled serum was compared before and after adsorption of the standard serum with live *M. catarrhalis*. The standard serum used has been described fully in the methods section (Chapter 2). Standard serum for the adsorption experiments was prepared by incubating an equal volume of serum and a bacterial pellet at either 37°C, 4°C or on ice for 3 hours. Following this incubation serum was centrifuged at 10,000g for 10 minutes and the serum recovered (adsorbed standard serum) was stored at -70°C.

Figure 4.4 shows that the binding of IgG antibodies from the pooled serum to the OMPs prepared from the two clinical isolates of *M. catarrhalis* (lanes b and c) was abolished (b) or reduced (c) following adsorption with live reference *M. catarrhalis*. In contrast, the binding to the pseudomonas antigen was not affected by the adsorption step suggesting that the inhibition of binding was due to specific removal of anti-*M. catarrhalis* IgG.
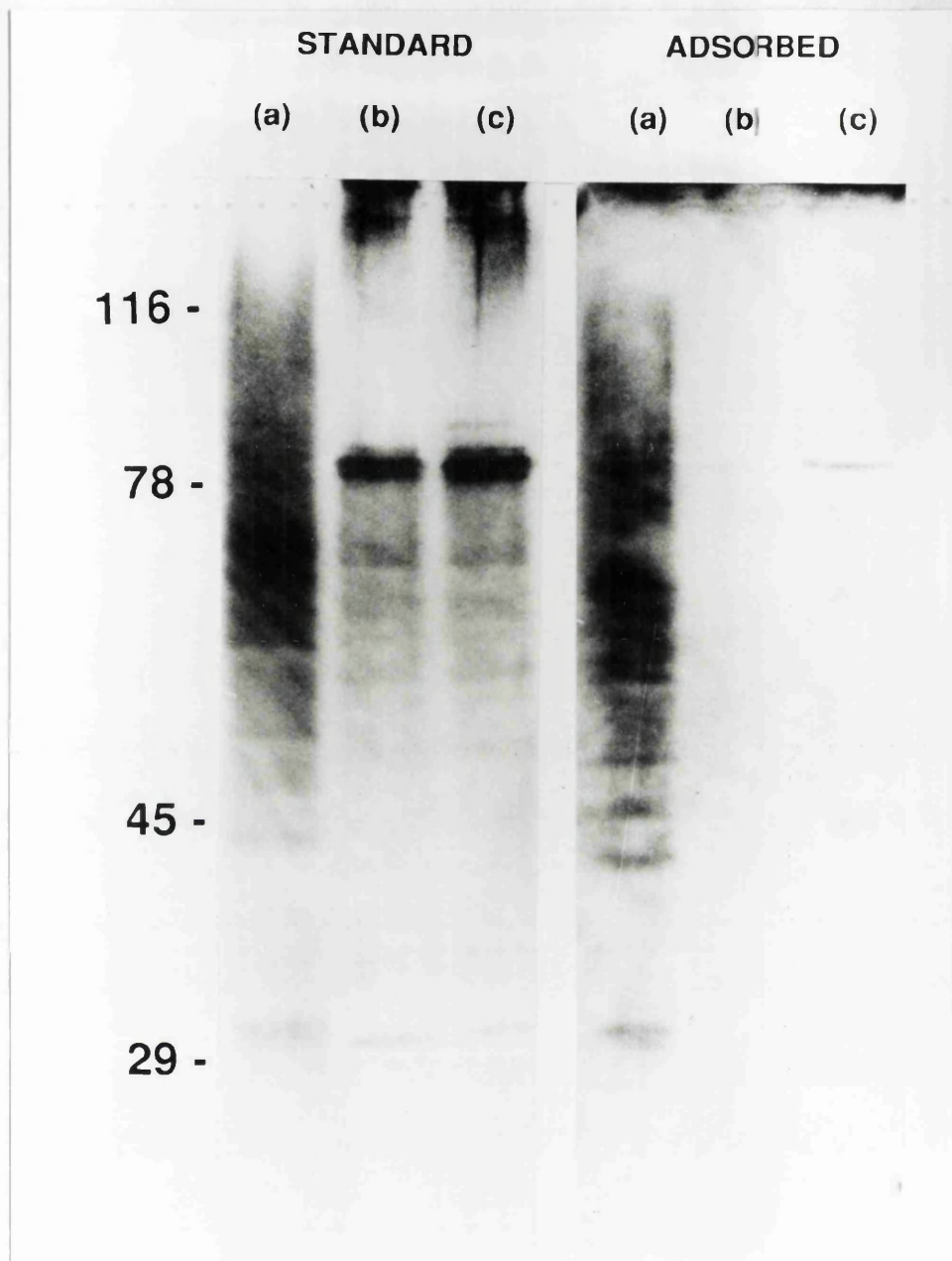
Figure 4.4: Immunoblots in which an irrelevant bacterial antigen {*Pseudomonas* antigen, (a)} and the OMPs of two clinical isolates (b and c) were incubated with standard pooled serum or standard pooled serum that had been adsorbed with a reference isolate of *M. catarrhalis* and then probed for the presence of IgG. Molecular mass standards are indicated at left in kilodaltons.

The OMPs in Figure 4.4 were isolated from the two clinical isolates and the

standard serum was adsorbed with the live reference organism. The ability of the

adsorption with the reference organism to remove binding of IgG to the clinical

OMPs suggests that in addition to the similar molecular weights of the OMPs of diverse organisms, the antigenicity of the proteins may be conserved between isolates.

While the same serum may recognise identical OMPs on diverse isolates it was important to establish whether different sera where able to recognise the same determinants. Figure 4.5 shows the patterns obtained following the incubation of standard pooled adult serum (1), standard serum adsorbed with outer membrane proteins (2), serum from a healthy adult (3) and serum from a nine month old child with a chest infection and *M. catarrhalis* cultured from the upper respiratory tract (4). Whole cell antigen was analysed in lane a and an outer membrane protein preparation was run in lane b. The antibody detected was total IgG. It is apparent that the adult standard serum and the healthy adult serum both recognised similar proteins. It can be seen in (2) that prior adsorption of the standard serum with an OMP preparation abolishes binding to the OMPs but only partially abolishes binding to the whole cell antigen. The serum from the nine month old child showed no binding to the outer membrane proteins. Serum from a patient with hypogammaglobulinaemia having immunoglobulins of IgG, 0.075 g/L, IgA 0.05 g/L, and IgM 1.0 g/L, showed no binding on immunoblotting (results not shown).
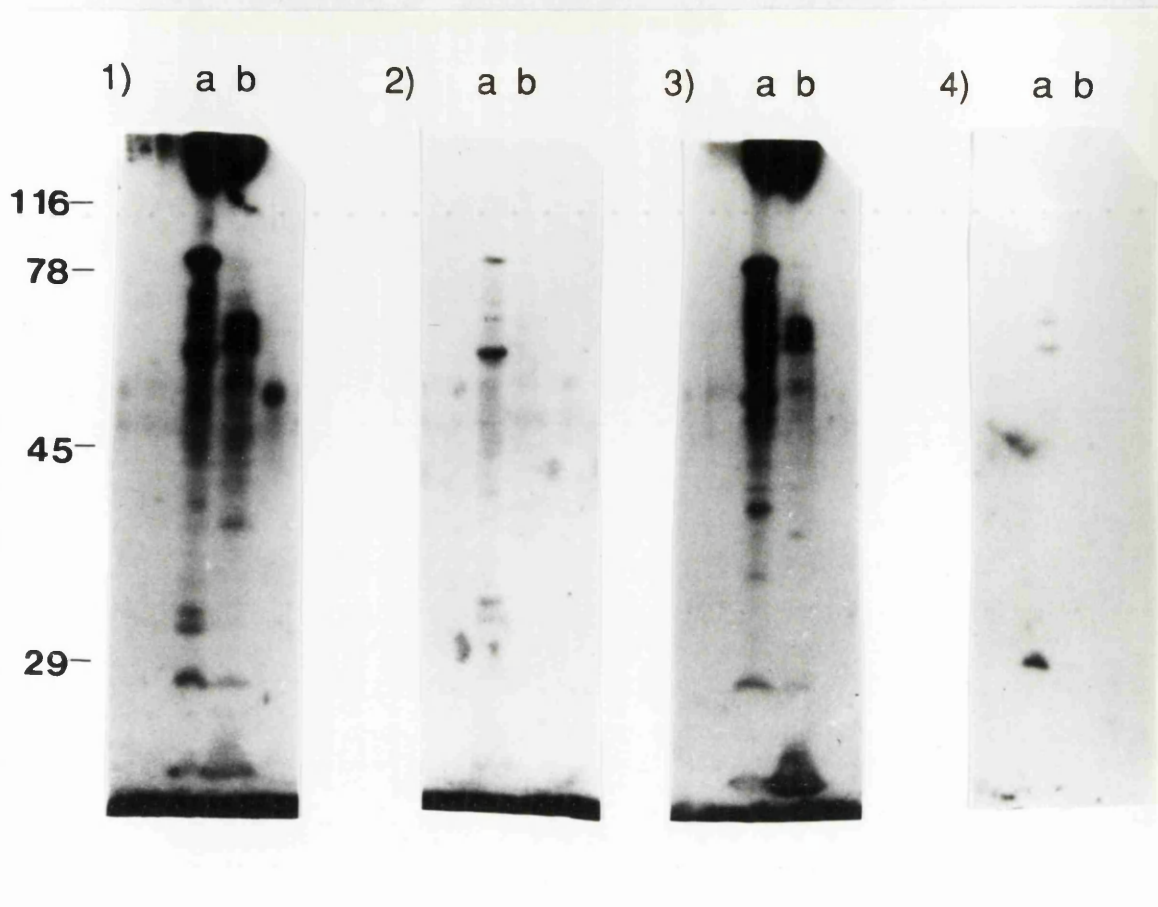
Figure 4.5: Immunoblots in which whole cell antigen (lane a) and outer membrane protein (lane b) were incubated with a standard adult serum (1), standard serum adsorbed with an OMP preparation (2), serum from a healthy adult (3) and serum from a nine month old child with sputum cultures positive for *M. catarrhalis* (4). Blots were probed with antibody to detect the presence of bound IgG. Molecular mass standards are indicated at left in kilodaltons.

In order to identify the IgG subclasses binding to the OMPs, specific anti-IgG subclass reagents were utilised. Initially non-biotinylated mouse anti-human IgG subclass reagents were used (Unipath, see chapter 2) and detected by an ^{125}I labelled anti-mouse antibody. Results from one such experiment are shown in Figure 4.6. Outer membrane proteins derived from the reference organism were blotted using the adult pooled standard serum and the IgG subclasses were then detected as described above. Adsorbed standard serum was included on each blot (Figure 4.6 lane b) to detect the specificity of binding of the individual IgG

subclasses detected. Some important differences were apparent in the binding of the four different subclasses. The binding of antibodies of the IgG3 subclass was predominant over that of the other subclasses. Binding of IgG1 and IgG4 antibodies was barely detectable apart from an interaction with an 82 kDa band that was apparently recognised by all 4 subclasses. The binding of IgG2, IgG3 and total IgG was again abolished by the prior adsorption of the serum with live whole bacteria. A band at +/- 60kDa to which IgG2 became bound, could not be adsorbed out by whole live bacteria possibly suggesting some non specific binding.
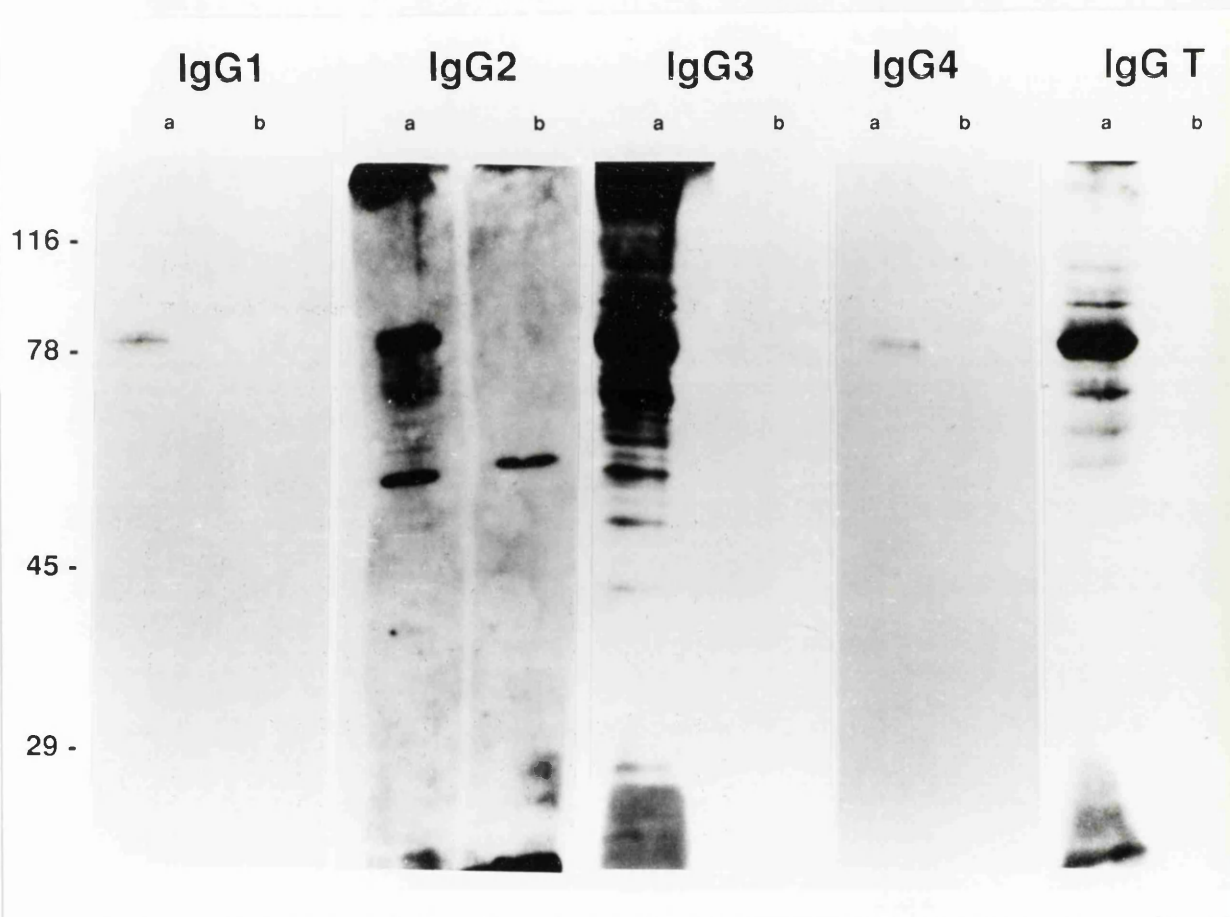
Figure 4.6: Immunoblots in which outer membrane proteins of a reference strain were incubated with standard serum (a) or standard serum adsorbed with live whole bacterial cells (b) and then probed for the presence of specific IgG subclasses or total IgG (IgGT), using Unipath mouse anti-human IgG subclass monoclonal antibodies followed by ^{125}I labelled anti-mouse antibody. Molecular mass standards are indicated at left in kilodaltons

To ensure that the binding patterns observed in the above experiment were not due to a particularly high affinity clone of the anti-IgG3 monoclonal, a different set of mouse anti-human IgG subclass monclonals were employed in a subsequent experiment. Biotinylated antibodies were obtained from Zymed laboratories and were detected by an ^{125}I labelled streptavidin peroxidase which gave the added advantage of amplifying the signal. Furthermore this experiment was designed to see whether the IgG subclass recognition by a single serum was similar for all of the OMPs from three diverse isolates of *M. catarrhalis*. Figure 4.7 shows the results following the blotting of the OMPs from the reference strain and two clinical isolates with the adult standard serum, detecting the bound IgG subclasses as described above.
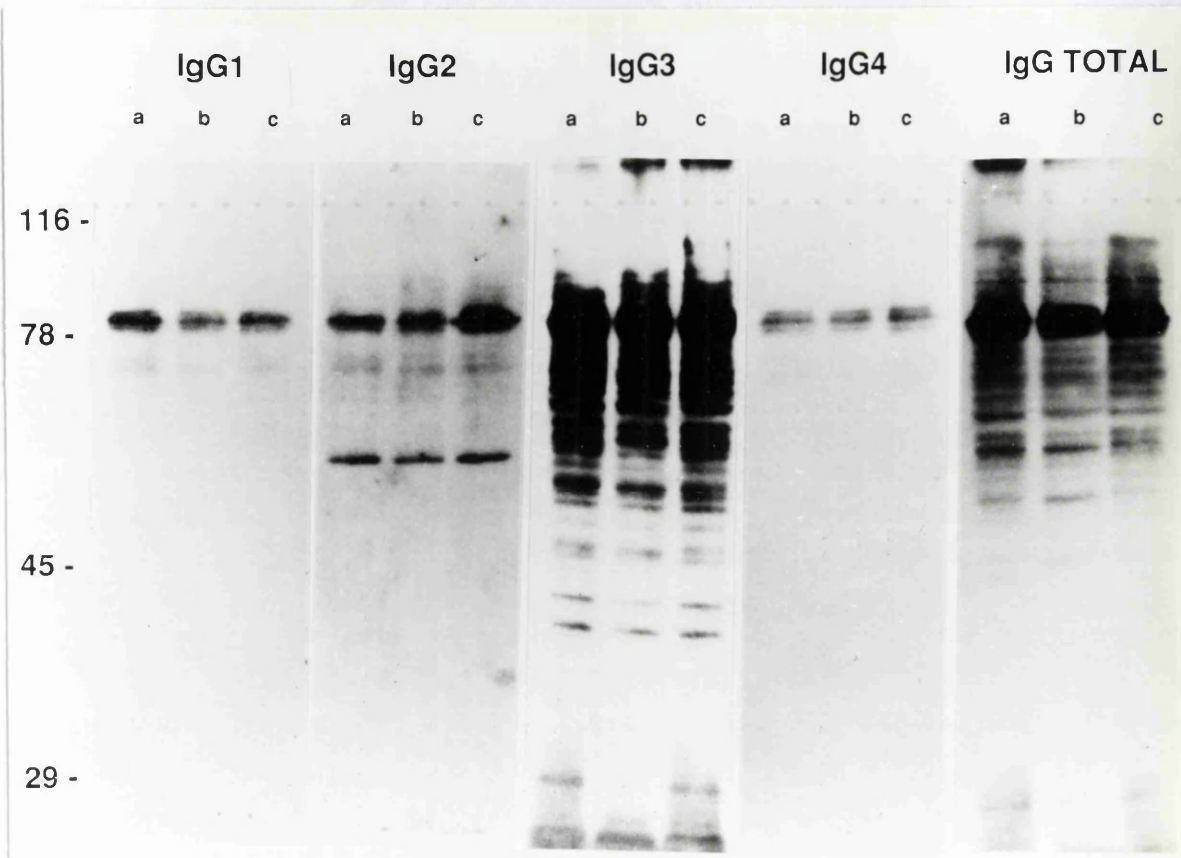
Figure 4.7: Immunoblots in which outer membrane proteins of a reference strain (a) and two clinical strains (b and c) of *M. catarrhalis* were incubated with a standard serum and then probed for the presence of IgG subclass binding utilising biotinylated mouse anti-human IgG subclass monoclonals and ^{125}I labelled streptavidin peroxidase for detection. Molecular mass standards are indicated at left in kilodalton.

As Figure 4.7 illustrates the binding pattern observed for the IgG subclasses appears to be independent of the detecting monoclonal antibody or system of detection and suggests that the binding of IgG3 is indeed more extensive than that of the other three subclasses. Furthermore, there is marked conservation in the recognition of any given protein independent of the source of the isolate from which the protein was derived.

(e) Discussion

In common with other gram negative bacteria, *M. catarrhalis* has OMPs present in the cell wall and secretes vesicles containing OMPs into the culture medium during growth [Devoe et al., 1973, Mustafa et al., 1989]. Murphy and Loeb [1989] have shown that the collection of these OMPs from the culture medium yields a preparation representative of the surface proteins that is free of cytoplasmic contamination. In addition, it has been shown that there is a striking degree of homology in the patterns of OMPs obtained from 50 diverse isolates [Bartos et al., 1988]. The similarity of OMP patterns has been confirmed for the isolates studied in this investigation.

The observed homology of OMPs derived from diverse isolates of *M. catarrhalis* contrasts with the heterogeneity of OMPs derived from organisms such as the nontypable *H. influenzae*. The OMP composition of nontypable *H. influenzae* is typical for that of gram-negative bacteria; approximately 20 proteins are present in the outer membrane, with four to six proteins accounting for most of the protein content. The OMPs in nontypable strains show much strain to strain variability, although two major protein bands located in the 32,000 to 42,000 dalton range are found regularly, each isolate of nontypable *H. influenzae* containing a major OMP in this range [Murphy and Apicella, 1987]. While the observed OMP heterogeneity permits typing of the nontypable *H. influenzae*, the similarity of the OMP patterns of diverse isolates of *M. catarrhalis* is striking and hence unlikely to be of use in studying the epidemiology of *M. catarrhalis* infections.

In addition to the heterogeneity of the patterns of OMPs derived from isolates of nontypable *H. influenzae*, animal models of experimental otitis media have provided evidence of antigenic heterogeneity. Chinchillas challenged with nontypable *H. infuenzae* mount an antibody response to the homologous strain and upon rechallenge are only protected from infection by the homologous strain

[Karasic et al., 1985]. From these studies it appears that immunity to nontypable *H. influenzae* is strain specific and several other studies have demonstrated that antibodies to the OMPs of nontypable *H. influenzae* are the targets of protective antibody [Gnehm et al., 1985, Murphy and Bartos, 1988]. The OMPs of other gram negative organisms have also been shown to induce an antibody response and it is this property of the OMPs of Group B *N. meningitides* coupled with the poor antigenicity of the Group B capsule that has led to the development of Group B vaccines containing outer membrane complexes [Mandrell and Zollinger, 1989].

The present results suggest that the OMPs of *M. catarrhalis* are recognised in a specific fashion by human antibodies, and that the majority of OMPs are not only conserved between strains as evidenced by similar patterns following SDS-PAGE and Coomassie blue staining, but bind antibodies on immunoblotting in a similar fashion. This antibody binding is specific for *M. catarrhalis* and can be completely abolished by the prior incubation of serum with live *M. catarrhalis* or OMPs derived from *M. catarrhalis*. Furthermore the ability to inhibit binding of antibody to OMPs is true not just for the homologous strain. Binding of antibody to the OMPs derived from the clinical isolates of *M. catarrhalis* can be completely abolished by the prior adsorbtion of serum with live reference isolates of *M. catarrhalis* suggesting that the immunogenicity of a given OMP is conserved between isolates. Murphy and Bartos [1989b] showed that following the immunisation of rabbits, antisera tested against a variety of strains appear to recognise conserved antigenic determinants common to most isolates. Further data on the specificity of recognition, patterns of binding of various different sera in an ELISA and on the recognition of non-homologous strains is given in Chapter 5. The data described in this chapter suggests that antibodies directed against whole organisms and OMPs may not be strain specific and that OMPs may be important constituents of the antigenic determinants on the surface of *M. catarrhalis*.

A striking feature of the antibody binding patterns seen on immunoblotting was the variation between IgG subclasses. This was not merely due to the affinity of the monoclonal detector antibodies since similar results were obtained with unrelated clones of antibodies (compare Figures 4.6 and 4.7). The IgG3 subclass was noted to bind to a broad range of OMPs whereas the binding of IgG1, IgG2 and IgG4 appeared to be restricted to the higher molecular weight proteins. In particular, an 82 kDa OMP appeared to bind all four subclasses. Prominent OMPs have been described for other organisms (cf P2 protein of nontypable *H. influenzae* [Murphy et al., 1988]) and further work is required to elucidate the significance of this 82 kDa protein.

To date, no studies have examined the IgG subclass response induced by exposure to this organism. An understanding of this may be important since the ability to mount an appropriate immune response to a given antigen may be subclass restricted and age dependent [Riesen et al., 1976, Hammarström et al., 1985]. Anti- carbohydrate antibodies are characteristically of the IgG2 isotype [Yount et al., 1968] and an inability to mount an appropriate immune response to carbohydrate antigens in early childhood (less than 2 years of age) limits the use of unconjugated polysaccharide vaccines (eg *H. influenzae* type b) in this age group [Peltola et al., 1977]. In contrast, protein antigens such as tetanus toxoid induce predominantly an IgG1 response with some IgG3 and occasionally IgG4 [Robin et al., 1986] and this response does not appear to be age restricted.

The relevance of the specific IgG3 response to the OMPs of *M. catarrhalis* is as yet unclear. IgG3 antibodies are known to be mounted in response to viral antigens and while IgG3 has been described as the dominant antibody in the responses to Herpes simplex virus type 1 and polio virus [Beck, 1981], it is usually IgG1 that dominates anti-viral responses. Bacterial targets for IgG3 have also been described and these include the streptococcal M associated protein [Mortimer et al.,

1979] and the major outer membrane proteins of Meningococci Group B [Wedege and Michaelson, 1987]. In the latter study, 33 adults were vaccinated with a noncovalent complex of Group B polysaccharide and outer membrane vesicles from serotype 2a *N. menigitidis*. The IgG subclass distribution of antibodies mounted in response to the components of the vaccine were measured both by ELISA and by immunoblotting. Meningococcal outer membrane proteins were shown to be targets of IgG1 and IgG3 antibodies and the strong binding to class 1 proteins correlated with high bactericidal activity in postvaccination sera. Furthermore, antibodies to the OMPs of nontypable *H. influenzae* have been shown to be protective [Murphy et al., 1987] and have been described as belonging to the IgG1 and IgG3 subclasses [Hammarström et al., 1986]. The present data suggests that part of the immune response to *M. catarrhalis* is mounted against the OMPs present in the cell wall. The importance of IgG3 as part of that immune response to *M. catarrhalis* is as yet unclear but the previously described binding of IgG3 to the OMPs of group B *N. meningitidis* and the correlation of IgG1 and IgG3 in the serum with postvaccination bactericidal activity suggests the potential importance of this finding, particularly as it is IgG3 alone that recognises a broad range of proteins whereas the binding of IgG1 is relatively restricted.

Finally, if a potential vaccine is envisaged [Murphy, 1989], it is important to ascertain which are the major antigenic determinants of this organism, whether they are recognised by protective antibody and whether they are immunogenic at all ages. The administration of a pneumococcal vaccine has been shown previously to reduce the frequency of attacks of otitis media attributable to *S. pneumoniae* serotypes represented in the vaccine [Mäkelä et al., 1980] and a further reduction in the frequency of otitis media may be achieved using an effective *M. catarrhalis* vaccine. However, in the same way that the use of polysaccharide vaccines is limited in young children because of the poor antigenicity of unconjugated

polysaccharides in this age group, it is possible that the OMPs towards which

protective antibody is directed may be those that stimulate only IgG3 responses.

Whether children under the age of four will be able to recognise such proteins is an

intriguing question. The 82 kDa protein which is recognised by all the subclasses

requires further characterisation since it is this protein which may be the best

potential vaccine candidate.

CHAPTER 5

Age related development and the affinity of anti-*M. catarrhalis* IgG subclass antibodies.

(a) Introduction

Despite the isolation of *M. catarrhalis* in clinical settings suggestive of infection, the interpretation of positive identification of the organism in cultures from the upper respiratory tract has always been complicated by its presence as a commensal in the mouth (Editorial, Lancet 1982). This has contributed to the uncertainty regarding the clinical relevance of this organism. In order to overcome this problem several groups have attempted to analyse the serology of patients thought to be infected with *M. catarrhalis*. To this end complement fixation (Brorson et al.,1976), immunodiffusion (Eliasson, 1980), ELISA (Leionen et al., 1981), bactericidal assays (Chapman et al.,1985), and immunofluorescent antibody techniques (Black et al., 1988) have all been used to determine total specific IgG.

Brorson et al. [1976] examined the serology of maxillary sinusitis by evaluating complement fixing and precipitating antibodies to a preparation of *M. catarrhalis* antigen. The antigen for immunodiffusion was prepared by culturing 10 clinical isolates of *M. catarrhalis* overnight on agar, washing the harvested colonies in PBS, physically disrupting the cells and then harvesting the supernatant following centrifugation. A control antigen was prepared in a similar fashion from 20 different isolates of *N. gonorrhoeae*. Bacteria for the complement fixation assay were harvested from the overnight culture on agar and heated to 56°C for 30 minutes and used together with a *N. gonorrhoeae* control. Rabbit were hyperimmunized with these crude extracts of both bacterial preparations and immunodiffusion with the respective hyperimmune sera revealed between 10 and 15 separate precipitation lines for each antigen although no cross precipitating antigens were found. Results from this study showed that 25 of 97 patients with radiological signs of maxillary sinusitis had complement fixing antibodies in their serum although the titres were low and only a small increase was seen between acute and convalescent (15 days later) serum. No bacteriology was obtained in this study so

the presence of complement fixing antibodies cannot be linked to the aetiology of

the sinusitis. However, in both patients and controls common antibodies to *M.*

catarrhalis were detected by immunodiffusion. Normal human serum was able to

kill 3 of the strains investigated in this way, and killing was abolished by heating

the serum. Leionen et al. [1981] developed an enzyme immunoassay to detect

antibodies to *M. catarrhalis* in the serum of children with acute otitis media. The

capture material for their assay was prepared by culturing 10 strains of *M.*

catarrhalis overnight on tryptic soy agar, then harvesting and washing the bacteria

before using as capture antigen. The specificity of their assay is not described hence

cross reacting antigens cannot be ruled out. Their results showed that children with

M. catarrhalis otitis media over the age of one year had IgG specific for *M.*

catarrhalis although in 5 of the 14 the levels dropped between the acute and

convalescent stage. Furthermore, 50% of the children with otitis media due to other

organisms also had antibodies to *M. catarrhalis* although the titre of these was said

to be lower and there was no change between acute and convalescent sera.

Chapman et al. [1985] looked for bactericidal activity in the sera of patients with

M. catarrhalis infections. They showed that normal human serum had no

bactericidal activity against 21 isolates of *M. catarrhalis,* whereas 7 of 19 acute and

18 of 20 convalescent sera from patients with *M. catarrhalis* infection demonstrated

significant bactericidal activity against the corresponding isolate, and a varied effect

against remote isolates. Selective blockade of the classical complement pathway and

heating of the sera both abolished this bactericidal activity from convalescent sera.

No control organisms were used in their experiments. More recently, Black and

Wilson [1988] used an immunofluorescence antibody test to investigate serum from

patients with acute bronchopulmonary infection in whose sputum *M. catarrhalis*

predominated or was the sole pathogen. *M. catarrhalis* antigen was prepared by

emulsifying equal quantities of an overnight culture of 10 different isolates of *M.*

catarrhalis, heating the PBS diluted suspension to 60°C and then vortexing to remove autoagglutination. A positive control using rabbit immune serum was included but the specificity of the binding to *M. catarrhalis* was not determined. While control sera contained antibody to *M. catarrhalis* the titres of antibody in the acute sera were higher and significantly higher still in the convalescent sera. The protein (P-protein) described by Eliasson [1980] has recently been used in an enzyme immunoassay. The original description was of a protein obtained from *M. catarrhalis* by sonication and hot acid extraction and visualised on double diffusion gels. The P-protein was precipitated by immune rabbit serum and found to be unique for *M. catarrhalis* with no cross reaction with various *Neisserial* species or *H. influenzae* strains. 69% of normal human sera derived from blood donors contained precipitating antibodies to this antigen. Subsequently this antigen was used by Chi et al. [1990] in the investigation of the serology of elderly patients with *M. catarrhalis* pneumonia or tracheobronchitis. They showed that both patients and controls had antibodies to the P-protein of *M. catarrhalis* but the levels in the patients were significantly higher than those of the controls. Furthermore 50% of the patients showed a rise in specific antibody levels between the acute and convalescent sera. While referring to the P-protein as an outer membrane protein, the authors provided no experimental evidence for this and have not responded to personal correspondence.

It is apparent from the published data that there is disagreement as to the presence or absence of antibodies to *M. catarrhalis* in the general population. The various methods used to assay antibodies to this organism have generally not rigorously excluded the possibility that the antibodies being measured might be cross reacting or non-specific antibodies. Few have looked systematically at children of different ages and no work has yet been published on the IgG subclass profile of the antibodies directed against this organism.

To this end, it was decided to develop immunoassays to measure antibodies to both the whole cell antigen (ie the whole organism) and the OMPs. This would permit the rapid investigation of large numbers of individuals using relatively small amounts of serum and would permit the investigation of antibody affinity, the importance of which was discussed in Chapter 1. This chapter therefore deals with the development of ELISA procedures to measure IgG subclasses specific for *M. catarrhalis*, the validation of these assays, their adaptation for the measurement of functional antibody affinity, and the application of these assays in the investigation of healthy adults, healthy children of various ages and adult patients with chronic rhinosinusitis.

(b) Development of enzyme-linked immunosorbent assays to measure IgG subclass antibodies with specificity for *M catarrhalis*

(i) Methods

Antigen

A whole cell suspension of *M. catarrhalis* was used as coating antigen and was prepared as follows: A reference isolate (code no. NC 11020) was obtained from the National Collection of Type Cultures, Colindale, London. The organism was grown on nutrient agar and subsequently stored on horse blood agar slopes. Prior to overnight culture the organism was plated out on horse blood agar and colony purity was checked after 18 hours. A colony scraping was then emulsified in 2 ml of broth and this suspension was then inoculated into Bactec aerobic culture vials (Becton Dickinson, Maryland, USA). Following an 18 hour culture at 37°C with agitation the culture medium was centrifuged at 10000g for 15 minutes at 4°C. The bacterial pellet was recovered and washed twice by spinning at 2500 rpm and resuspending in phosphate buffered saline pH 7.4 (PBS). After washing, the bacterial pellet was finally resuspended to an optical density of 0.9 at 540nm. This

suspension was then referred to as whole cell antigen and was used as the solid

phase antigen in the ELISA described fully in Chapter 2 and outlined in the flow

diagram below. The levels of antibody in an unknown serum were interpolated

from a standard curve derived from a standard serum (see Chapter 2), and

expressed as a percentage of the bound standard. An adult range was obtained by

analysis of the serum of forty apparently healthy laboratory and hospital workers.

General Scheme For Antigen Specific ELISA

CAPTURE	Coat Antigen on Dynatech IMMULON plates (24 hrs RT/48 hrs 4°C) ↓
SERA	Sera diluted in 1% BSA PBS-T (2 hrs RT) ↓
Wash	*PBS T (x4)* ↓
DETECTION ANTIBODY	Biotinylated anti-Hu IgG subclass antibodies (2 hrs RT) ↓
Wash	*PBS-T (x4)* ↓
STREPTAVIDIN	Streptavidin Peroxidase in PBS-T (1 hr RT) ↓
Wash	*PBS-T (x4)* ↓
SUBSTRATE	0.05% OPD in PO_4/citrate H_2O_2 ↓
STOP	4N H_2SO_4 ↓
COLOUR	Absorbance 492nm

Pediatric test sera: Clotted venous blood was obtained from 53 children of various ages all of whom had been admitted to The Hospital for Sick Children, Great Ormond Street for elective surgical procedures and whose case notes revealed no family history or clinical condition that would affect their immunity. The serum obtained was stored at -70°C until analysed.

(ii) Results

Antigen binding to solid phase

Several methods were compared for the binding of whole *M. catarrhalis* to the solid phase. These included diluting the antigen suspension in methyl glyoxal or

poly-1-lysine prior to coating the plates as described by Czerkinsky et al., (1983).

Neither method showed any advantage over the use of carbonate-bicarbonate buffer.

Both live and formaldehyde treated bacterial preparations were used to coat the

plates and the use of live bacteria independent of the diluent showed no advantage

over killed bacteria (results not shown).

Detector antibody binding

Optimal dilutions of the detector monoclonal antibodies were achieved when

binding resulted in optical densities within a similar range. Within each isotype

specific ELISA the dilution curves for test sera were all approximately parallel both

to each other and to the standard curve. No attempt was made to quantify

gravimetrically the amounts of specific antibody binding to the solid phase. Values

for unknown sera were expressed as a percentage of the standard serum bound at

the same dilution. Values for unknown sera were rejected if their absorbance was

less than that of four times the background absorbance. Background absorbance was

generally low except for the IgG2 assay. The standard curves for IgG1, IgG2,

IgG3, IgG4 and total IgG are shown in Figure 5.1. It was not possible to detect

bound IgG4 in the standard serum.
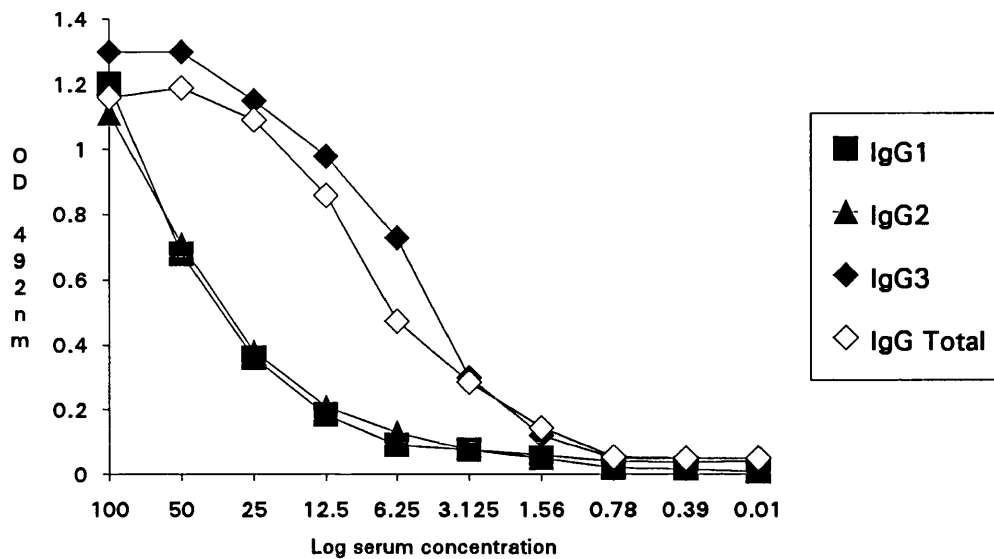
Figure 5.1. ELISA procedure for the detection of anti-*M. catarrhalis* antibodies. Standard curves obtained for the binding of standard serum (doubling dilutions ranging from $1/40$ to $1/10240$) to whole *M. catarrhalis*. Specific antibodies were detected using mouse anti-human IgG subclass monoclonals (see legend).

Assay Specificity

To determine the specificity of binding of IgG to antigen on the solid phase the subsequent experiment was performed. Doubling dilutions of a live suspension of *M. catarrhalis* in 1% BSA PBS-T was prepared. The concentration of the undiluted bacterial suspension was referred to as 100 and subsequent dilutions as 50, 25 etc (see x axis in Figure 5.2). An equal volume of each diltuion of the bacterial suspension was then added to a constant dilution of standard serum (40μl) in each well. As shown in Figure 5.2 there was complete inhibition of binding of specific IgG3 to the solid phase and partial inhibition of IgG1, IgG2 and total IgG by the undiluted bacterial suspension.

(a)

(b)

(c)

(d)
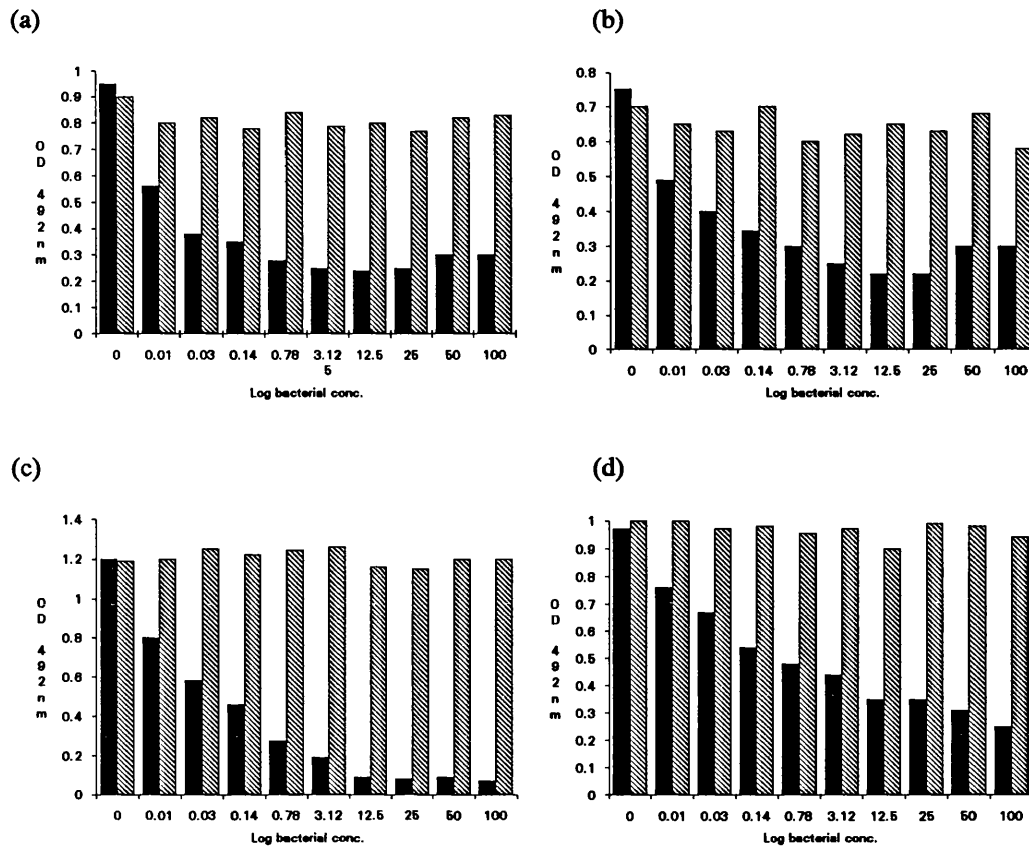
Figure 5.2. Inhibition of binding of specific anti-*M. catarrhalis* IgG1 (a), IgG2 (b), IgG3 (c) and IgG Total (d) antibodies to the solid phase (whole cell antigen). A suspension of live *M. catarrhalis* was added in increasing concentrations (see text) to 40 μl of a pooled standard serum at a concentration of $^1/_{40}$ (IgG1, IgG2) or $^1/_{80}$ (IgG3 and total IgG). Solid bars represent the absorbance readings from wells to which bacteria were added and cross hatched bars represent values for wells with serum alone.

In order to determine whether binding to the solid phase was due to cross-reacting antibodies, aliquots of standard serum were adsorbed individually with a variety of bacteria by incubating the serum and bacteria together at 37°C for 2 hours and then centrifuging at 10,000g to remove the bacteria. The bacteria used for adsorption included *N. meningitidis* group B, a nontypeable *H. influenza*, *Pasteurella multocida*, *E. coli*, *Staphylococcus aureus* and *M. catarrhalis*. As shown in Figure 5.3 there was little inhibition of IgG3 and total IgG binding to the solid phase in sera adsorbed with bacteria other than *M. catarrhalis*. The IgG3

assay again showed maximal inhibition exclusively by *M. catarrhalis*. The IgG2

assay Figure 5.3(b) showed some degree of cross-reaction with *Pastuerella*

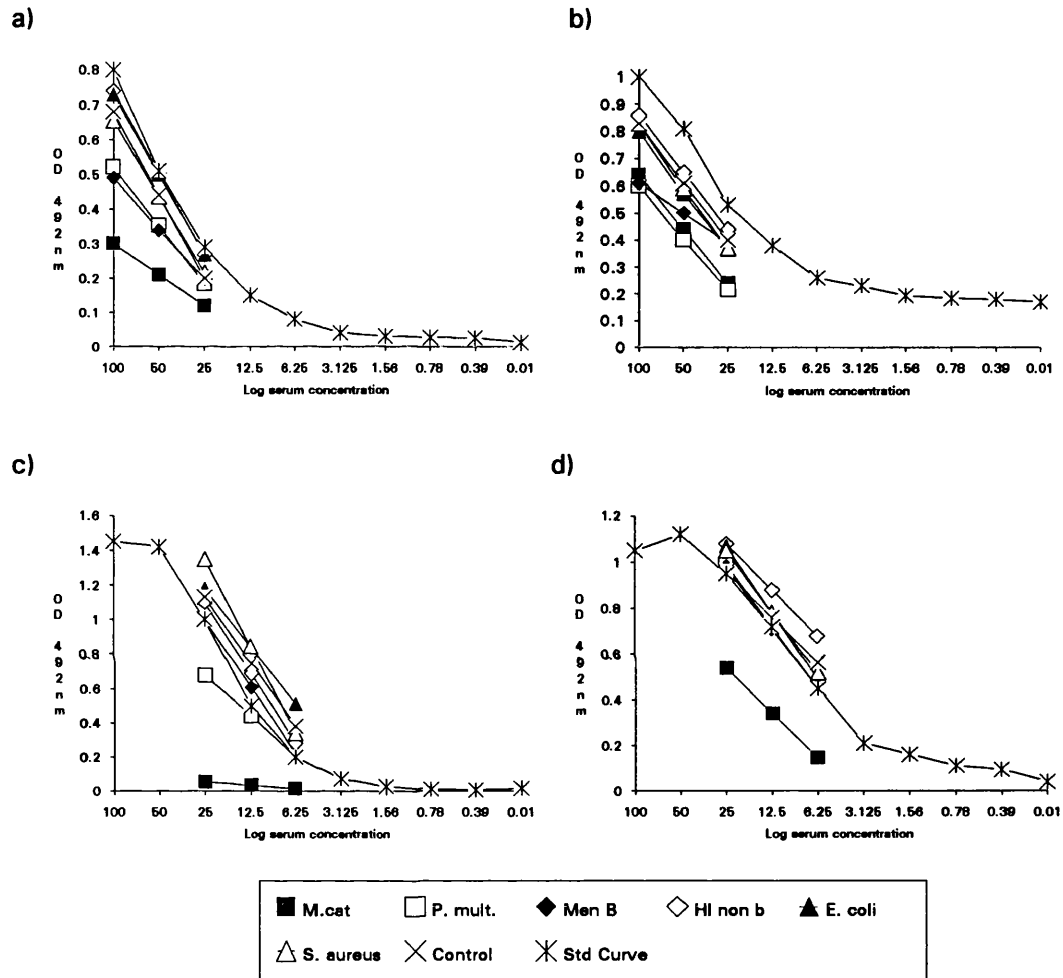*multocida* and *N. meningitidis* group B.

Figure 5.3 (a-d) Specificity of the binding of anti-*M. catarrhalis* IgG1 (a), IgG2 (b), IgG3 (c) and IgG Total (d) antibody to the solid phase. Standard serum was adsorbed with live *M.catarrhalis* or a variety of other live bacteria (Std: standard curve, M. Cat: *M. catarrhalis*, Past.: *Pastuerella multocida*, Men. B.: *Neisseria Meningitidis* group B, HI non b: *Haemophilus influenza* non type b, E. Col: *Escherichia coli*, Staph: *Staphylococcus aureus*). Curves obtained for the binding of specific anti-*M. catarrhalis* antibody of the IgG1, IgG2 and IgG3 subclasses and total IgG were then generated using the ELISA procedure.

Assay variation

Inter-assay variation was measured by assaying a known high titre and a known low titre serum in addition to the standard serum on the same batch of antigen coated plates on four different days. Results were expressed as the coefficient of variation for both the high and the low titre serum and are shown in Table 5.1(a). Intra-assay variation was measured by assaying a known high titre and a known low titre serum in twelve replicates on the same plate together with a dilution curve for the standard serum. This same experiment was repeated on three consecutive days and the results are expressed as the mean and standard deviation of the coefficient of variation obtained from the replicates over the three days. Table 5.1(b) shows that while the intra-assay variation differed for the four assays it was acceptably low overall.

Table 5.1(a) Inter-assay variation for IgG1, IgG2, IgG3 and total IgG as measured by ELISA (see text for details)

| | Coefficient of Variation | |
	High level serum	Low level serum
IgG1	6.28	10.08
IgG2	5.73	8.78
IgG3	5.59	6.7
IgG (Total)	6.63	14.7

Table 5.1(b) Intra-assay variation for IgG1, IgG2, IgG3 and total IgG as measured by ELISA (see text for details)

| | Coefficient of variation | | | |
| | High level serum | | Low level serum | |
	Mean	SD	Mean	SD
IgG1	10.78	3.12	12.14	2.79
IgG2	11.72	2.08	14.35	4.25
IgG3	3.35	1.99	5.28	0.94
IgG (Total)	3.64	0.55	6.63	2.6

Binding of the standard serum to the solid phase was similar irrespective of the source of antigen used on the solid phase. Figures 5(a)-5(d) show standard curves obtained for the binding of specific IgG1, IgG2, IgG3 and total IgG binding to a reference isolate of *M. catarrhalis*, two clinical isolates of *M. catarrhalis* and an OMP preparation obtained from the culture supernatant of the reference isolate. Differing amounts of coating antigen resulted in minor variability in the standard curves although overall the curves obtained showed parallelism and gave similar IgG subclass profiles. In addition, specific antibody levels in any given test sera were similar regardless of the source of capture antigen. Furthermore, serum adsorbed with the reference organism reduced (IgG1, IgG2 and total IgG) or completely abrogated (IgG3) the binding of the antibody to the solid phase irrespective of both the source of capture antigen and the type of antigen (ie whole cell versus OMP). It should be noted that the unique nature of the monoclonal reagents used in assays of this type preclude comparisons between subclasses as regards gravimetric quantitation.
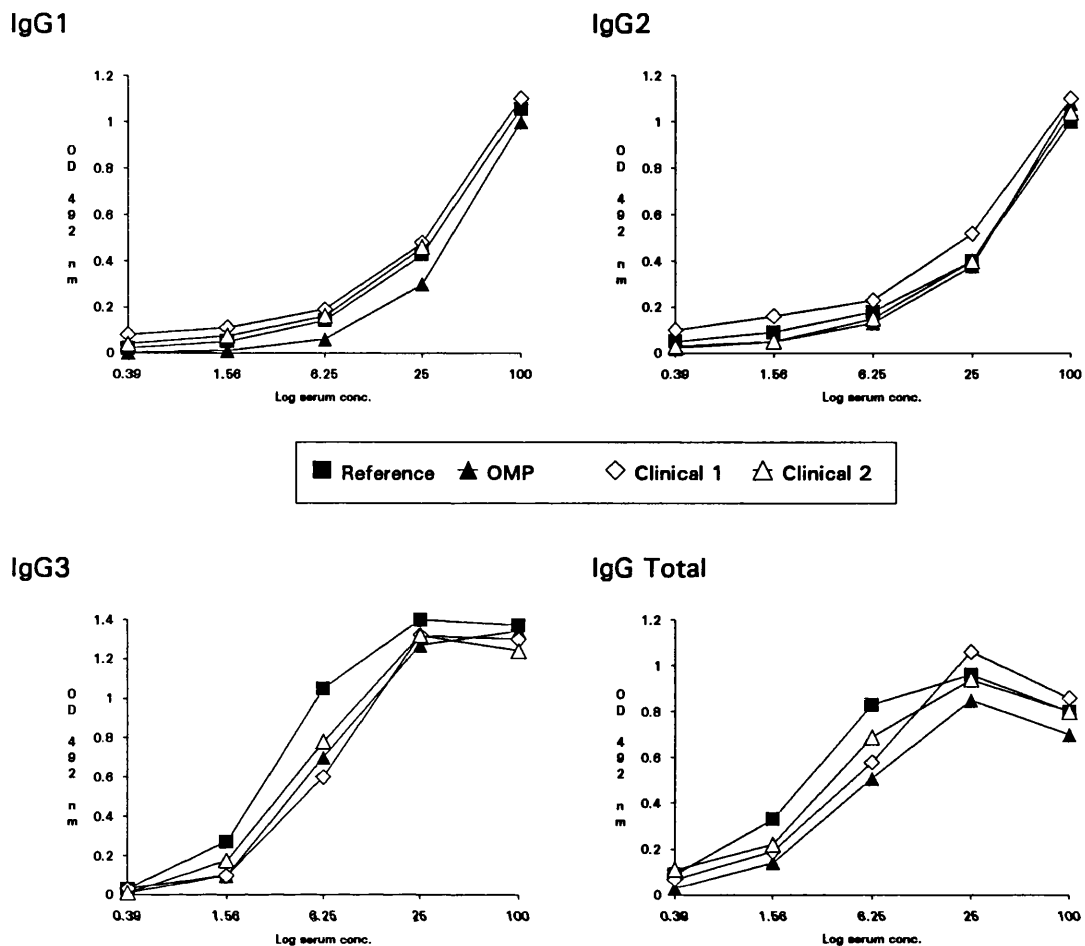
Figure 5.4. Comparison of different coating antigens for the anti-*M. catarrhalis* isotype specific ELISA. A preparation of whole cell *M. catarrhalis* antigen derived from a reference organism or two clinical isolates as well as an outer membrane preparation derived from the culture supernatant of the reference organism were compared as capture antigen in the ELISA (see methods section). Curves obtained for the binding of specific anti-*M. catarrhalis* antibody of the IgG1, IgG2 and IgG3 subclasses and total IgG derived from the standard serum were detected using mouse anti- human IgG monoclonals.

(c) *M. catarrhalis* specific IgG subclass affinity assays

(i) Methods

A modification of the ELISA as described above permitted measurements of the functional affinity of the individual antigen specific isotypes [Devey et al., 1988, Rath et al., 1988]. The first method involved performing the ELISA in the presence of a chaotropic agent which disrupts antibody-antigen binding. Several

chaotropic agents have been used in such assays including 0.5 or 1.0M guanidine hydrochloride [Inouye et al., 1984], thiocyanate [Pullen et al., 1986], and Diethylamine (DEA) [Devey et al., 1988]. Serum for analysis was diluted in 1% BSA PBS-T with or without 20mM DEA and the ELISA was performed over a range of serum dilutions from which binding curves were constructed. The left shift of the binding curve noted in the presence of DEA was calculated at an OD 50% of maximum, and the magnitude of the shift was compared between the various isotypes. Low affinity antibody binding is more affected by the presence of DEA than is the binding of high affinity antibodies. Therefore, there will be a greater leftward shift of the binding curve for low affinity than for high affinity antibodies. Using both methods it is possible to compare the affinity of various isotypes for the same antigen and this permits a ranking of antibody affinity rather than absolute measures of affinity.

The second method for the estimation of high affinity antibodies by competitive inhibition was performed as follows: a live *M. catarrhalis* preparation was prepared as described above and adjusted to an OD of 0.9 at 540nm. Doubling dilutions of this preparation were made and 40 μl of each dilution were added (in duplicate) to wells of an ELISA plate that had been precoated with *M. catarrhalis* antigen. 40 μl of serum at a constant dilution were then added to each well and the isotype specific assay performed without modification as described above. Results were then calculated by determining the % inhibition using the following formula:

$$\% \text{ inhibition} = 100 - \left\{ \text{OD at concn } x \text{ of free antigen} / \text{OD with no free antigen} \times 100 \right\}$$

High affinity antibodies require less free antigen to inhibit binding to the solid phase than low affinity antibodies and the % inhibition of high affinity antibody will then approach 100.

(ii) Results

Experiments were carried out to determine the ideal molar concentration of DEA required to discriminate between the affinities of the various subclasses. Figure 5.5 shows the results of such an experiment where standard curves were constructed for the binding of standard serum to *M. catarrhalis* in the presence of increasing concentration of DEA. As can be seen, at 20mM DEA there is good discrimination between the different subclasses, this concentration of DEA being the same as that chosen by Devey et al. [1988] for the determination of the functional affinity of tetanus toxoid antibodies. While 20mM DEA has little effect on the binding of IgG3 to the solid phase, the binding curves of the IgG1 and IgG2 antibodies are shifted to the left by the chaotropic ion suggesting that the affinities for specific anti-*M. catarrhalis* antibodies rank IgG3 > IgG1 >/= IgG2.

IgG1   IgG2

IgG3   IgG Total
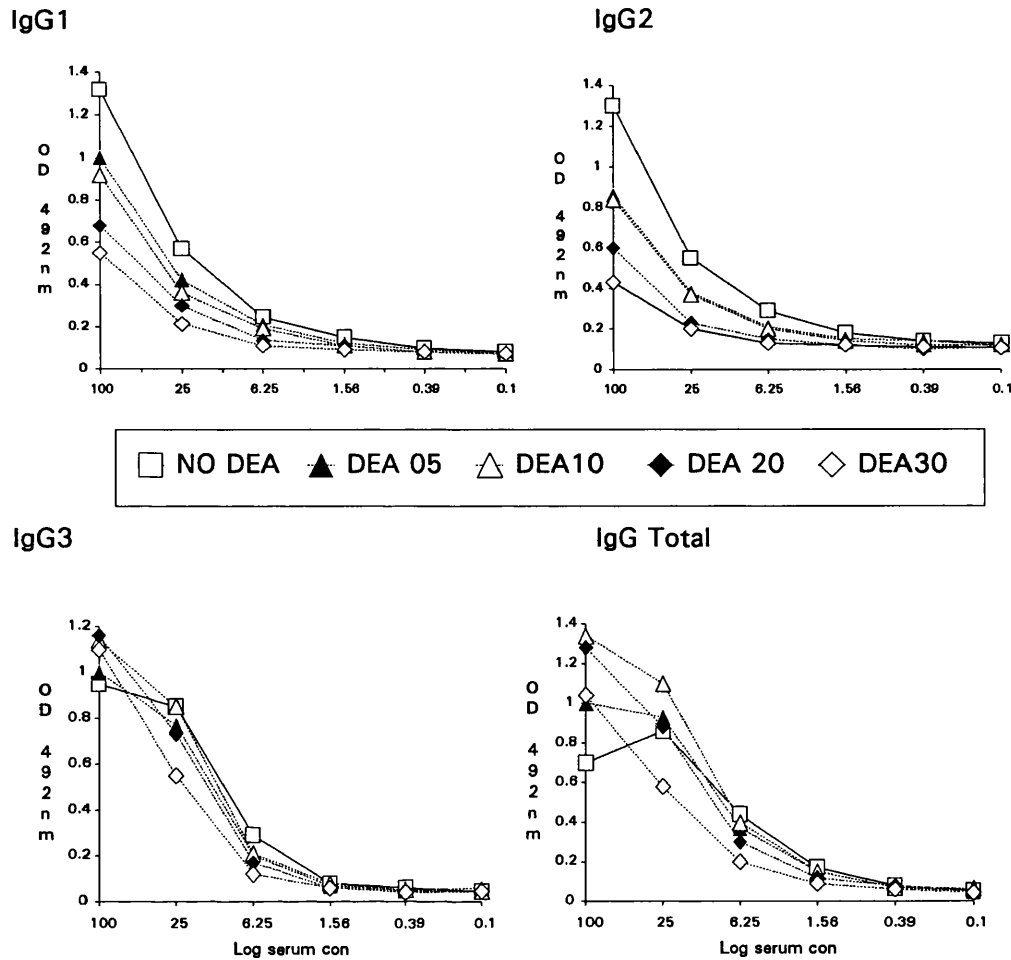
Figure 5.5: Functional affinity (avidity) of anti-*M. catarrhalis* antibodies as measured by ELISA in the absence (solid line) or presence (dotted lines) of increasing concentrations (see key: figures in mM DEA)) of a chaotropic ion, diethylamine. Low affinity antibody binding is more easily disrupted than high affinity antigen-antibody interactions leading to greater left shift for low affinity antibody The absence of any significant deviation in the case of IgG3 suggests higher functional affinity

To confirm these observations a competitive inhibition assay was performed using live whole *M. catarrhalis* as free antigen. The results from this experiment can be seen in Figure 5.6. A lower concentration of free antigen is required to inhibit the binding of IgG3 to the solid phase as compared to that required to inhibit the binding of IgG1 and IgG2. This result confirms the ranking order of the affinities for the subclasses obtained in the DEA ELISA. Furthermore the affinity

of total IgG in the competitive inhibition assay lies somewhere between the

affinities of IgG3 and IgG1/IgG2.
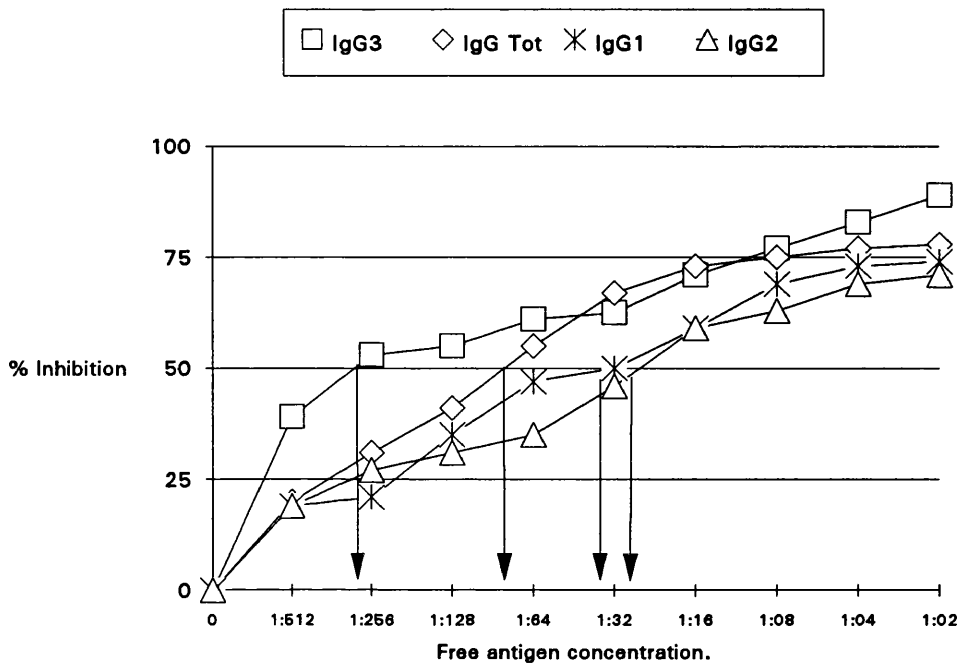
Figure 5.6: Functional affinity (avidity) of anti-*M. catarrhalis* antibodies as measured by a competitive inhibition assay. An increasing concentration of free antigen (live *M. catarrhalis*) was added to serum of a fixed concentration. The amount of antigen required to produce 50% inhibition was compared for the three subclasses. Less free antigen is required to inhibit the binding of high affinity than low affinity antibodies, and for high affinity antibodies inhibition approaches 100%

Anti-*M. catarrhalis* antibody affinities were measured in a group of healthy adults

and confirmed that in general the affinities ranked IgG3 > IgG1 > / = IgG2.

However as can be seen from Figure 5.7 the amount of deviation obtained in the

presence of 20mM DEA varied from patient to patient suggesting that the measures

of absolute affinity might vary between individuals.

Patient A

IgG1
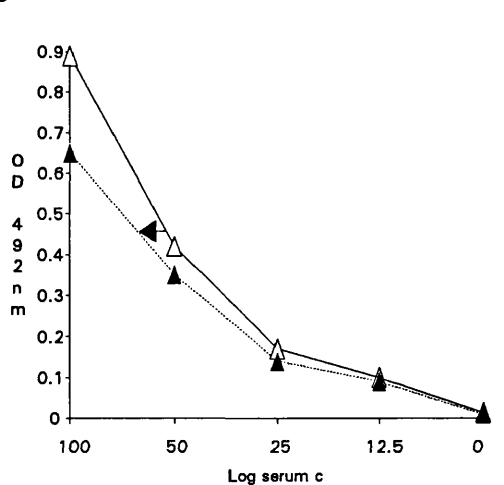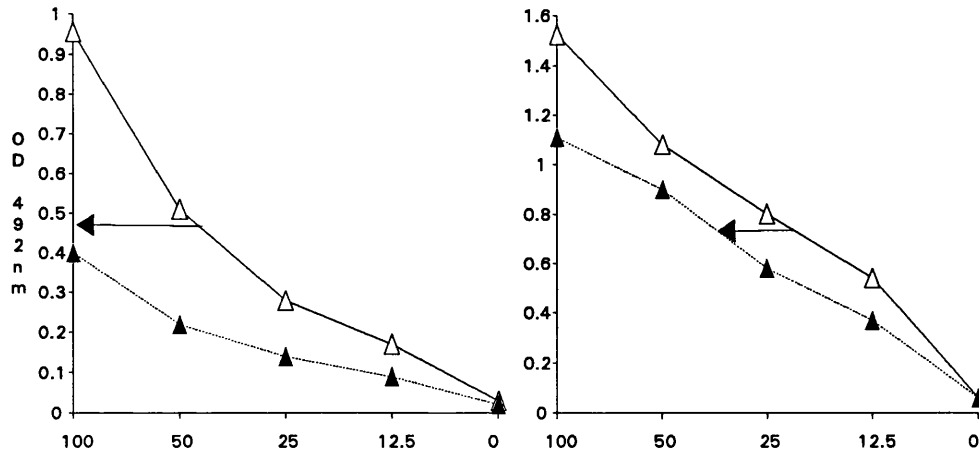

IgG2

IgG3

Log serum c
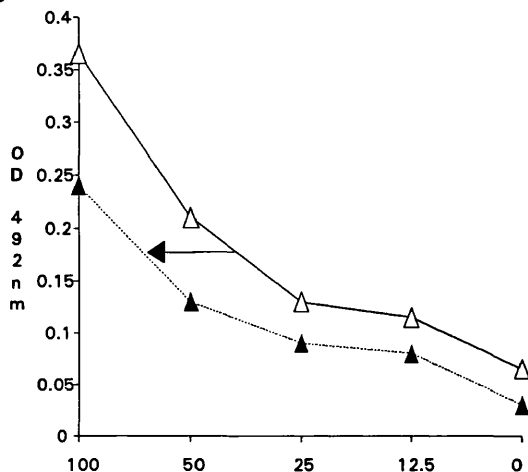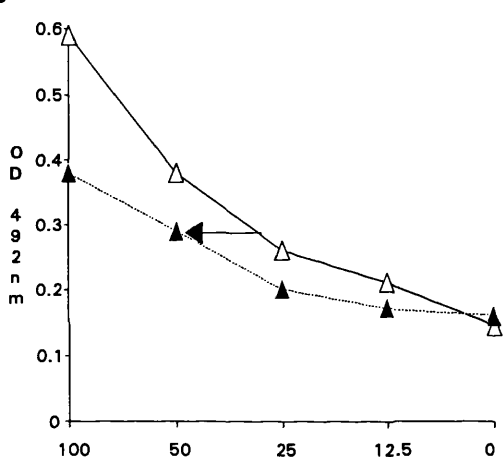
Figure 5.7: DEA-ELISA affinity measurements for anti *M. catarrhalis* whole cell IgG1, IgG2 and IgG3 antibodies of three representative healthy adults (A, this page, B & C overleaf). Open triangles represent binding in the absence of DEA and closed triangles represent binding in the presence of 20mM DEA. While the rank of antibody affinity is IgG3>IgG1>/=IgG2 in all three, for IgG3, the amount of left shift produced by DEA at 50% of the maximal OD (see arrows) correlating with the absolute affinity of IgG3, varies between the individuals.
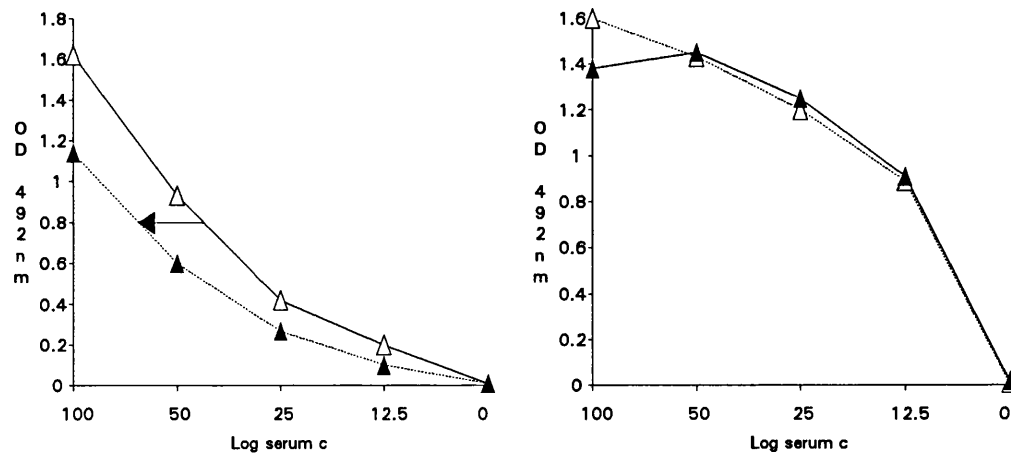
Patient B

IgG1

IgG2

IgG3

Patient C
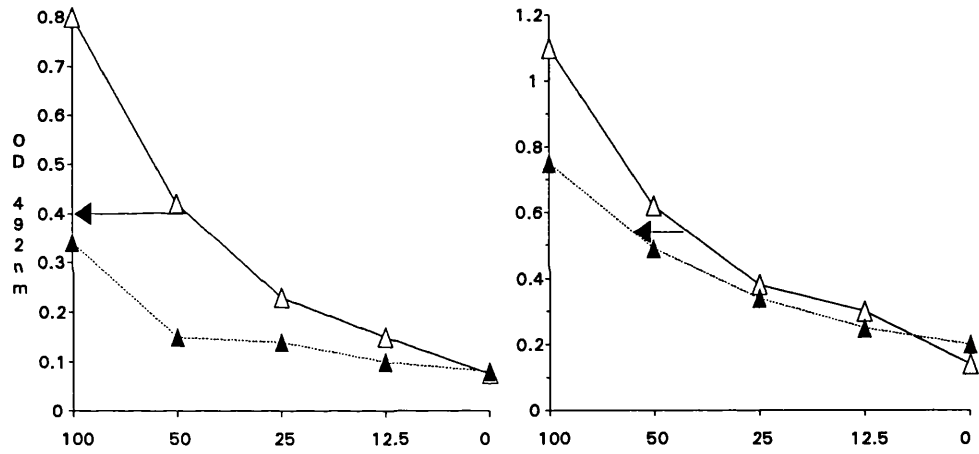
(d) IgG subclass antibodies to *M. catarrhalis* in healthy adults and children

Sera from 40 healthy adults were assayed to establish the range of naturally occurring specific anti-*M. catarrhalis* present in adult sera. All sera had detectable IgG specific for *M. catarrhalis* although the subclass pattern differed from individual to individual. The binding activity for a group of apparently healthy adults is shown in Figure 5.8. IgG4 antibodies could only be detected in a minority (17%) of the sera tested.
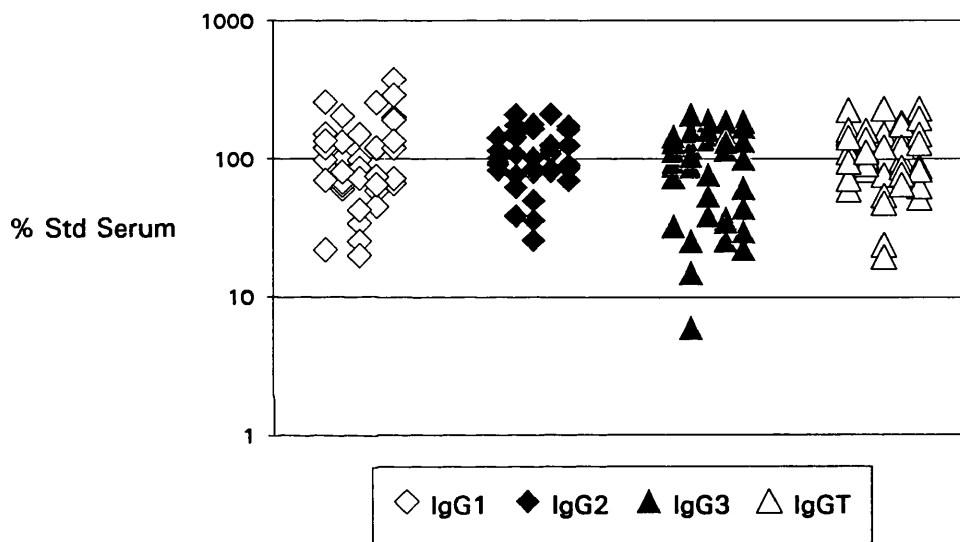

Figure 5.8. Range for anti-*Branhamella catarrhalis* antibodies detected in the sera of 40 apparently healthy adults.

Figure 5.9 (overleaf) shows the pediatric age related range for specific anti-*M. catarrhalis* total IgG and IgG1, IgG2 and IgG3. IgG4 could only be detected in a minority of children (13%). Antibodies of the IgG1 and IgG2 isotype were detected in most younger infants and those of the IgG1 isotype rose to adult levels (ie close to 100% of the standard serum) between the ages of two and three. In contrast, levels of IgG3 antibodies were less than 9% of that of the pooled standard in patients under the age of four, but rose rapidly thereafter.
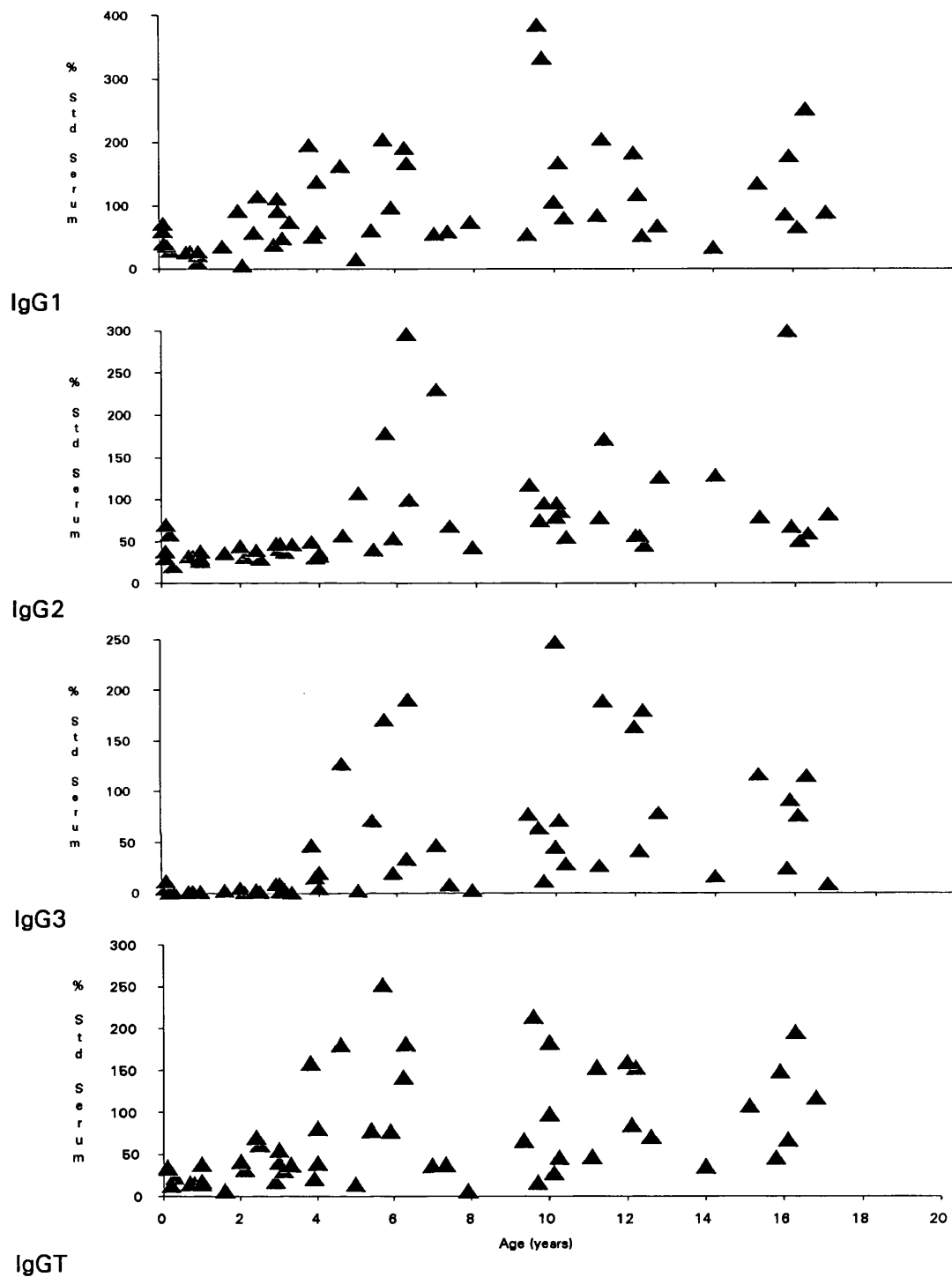
IgG1

IgG2

IgG3

IgGT

Figure 5.9 The range of age related naturally occurring anti-*M. catarrhalis* IgG subclass and total IgG (IgGT) antibodies in children undergoing elective surgical procedures. Each triangle represents an individual child (n = 53) and the levels, determined in an ELISA procedure using whole cell antigen as capture, are expressed as a percentage of the level of antibody found in a pooled standard prepared from 60 apparently healthy adults (see text).

Serum was available for analysis of total serum IgG3 levels (mg/dl) in 42 of the 53 children whose anti-*M. catarrhalis* levels are shown in the previous page. Total serum IgG3 levels were normal for the majority of these children and are shown in Figure 5.10. A table of levels of specific *M. catarrhalis* antibodies and total IgG3 in the children undergoing cold surgical procedures are shown in Appendix C.
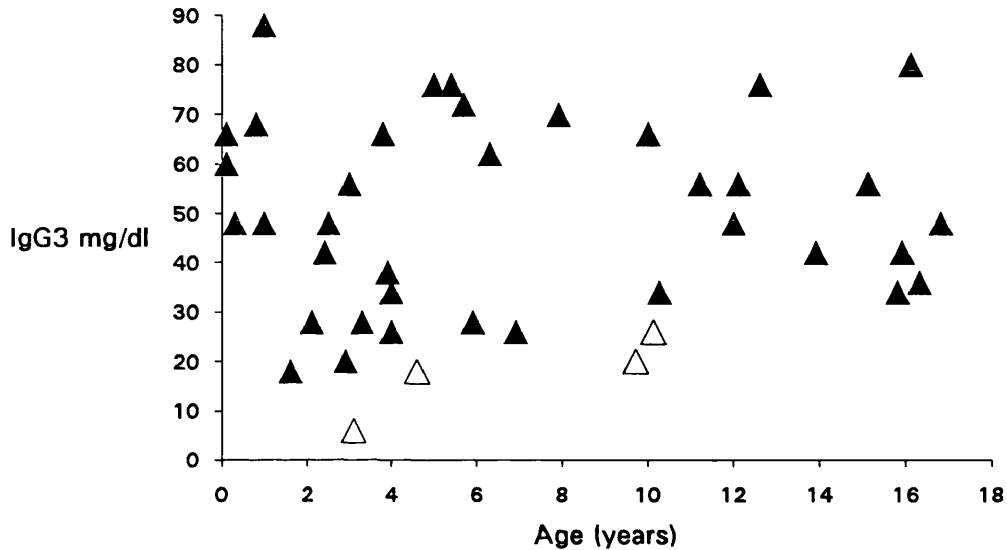

Figure 5.10: Levels of serum IgG3 (mg/dl) in 42 of 53 children undergoing elective surgery. Levels of IgG3 which fell below 2SD of an age matched normal range are indicated by open triangles.

(e) Titre, affinity and Gm allotype of anti-*M. catarrhalis* antibodies in adults with chronic rhinosinusitis

Adults with chronic or recurring infection of the nose and/or sinuses may have a minor immunodeficiency underlying their susceptibility to frequent infection. A recent study of 26 such adults showed that IgG3 deficiency was the commonest IgG subclass deficiency in the group as a whole [Scadding GK, personal communication]. To investigate whether this low level of serum IgG3 was a pointer to an inability to respond appropriately to an antigen specific anti-*M. catarrhalis* antibodies were measured in this group and compared with the levels in a healthy control group. Figure 5.11 shows the levels of specific IgG3 in the 47 adult patients and in the controls. Statistical analysis of the difference between the two groups showed the levels of specific anti-*M. catarrhalis* IgG3 to be significantly lower in the patient group (p=0.016) by the paired two sample t test.
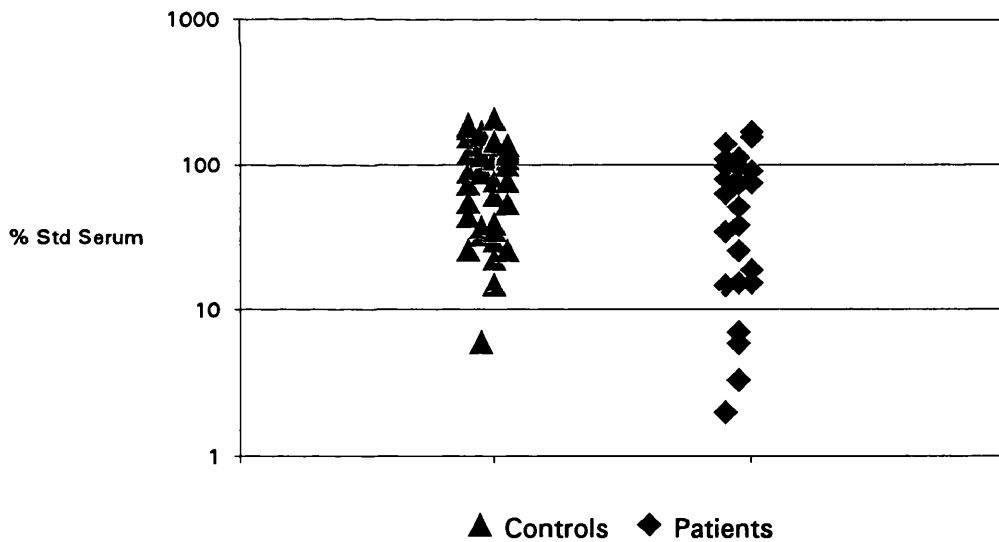

Figure 5.11: Anti-*M. catarrhalis* IgG3 antibodies in patients with recurrent or chronic infections of the nose and/or sinuses and healthy controls. Levels are expressed as a percentage of the level of antibody in a standard serum. Levels of specific IgG3 were significantly lower in the patient group (p=0.016) when tested on a two sample t test.

Since the patient group had been identified as having IgG3 deficiency we

investigated the relationship between the serum IgG3 and specific IgG3 levels.

When the level of specific anti-*M. catarrhalis* IgG3 was compared to the total

serum IgG3 there was no positive correlation (Figure 5.12) suggesting that the

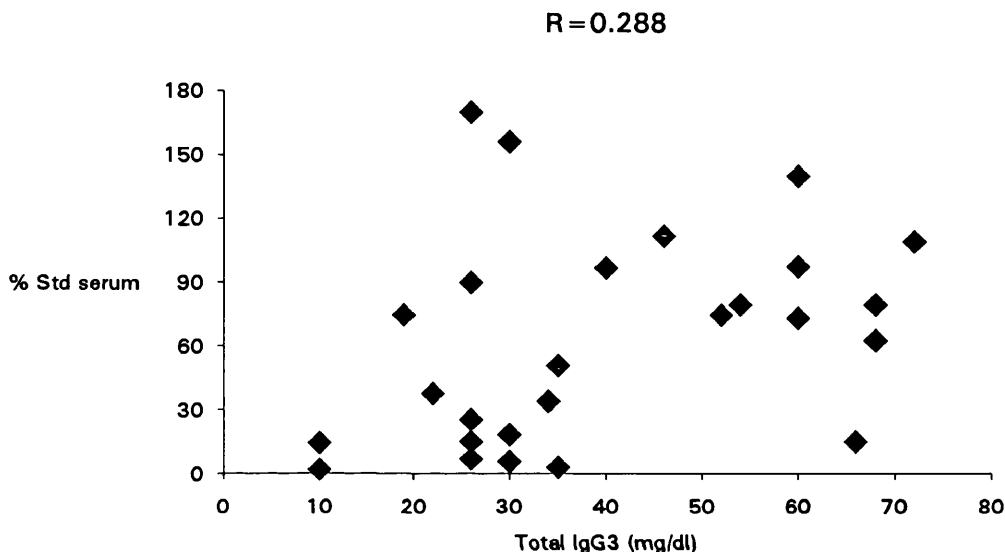lower level of specific IgG3 in the patient group was independent of the total.

R = 0.288

Figure 5.12: Correlation between the specific anti-*M. catarrhalis* IgG3 antibody levels in patients with chronic or recurring rhinosinusitis and their total serum IgG3 levels. Levels of specific IgG3 are expressed as a percentage of a pooled standard. The low levels of specific IgG3 are essentially independent of the serum IgG3 as shown by the correlation coefficient of 0.288.

Furthermore when the affinity of anti-*M. catarrhalis* IgG3 antibodies in these

patients was measured there was a range of affinity values for IgG3 although once

again, for the group as a whole, the affinity ranked IgG3 > IgG1 > / = IgG2. Figure

5.13 shows the affinity of the specific IgG3 antibody in four of the patients with

chronic rhinosinusitis as measured by the DEA assay. In addition to the individual

variation in affinity it was again apparent that there was no correlation between the

absolute level of antibody measured and affinity, ie. patients with high levels of

antibody may have IgG3 of relatively lower affinity than patients with lower levels
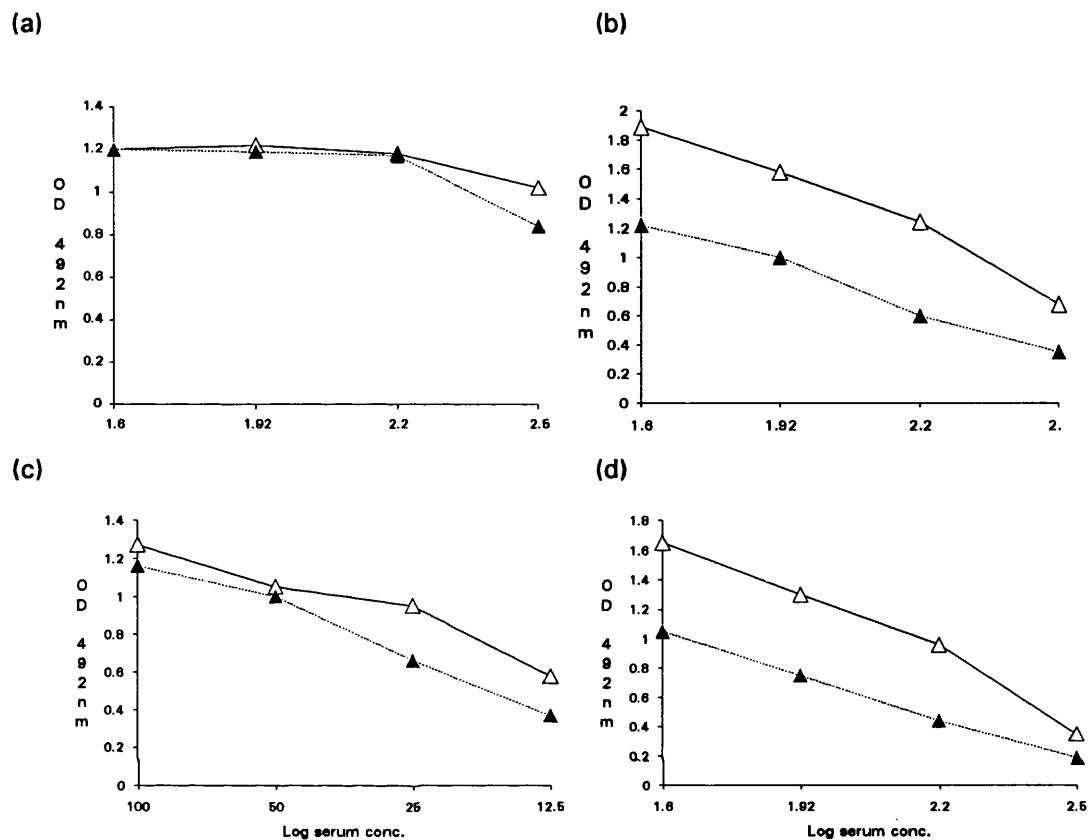of specific IgG3.

(a)

(b)

(c)

(d)

Log serum conc.

Log serum conc.

Figure 5.13: DEA affinity measurements of anti-*M. catarrhalis* IgG3 antibodies in four representative adults with chronic rhinosinusitis. Open triangles represent serum in the absence of DEA, closed triangles represent serum + DEA. As can be seen the affinity of IgG3 differs between the patients although the IgG3 antibody was, in each case, of higher affinity than the IgG1 and IgG2 (data not shown). Specific anti-*M. catarrhalis* IgG3 antibody levels in the four individuals were (a)156.1, (b) 96.6, (c) 74.6 and (d) 50.8% of the standard serum respectively.

Finally to explore the effect of Gm allotype on the total IgG3 levels and on the ability to produce specific anti-*M. catarrhalis* IgG antibodies, serum from 12 of the adults with chronic rhinosinusitis and 12 healthy adult controls was sent to Prof JP Pandey at the University of South Carolina, Charleston, SC, USA, for the measurement of Gm allotype profiles. These results together with Km allotyping of immunoglobulin light chains are shown in Table 5.2. As can be seen from the table

there were no major differences between the two groups (patients vs controls)

although the numbers of individuals investigated were small and the study should be

extended.

Table 5.2: Gm and Km allotyping in 12 adult patients with chronic/recurrent rhinosinusitis and 12 healthy adult controls (kindly performed by Prof JP Pandey, Medical University of South Carolina)

	G1m(a)	G1m(x)	G1m(f)	G1m(z)	G3m(b)	G3m(g)	Km1	Km3
Patients								
1	-	-	+	-	+	-	-	+
2	+	-	+	+	+	+	+	+
3	+	-	+	+	+	+	-	+
4	-	-	+	-	+	-	-	+
5	-	-	+	-	+	-	-	+
6	+	+	-	+	-	+	-	-
7	-	-	+	-	+	-	-	+
8	+	-	-	+	-	+	+	+
9	+	-	+	+	+	+	-	+
10	-	-	+	-	+	-	+	+
11	-	-	+	-	-	-	-	+
12	+	-	+	+	+	+	-	+
Controls								
1	+	-	+	+	+	+	+	+
2	-	-	+	-	+	-	-	+
3	-	-	+	-	+	-	-	+
4	+	-	+	+	+	+	-	+
5	+	-	-	+	+	+	+	+
6	+	+	+	+	+	+	-	+
7	+	-	-	+	-	+	+	+
8	+	-	+	+	+	+	-	+
9	-	-	+	-	+	-	-	+
10	+	-	-	+	-	+	-	+
11	-	-	+	-	+	-	-	+
12	+	+	-	+	-	+	-	+

(f) Discussion

Reliable binding of *M. catarrhalis* antigen to polystyrene microtitre plates was achieved using a standard carbonate buffer coating procedure and it was found that the antigen, once bound, was stable for several months when coated plates were stored in the dark at 4°C. There was parallelism in the binding of the detector antibody used in the IgG1, IgG2 and IgG3 subclass assays and the optical densities obtained were in roughly the same range. The assays described were shown to be specific for *M. catarrhalis* and antibody binding was not due to cross-reactive antibodies. Inter- and intra-assay variation was found to be acceptably low for all the assays. The binding patterns of antibodies derived from the standard serum and from clinical samples was identical irrespective of the source of the whole cell antigen and similar for whole cell antigen or outer membrane protein. The difficulty with quantifying small amounts of the OMPs led to the use of whole cell antigen routinely as the capture in the ELISA.

All adult sera tested had demonstrable levels of *M. catarrhalis* specific IgG1, IgG2, IgG3 and total IgG although only a minority had detectable IgG4. Since *M. catarrhalis* is an organism found in the upper respiratory tract of humans it is likely that its presence provides ongoing antigenic stimulation to the immune system, and this may explain the presence of antibodies in all of the adults tested.

While all adults have antibodies to *M. catarrhalis*, the acquisition of antibodies in childhood was found to be age related. IgG1 and IgG2 antibodies were detected earlier than specific IgG3 which was absent under the age of four. The reason for the absence of specific antibody of this isotype during the first few years of life and the apparent "switch" to significant levels of synthesis during the fourth year of life is unclear since the synthesis of IgG3 begins in the first year of life and by the age of 2 is already approaching adult levels [Lee et al., 1986]. Age related delay in the appearance of IgG subclasses has been described previously, but only

for IgG2 antibodies specific for carbohydrate antigens. The use of unconjugated carbohydrate vaccines such as those containing pneumococcal capsular polysaccharide or *H. influenzae* polyribosephosphate (PRP), are limited to children over the age of 2 because of their poor immunogenicity in younger children. In contrast, protein antigens which induce predominantly IgG1 and IgG3 responses, have previously been considered to be adequately immunogenic even in young children, hence the success of various vaccination schedules using protein antigens in infants (cf tetanus toxoid, diphtheria toxoid, hepatitis B vaccine, pertussis etc.). Newer Hib vaccines now contain PRP conjugated to a protein carrier such as diphtheria or tetanus toxoid, and these newer vaccines appear to be immunogenic even in very young infants [Booy et al., 1991].

Non-exposure to *M. catarrhalis* is an unlikely explanation for the absence of specific IgG3 since nasopharyngeal carriage rates for this organism have been reported in as many as 46% of children (age range 2 months to 18 years) attending the well-child care and pediatric outpatient department of the Cleveland Metropolitan General Hospital, Cleveland, Ohio [Van Hare et al., 1987]. Furthermore total specific IgG levels rise from the age of one year onwards and this, together with data showing that all adults have specific anti-*M. catarrhalis*, antibody suggests that the presence of the organism in the upper respiratory tract may provide a stimulus to antibody production. Whether the mere presence of the organism in the URT is sufficient to induce a systemic immune response is unclear. Hietala et al. [1989] have recently shown that children presenting with documented viral infection may show a convalescent rise in anti-bacterial titres suggesting mixed bacterial and viral infection. Of 51 children presenting with a proven viral infection and no obvious culture confirmed bacterial infection, 37% (19 patients) had a convalescent rise in antibody titre to various bacteria. Seven patients (14%) showed a "diagnostic" rise (> 3 fold increase) in antibody levels in their convalescent

serum to *H. influenzae* and 8 patients (16%) showed a similar rise to *M. catarrhalis*. The assay used to measure the anti-*M. catarrhalis* antibodies is that previously described by Leinonen et al. [1981]. While these antibody responses, in association with negative bacterial cultures, could be attributed to polyclonal B cell stimulation, viral infection may compromise the local factors in the URT which usually prevent invasion by commensal organisms, allowing contact between the immune system and the relevant organism. Read et al. [1991] have recently shown in a human respiratory epithelium organ culture model, that nontypeable *H. influenzae* associate predominantly with damaged cells in the epithelium, suggesting that a preceding viral infection and consequent damage to the epithelium may well facilitate bacterial adhesion. This may be one explanation for the levels of anti-*M. catarrhalis* antibodies found in most adults. Regular viral infections may account for contact between the organism and the immune system maintaining high titres of antibody which protect from invasive infection.

The finding that specific IgG3 antibody was of higher affinity than IgG1 and IgG2 is interesting. The methods used compare favourably with traditional methods of affinity determination and the DEA ELISA has been shown to rank monoclonal antibodies to DNP and polyclonal antibodies to HSA in the same order as ammonium sulphate precipitation [Devey et al., 1988]. As IgG3 only comprises 5-8% of total serum IgG it is unlikely that the effect seen is due to quantitative bias and unfair competition for binding sites of a large amount of IgG3. While the affinity of the anti-*M. catarrhalis* antibodies consistently rank IgG3 > IgG1 >/= IgG2 there is clearly heterogeneity of IgG3 affinity between individuals. Whether this heterogeneity has consequences for protection is unclear but is an ongoing source of investigation in our laboratory, as too is the relationship between age and the ability to develop high affinity responses.

It is interesting to note that in adults who suffer from recurrent or chronic non-allergic rhinosinusitis, levels of specific anti-*M. catarrhalis* IgG3 were lower than a group of healthy controls. While this group of patients have been noted to have a high incidence of total serum IgG3 deficiency [Scadding Personal communication], the lower specific IgG3 levels in these patients did not correlate with their total serum level of IgG3. This finding is analogous to the recent work by Smith et al. [1990] who investigated the relationship between serum IgG2 concentrations and antibody responses to pneumococcal polysaccharides in children with chronic chest symptoms. They showed that antibody increases after vaccination were not affected by the overall levels of IgG2 and hence total serum IgG2 could not be used to predict the antibody response. In the same way, serum IgG3 does not predict the level of specific anti-*M. catarrhalis* IgG3. Certain individuals with levels of IgG3 at the lower end of the normal range were found to have high levels of specific anti-*M. catarrhalis* IgG3. Analysis of the affinity of the specific anti-*M. catarrhalis* antibodies showed too that there was no simple relationship between the level of antibody and the affinity of antibody. Patients with different levels of specific IgG3 were found to have similar antibody affinities although in general there was heterogeneity of antibody affinity amongst the group as shown for the control group. Gm allotyping revealed no obvious difference between the patients and the controls although the numbers were small and larger studies will need to be performed.

The relevance of the specific IgG3 response may well be clarified following further elucidation of the antigenic determinants of this bacterium. While IgG3 antibodies are usually elicited by viral antigens, bacterial proteins such as the OMPs of nontypeable *Haemophilus influenzae* may, nevertheless, be potently immunogenic for this subclass. Antibodies to the OMPs of nontypeable *Haemophilus influenzae* have been shown to be protective [Murphy et al., 1987]

and have been described as belonging to the IgG1 and IgG3 subclasses
[Hammarström et al., 1986]. Our data shows that part of the immune response to
M. catarrhalis is mounted against the OMPs present in the cell wall and it is
possible that the importance of IgG3 in response to this organism is analogous to
that described for nontypeable *Haemophilus influenzae*.

Interestingly, it has been noted that in vitro, *M. catarrhalis* acts as a B-cell
mitogen [Banck et al., 1978] and appears to stimulate preferentially an IgG3
response as shown by increased intracytoplasmic IgG3 expression [Walker et al.,
1983] and secretion of IgG3 into the culture supernatants [Huston et al., 1989]. The
relationship between this phenomenon and the *in-vivo* observation of the importance
of IgG3 is unclear although undergoing further investigation in our laboratory. For
further discussion of this see Chapter 6.

CHAPTER 6

General Discussion

(a) IgG Subclass Deficiencies

Patients with low or undetectable levels of IgG subclasses in the serum can be divided into two broad groups; those with undetectable levels of IgG subclasses secondary to immunoglobulin heavy chain constant region gene deletions (the minority) and those with low or undetectable levels of IgG subclasses but normal constant region genes in whom the mechanism for the deficiency is unknown. Since the original description of an inherited deletion of immunoglobulin heavy chain constant region genes in normal human individuals (Le Franc et al., 1982), several other deletions have been described and are illustrated in Figure 6.1.
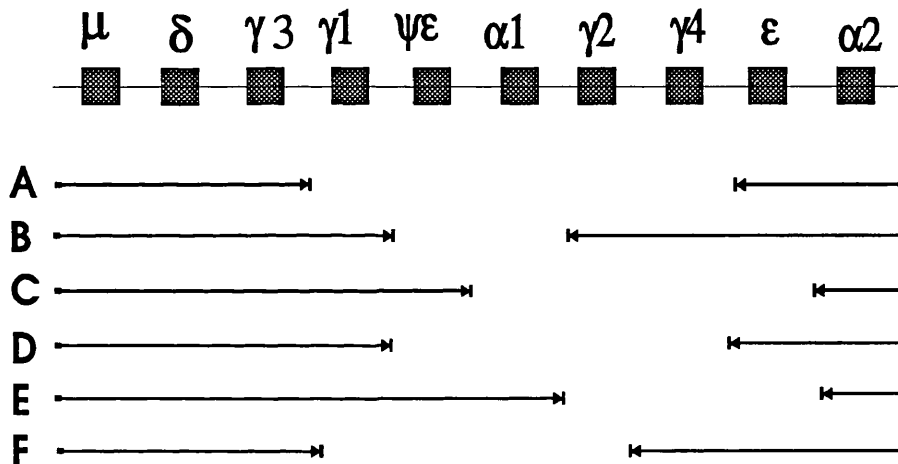

Figure 6.1: Multigene deletions of the human immunoglobulin heavy chain constant region locus on chromosome 14 as reported chronologically; A [Lefranc et al., 1980], B [Lefranc et al., 1983], C [Migone et al., 1984], D [Chaabani et al., 1985], E [Bottaro et al., 1989], F [Smith et al., 1989] (after Lefranc et al., 1990)

Lefranc's original description of a healthy 75 year old Tunisian woman with a constant region gene deletion (A in Figure 6.1) was followed by analysis of her immunological function. Her serum contained only IgM, IgD, IgG3 IgA2 and IgE. Levels of IgG3 were high (2.41 mg/ml) [Lefranc et al., 1983] and subsequent analysis of both her immune function and that of several similar individuals, showed that antibodies against protein and carbohydrate antigens

were present but were all restricted to the only IgG subclass present in the serum ie IgG3 [Hammarström, 1987]. In the two heterozygote daughters of this Tunisian woman, relatively normal distributions of antigen specific IgG subclasses were noted. The ability of individuals completely lacking one or a combination of IgG subclasses to resist infection, suggests that some functional compensation may occur between the subclasses. The mechanism by which such individuals overcome the antigen restriction of the IgG subclass response, and produce antibodies against a range of antigens of a single subclass, is unknown.

While the study of individuals with constant region gene deletions is of interest to those studying IgG subclasses, the fast majority of patients with abnormal IgG subclasses have low rather than undetectable levels of serum IgG subclasses, and hence do not have gene deletions underlying their abnormality. The categorisation of patients who have detectable but low levels of IgG subclasses as "IgG subclass deficient" is complicated by the definition of deficiency; when is a low level of a given subclass indicative of a deficiency? While low serum levels of one or a combination of IgG subclasses appears to be a fairly common finding (45% of the highly selected group of patients described in Chapter 3), the difficulty of defining IgG subclass deficiency is just one of the factors that complicates the whole field of clinical research in this area. The problems of comparing studies of subclass deficiencies from different laboratories is compounded by a lack of standardisation of the age related normal ranges. Normal ranges show wide variations between laboratories as indicated by Figures 6.2(a) and 6.2 (b), hence individuals classified as "deficient" in one laboratory may well fall within the normal range of a second laboratory.
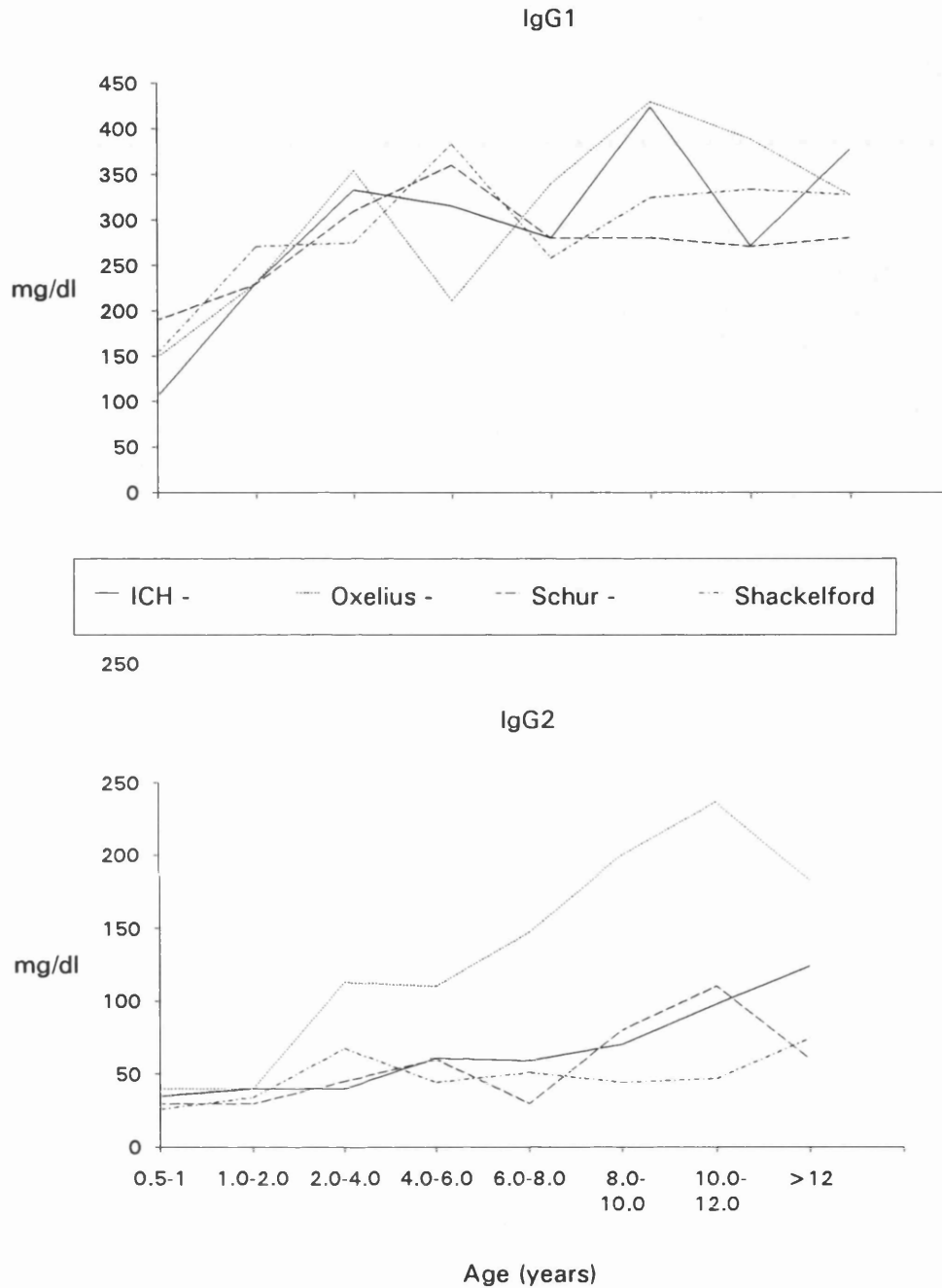
Figure 6.2(a): Comparison of the lower limits of IgG1 and IgG2 age related normal ranges derived from the literature as published by three different laboratories [Oxelius 1979, Schur et al., 1979, Shackleford et al., 1985b] and compared to our own laboratory normal ranges (ICH).
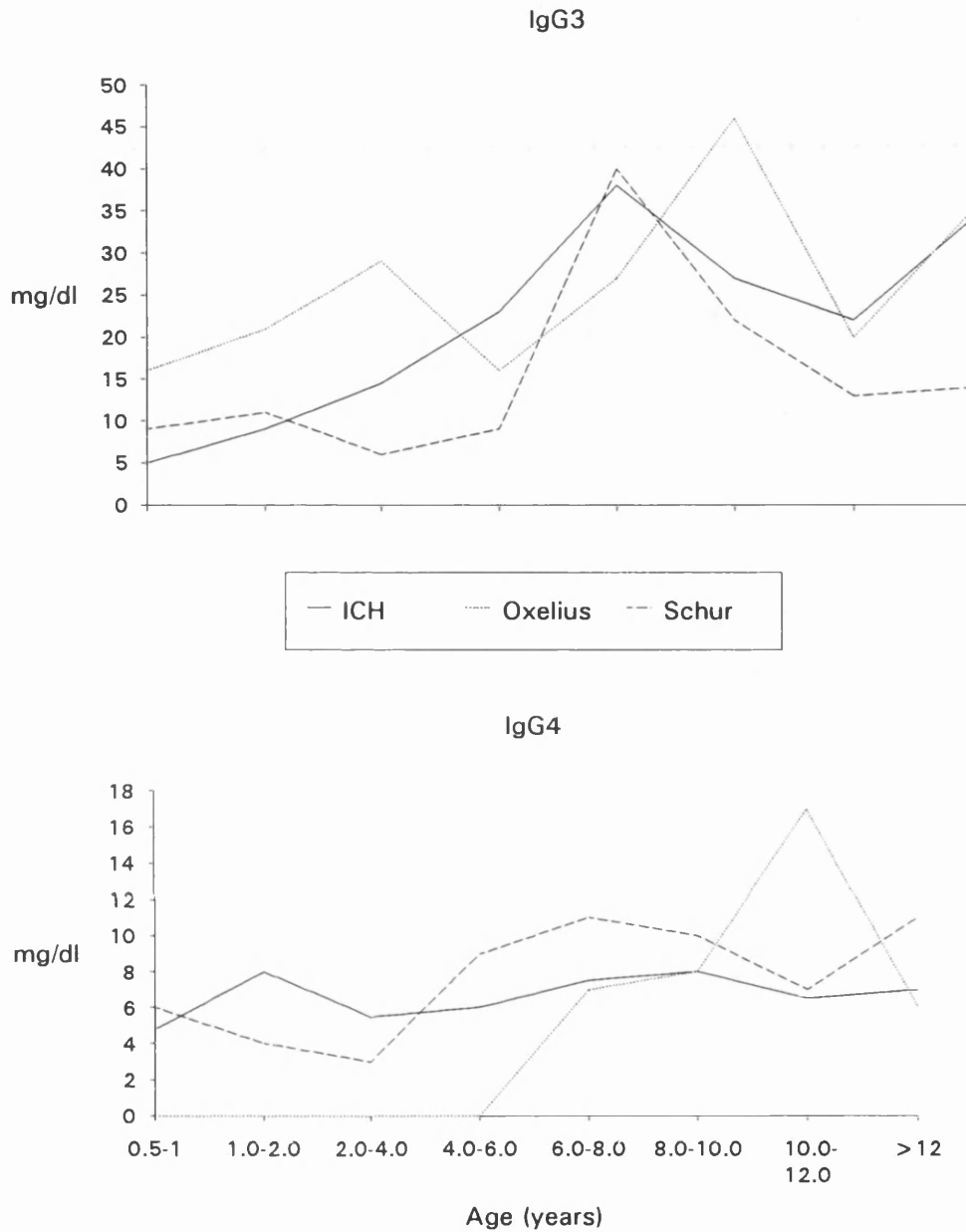
IgG3

IgG4

Figure 6.2(b): Comparison of the lower limits of IgG3 and IgG4 age related normal ranges derived from the literature as published by two different laboratories [Oxelius 1979, Schur et al., 1979] and compared to our own laboratory normal ranges (ICH).

The reason for these differences between laboratories are undoubtedly multifactorial and over and above differences in methodology, may reflect differences in the populations used for determining the normal range, in particular, race and Gm allotype [Shackleford et al., 1985b]. In the light of these inter-laboratory differences, generalisations regarding the clinical

presentation of IgG subclass "deficiencies" and comparison of patient groups from different centres become difficult. In particular, caution is needed when interpreting IgG subclass levels measured in one laboratory against the normal range derived in a different laboratory [Beard LJ et al., 1990]. Even when a similar technology is used there are good scientific reasons for avoiding such comparisons. Nevertheless a recent multicentre collaborative study [Kemeny DM et al., personal communication] comparing IgG subclass measurements from different laboratories around the world using international reference preparations has shown that results between laboratories may be comparable. There was generally agreement within 36% between the laboratories for the measurement of the four IgG subclasses and the agreement between RID and ELISA was good.

Notwithstanding the difficulties of defining IgG subclass deficiencies, the problem of linking the clinical presentation with the observed low level of IgG subclasses remains. The fact that individuals with gene deletions and absent IgG subclasses may be entirely healthy does not rule out a link between IgG subclass deficiencies in general and clinical disease. The mechanisms underlying subclass deficiency in individuals without gene deletions may be critical in understanding the pathogenesis of their clinical presentation. IgG subclass deficiency in non-deleted individuals may reflect abnormal regulation of IgG subclass production, and this may prevent the individuals from compensating, as those with deletions do, by making antibodies of a different subclass. This is borne out to some extent by the description of patients with frequent infection and IgG2 deficiency who mount a weak IgG2 restricted response to polysaccharide antigens [Siber et al., 1980, Umetsu et al., 1985], in contrast to patients with gene deletions who, despite no IgG2, make vigorous anti-carbohydrate antibodies of other subclasses. While IgG2 is the predominant subclass mounted in response to carbohydrate antigens in the adult, IgG1 is produced in younger children and more recently has also been recognised as part of the adult anti-carbohydrate

response [Shackleford et al., 1987]. Despite this property of IgG1 the patients described above appear unable to compensate for the lack of IgG2 production.

An added complication in the interpretation of the finding of a low level of an IgG subclass is that such individuals may, like the patients with gene deletions mentioned above, be completely healthy. Shackleford et al. [1989] measured IgG subclass levels in 575 healthy children and found that 11 had IgG2 levels >2SD below the age matched normal range. The IgG2 levels were similar to those of a group with frequent infection, yet these 11 children were entirely healthy and mounted normal IgG2 responses to polysaccharide vaccines. Relatives of patients with IgG3 deficiency and frequent infection have also been found to be low in this subclass yet entirely healthy [Oxelius et al., 1986]. It is possible that the low level of IgG3 in the family may be related to genetic factors such as the Gm allotype and the frequent infection in the symptomatic relative may be unrelated to the low level of IgG3. In certain circumstances however, the subclass deficiency may be an indication of developing hypogammaglobulinaemia as described by Sanders et al. [1991] in a follow up study of a group of subclass deficient patients.

Underlying this discussion is the fact that population based studies of IgG subclass levels of large numbers of adults and children, measured using standardised reagents and analysed on locally produced age matched normal ranges with a universally accepted definition of IgG subclass deficiency do not exist. In the absence of this idealised situation, the analysis and interpretation of IgG subclass serum levels remains fraught with difficulty.

The most useful model for studying IgG subclass regulation and function may well be that which concentrates on IgG subclass production in response to particular antigens rather than focussing on antibody levels in the serum. Patients who, despite normal levels of immunoglobulins (including IgG subclasses), suffer from frequent infections and an unresponsiveness to vaccines may provide such an opportunity. Ambrosino et al. [1987] first reported such a

patient, a 30 year old man who suffered from recurrent pneumococcal pneumonia. All tests of his humoral and cell mediated immune function were normal except for his unresponsiveness to polysaccharide vaccines, including those containing the polysaccharide capsules of *S.pneumoniae,* Hib and *N. meningitides* group A and C. Gilgliotti et al. [1988] subsequently reported a 7½ year old boy who was investigated for recurrent pneumococcal septicaemia. He too had a normal immunological profile except for an isolated inability to respond to capsular polysaccharide vaccines. Interestingly he did respond when vaccinated with a protein conjugated Hib vaccine (having failed to respond to pure PRP), suggesting that the nature of the antigen was important in the pathogenesis of the frequent infections. Ambrosino et al. [1988] followed up their original description (see above) with a study of 15 children who had recurrent URTI (otitis, sinusitis and pneumonia) of a frequency similar to that of children diagnosed as IgG2 subclass deficient, yet with normal IgG subclass serum levels. Analysis of their response to Hib PRP vaccine showed a poor response in comparison with healthy age matched controls, yet a normal response to diphtheria toxoid. This inability to produce IgG of any subclass against selected polysaccharides, suggests that certain properties of the antigen are critical, while at the same time the lack of compensatory antibody of a subclass other than IgG2 suggests that unlike patients with gene deletions, a regulatory abnormality prevents antibody responses of a different subclass. The abnormality described in the patients above has some similarities to the normal situation in children under the age of two. Unlike adults, they are unable to mount IgG2 responses to polysaccharide antigens (although do make some IgG1 antibodies). While the defect in the patients described above may represent the delayed maturation of the IgG2 anti-polysaccharide response, the failure to mount any antibodies at all to polysaccharides still requires explanation.

Similarities with the situation in young children are apparent however when responses to protein-polysaccharide conjugate vaccines are analysed. The

conjugation of Hib capsular polysaccharide (PRP) to protein changes the immunogenicity of the polysaccharide, possibly changing it from a T cell independent to a T cell dependent antigen. Young children, whose response to T cell dependent antigens is thought to be adequate, mount an antibody response to the conjugate vaccine, as do the patients described above. Protein-polysaccharide conjugate vaccines induce IgG1 antibodies in those under the age of 18 months [Ahonkhai et al., 1990], predominantly IgG1 responses in those ages 18-23 months [Claesson et al., 1988] and equal amounts of IgG1 and IgG2 in adults [Seppälä et al., 1988]. The ability of these IgG1 antibodies to protect from invasive disease is as yet unproven, although some experimental data suggests that anti-Hib IgG1 is able to mediate complement dependent killing of Hib and provides passive protection of infant rats [Weinberg et al., 1986].

It is apparent, therefore, that the information derived from the measurement of IgG subclasss levels in the serum requires analysis in the light of many factors including the methodology used to measure the levels, the normal range used and individual considerations such as Gm allotype. The interpretation of low levels of a subclass in the light of the clinical presentation still requires further elucidation of what is undoubtedly a complex set of relationships. However, the antigen specific nature of certain subclass responses and the identification of individuals with "blind windows" for polysaccharide antigens provides an exciting opportunity to study the underlying mechanisms of IgG2 production. In addition, the analysis of an absent antibody response in the face of an antigen challenge in a patient with frequent infection provides a useful correlation between disease susceptibility and an absent immune response. It is, of course, also possible that following more extensive investigation healthy individuals unable to respond to vaccines will be identified and this would further complicate the dissection of these problems.

(b) Outer Membrane Proteins and the IgG subclass response

Several infections that are predominantly found in younger children have been associated with the inability of the immature immune system to cope with the particular infectious agent. To date the best example of this is the susceptibility of young children to infection with encapsulated organisms such as Hib, pneumococcus and meningococcus. The link with the inability of such children to produce IgG2 has already been discussed. Patients post-bone marrow transplantation [Aucouturier et al., 1987], those with ataxia telangiectasia [Oxelius, 1986a] and those with HIV infection [Parkin et al., 1989] are all susceptible to infection with such encapsulated organisms, possibly due to the same underlying pathogenesis ie the inability to respond to polysaccharide antigens. Interestingly, the three situations described above have also all been linked with IgG2 deficiency. In contrast to carbohydrates, the ability to mount antibody responses to proteins does not appear to be age restricted or problematic in the patients falling into the categories mentioned above. Infants vaccinated with a variety of protein antigens (tetanus, diphtheria, pertussis, hepatitis B etc) appear able to mount antibody responses that confer protection from early life. The IgG subclass mounted in response to these antigens is predominantly IgG1 and to a lesser extent IgG3. No examples of delayed maturation of anti-protein responses analogous to those described for anti-carbohydrate responses have been reported, and the anti-protein responses of the patients with an inability to respond to polysaccharide antigens have, on the whole, been shown to be normal. However, one study by French and Harrison [1984] did measure anti-tetanus toxoid responses in 45 adults with chronic lung disease and 18 with recurrent chest infections. None of the adults in this study had IgG abnormalities (IgG subclasses were not measured) but several had IgA deficiency. 18% of those with chronic chest infections had poor anti-tetanus responses and 3 of the recurrent infection patients, all with IgA deficiency, showed poor anti-tetanus responses.

The studies described in this thesis have originated from the same observation noted initially for the encapsulated organisms; ie the particular susceptibility of young, otherwise healthy children to infection with *M. catarrhalis*, the relative immunity of healthy adults and the susceptibility of adults once again to infection with this organism, when generally immunocompromised or when local lung defences are damaged. However, as a non-encapsulated gram-negative organism, it was unlikely that the major antigens of *M. catarrhalis* would be carbohydrate, hence the analysis of the antigens on the surface of the organism. The recognition of OMPs, conserved between isolates and targets for human antibody provides an opportunity to investigate in some detail, anti-protein responses.

While the polysaccharide antigens of various organisms have received much attention, the role of OMPs of a variety of gram-negative organisms have also begun to stimulate interest. Much attention is focussed on the polysaccharide capsule of Hib which is a virulence factor and a target for bactericidal antibodies, yet the OMPs of Hib are also clearly important antigens. Several studies involving experimental models of Hib infection have shown that antibody to OMP can be protective [Kimura et al., 1985, Munson et al., 1985]. In addition, studies in man indicate that antibody to non-capsular antigens participate in immunity to Hib disease and that antibody to OMPs can be detected in convalescent sera of both adults and children recovering from Hib infection [Gulig et al., 1982, Lagergård et al., 1984]. The OMPs of group B *N. meningitidis* have been used in vaccine formulations because the capsular polysaccharide of group B is a poor immunogen [Wyle et al., 1982]. However, field trials of an OMP vaccine formulation have unfortunately been disappointing to date. The OMPs of group B *N. meningitidis* have also been conjugated to the PRP of Hib to make the Hib vaccine more immunogenic [Santosham et al., 1991]. While this formulation does appear to increase the immunogenicity of the vaccine in young children, little is known of the immune

response to the group B OMPs. The OMPs of nontypable *H. influenzae* are targets of bactericidal antibody but because they differ from strain to strain, a vaccine based on the OMPs will require the identification of an OMP common to all strains or the inclusion of a large number of OMPs in the vaccine.

The reason for the interest in a vaccine for nontypable *H. influenzae*, is because it is the most frequent bacterial cause of acute otitis media, a disease which afflicts 75% of children by 6 years of age [Hendrickse et al., 1988]. More importantly, 50% of those who have acute otitis media in the first year of life will have recurrent disease, and recurrent otitis is the principal reason for seeking medical attention for a child in the USA. Vaccination of children in Norway to try and reduce the incidence of pneumococcal otitis media (*S. pneumoniae* is the second most common bacterial pathogen in childhood otitis media) has already been evaluated. Mäkelä et al. [1980] vaccinated 827 children aged 3 months to six years with pneumococcal vaccine or a control vaccine (Hib) following an attack of acute otitis. Children aged over 6 months who received the pneumococcal vaccine were protected from recurrence of otitis media due to pneumococci which were represented in the vaccine. The protective efficacy of the vaccine (57% overall) was somewhat better in children over the age of two.

It is conceivable that the third most common cause of bacterial otitis media ie *M. catarrhalis* may also become a candidate for vaccine development in which case a comprehensive understanding of the surface antigens would be crucial. Presumably, the ideal candidate antigen would be immunogenic for all four subclasses. The model of *M. catarrhalis* described in this thesis raises the possibility that the choice of an OMP recognised only by IgG3 may fail to stimulate an immune response in those recipients under the age of 4. Interestingly though, antigen specific IgG subclass responses may differ depending on the route of antigen administration. In a study investigating the IgG subclass composition of the response to natural pertussis infection and the

pertussis vaccine, differences were found between the two [Zackrisson et al., 1989]. IgG1 and IgG3 were detected in 92 and 42% respectively of samples from patients with natural pertussis infection while IgG2 and IgG4 were rarely detected. In contrast, children vaccinated with an aluminium-adsorbed pertussis toxoid, mounted IgG1 and IgG4 responses, with some low levels of IgG2, whereas IgG3 was rarely detected. Unfortunately the patients in the two groups were not age matched, a fact which might be crucial to the interpretation as the vaccinated children were all between 18 and 24 months while the natural infection group consisted of predominantly older children and adults. This may in part account for the absence of IgG3 in the vaccinated recipients. However, the identical subclass pattern has been shown following hepatitis B vaccination. Natural infection in adults induces IgG1 and IgG3 antibodies specific for the hepatitis B surface antigen, whereas adult vaccinees mount IgG1 and IgG4 antibodies [Mattila et al., 1986, Persson et al., 1988]. The route of antigen presentation appears to influence the subclass profile of the antibodies produced and there may be crucial differences in the response to a live virus as opposed to a protein sub-unit. It is possible therefore that the subclass response to injected *M. catarrhalis* antigens may differ to that seen naturally. As *M. catarrhalis* is a commensal that enjoys the warm moist environment of the upper respiratory tract mucosae, ongoing contact occurs at a mucosal level and it is presumably rare for haematogenous dissemination to occur. While IgA may therefore be important, the study of nontypable *H. influenzae*, an organism with many similarities to *M. catarrhalis*, has shown that protection from middle ear infection with this organism correlates directly with levels of specific IgG in serum and middle ear secretions rather than with IgA. Furthermore IgG antibodies persist following infection [Faden et al., 1989].

Analysis of the immunoblots of *M. catarrhalis* OMPs would suggest that the 82 kDa protein identified after silver staining and recognised by all four IgG subclasses may well be a candidate antigen should vaccine development become

a future priority. Whether the four subclasses have equivalent effector functions is unknown but seems unlikely since we know that IgG4 cannot bind complement. However, despite the fact that IgG2 is said to bind complement poorly, it is the subclass of choice mounted in response to polysaccharides and clearly protects from invasive infection with encapsulated organisms. This discrepancy between defined effector function and *in-vivo* performance is just one of the many intriguing questions regarding IgG subclasses that remains unanswered.

Finally, the role of OMPs on the surface of the organism are also unknown. They may be crucial to the process of colonisation of the airways which is thought to involve specific interaction between bacterial adhesins and complementary epithelial receptors [Beachey 1981]. Read et al. [1991] have recently showed that nontypable *H. influenzae* will only associate with human respiratory epithelium that is structurally damaged. Preliminary data from immunofluorescent staining experiments suggests that *M. catarrhalis* binds to the submucosal rather then the epithelial layer of fixed sections of human turbinates (data not shown). Evidence for bacterial superinfection by *M. catarrhalis* following viral infection as determined by a rise in anti-*M. catarrhalis* antibodies [Hietala et al., 1989], suggests that viral infection may well render the usually resistant upper respiratory tract epithelium susceptible to infection with *M. catarrhalis*. The mechanism by which this is achieved is unclear as is the role of the OMPs in this process. Interestingly, Mbaki et al. [1987] have demonstrated that *M. catarrhalis* shows a seasonal increased adherence to oropharyngeal cells, with adherence peaking in the winter, a pattern which coincides with the observed peak of infection due to *M. catarrhalis*. OMPs may be candidates for receptor mediated binding in the same way that an 82kDa OMP on the surface of *Treponema pallidum* has been identified as a fibronectin binding protein [Thomas et al., 1985]. As *M. catarrhalis* has a prominent 82 kDa OMP, the potential that this OMP was

analogous to the *T pallidum* OMP has been investigated in collaboration with Dr N Klein. Exploiting the fact that human umbilical vein endothelium (HUVEC) is coated with fibronectin, the binding of *M. catarrhalis* to HUVEC cultures was explored. In summary, while *M. catarrhalis* was found to bind to HUVECs, neither fibronectin antibodies, nor the preincubation of *M. catarrhalis* with fibronectin could block attachment of *M. catarrhalis*. A role for the OMPs and possible mechanisms by which antibody binding to these OMPs prevents infection requires further study. Finally, some isolates of *M. catarrhalis* have poorly characterised fimbriae on their surface although their role is unclear. While fimbriae may in general be involved in adherence, the degree of fimbriation of other organsims such as nontypable *H. influenzae* does not necessarily correlate with increased adherence [Bakaletz et al., 1988].

(c) IgG3: Aspects of control and production

Although IgG3 is structurally a unique molecule it does not appear to play as prominent a role in protection from infection as IgG1 and IgG2. IgG3 antibodies are described in association with anti-protein responses, but they are usually present together with IgG1. A good example of this is the study by Wedege and Michaelsen [1987] who showed by means of immunoblotting that antibodies to the OMPs of group B *N. meningitidis* were, following vaccination, initially of the IgG1 and IgG3 isotype but that levels of IgG3 had dropped by 25 weeks post-vaccination. Functionally IgG3 and IgG1 were shown to be synergistic in *in-vitro* experiments in which IgG1 and IgG3 monoclonal anti-D antibodies were used to sensitise red cells in monocyte stimulation assays [Hadley and Kumpel, 1989]. The metabolic response of the monocytes to the red cells as measured by chemiluminescence, was greatest when the red cells were sensitised by mixtures of the monoclonals. The finding that IgG3 was such a prominent part of the anti *M. catarrhalis* response was particularly interesting since all adult serum contained antibody, presumably as discussed before,

secondary to ongoing stimulation. The absence of IgG3 under the age of four, the very age group which appears to be particularly susceptible to infection with this organism, suggests that this isotype may in some way be critical for protection from infection, and is the first description of delayed maturation of anti-protein responses.

What this observation also does is provide the opportunity to investigate mechanisms underlying the control of IgG3 production. For many years *M. catarrhalis* has been recognised as a B cell mitogen [Banck and Forsgren, 1978]. Walker et al. [1983] investigated the isotype specific responses of the B cells stimulated by *M. catarrhalis* by staining the cytoplasm for IgG subclasses. In comparison with B cells stimulated by a range of other mitogens (PWM, LPS, EBV, *S. Aureus*), the isotype profile of the *M. catarrhalis* stimulated B cells showed that all subclasses were stimulated but a preferential stimulation of IgG3 was noted. This is in contrast to the predominantly IgG2 response to LPS and *S. aureus,* and in contrast to PWM induced responses in which the IgG subclass antibody profile reflects that seen in peripheral blood ie
IgG1 > IgG2 > IgG3 > IgG4.

This IgG3 predominant response of B cells to *M. catarrhalis* may be linked in some way to the *in-vivo* production of anti-*M. catarrhalis* IgG3. The stimulus provided *in-vitro* by *M. catarrhalis* is polyclonal rather than antigen specific. The polyclonal production of IgG3 may be due to the fact that a particular subpopulation of B cells is being stimulated preferentially by *M. catarrhalis,* perhaps as a result of cell surface antigen binding. *M. catarrhalis* has been shown to activate human B cells by binding to surface IgD and Class I MHC antigens [Forsgren et al., 1988] but this is unlikely to be the mechanism for preferential IgG3 stimulation. The addition of antibodies reactive with IgD and HLA Class I antigens inhibited B-lymphocyte stimulation by *M. catarrhalis* but not by anti-IgM, *S. aureus* or EBV. Preliminary studies using *M. catarrhalis* as a mitogen have confirmed that it is able to stimulate PBMCs (mixed T and B

cells) to produce IgM and IgG (data not shown). Both IgG1 and IgG3 are produced although this appears to vary depending on the source of B cells. Antigen specific stimulation of B cells by *M. catarrhalis* has so far not been successfully accomplished. Interestingly Calvert and Calogeras [1986] have characterised *M. catarrhalis* as a T cell independent antigen although the reported Ig isotype profile following stimulation is dissimilar to other T cell independent antigens which stimulate preferentially IgG2. It would be interesting to analyse the responses of B cells to whole *M. catarrhalis* as compared to purified OMPs in order to establish whether they alone are capable of stimulating B cells and whether the Ig profile is similar.

Individuals with low anti-*M. catarrhalis* antibodies, specifically low IgG3 levels, may be a valuable source of material for the studies of those factors that may influence isotype switching. Experiments with both murine and, more recently, human lymphocytes indicate that T cells and their cytokines are important regulators of isotype production [Coffman et al., 1988, Thyphronitis et al., 1989, Ishizaka et al., 1990]. B cells from patients with IgG3 deficiency could be used to study the effect of *M. catarrhalis* as a polyclonal mitogen while B cells from patients with low anti-*M. catarrhalis* antibodies could be used to study antigen specific responses. The role of cytokines in the production of IgG3 may then be studied in the two systems outlined above. Recently Benson et al. [1990] have explored the role of IL5 in stimulating human B cells. They have shown that IL5 added to *M. catarrhalis* activated B cells enhances IgM and IgA secretion although their data may be influenced by possible T cell contamination as indicated by the production of IgG from B cell cultures with *M. catarrhalis* but with no added IL5. In addition, Huston DM and Huston MM at Baylor College of Medicine, Houston, Texas [personal communication] have shown that IL5 added to *M. catarrhalis* activated pure B cells enhances IgM and IgA production and that *M. catarrhalis* alone in highly purified B cell cultures has no effect on immunoglobulin production, suggesting T dependent rather than T

independent mitogenic properties. These results are of interest since the role of IL5 in humans is unknown. In mice, IL5 was initially described as a switch factor for IgA production although more recently has been described as a terminal differentiation factor for murine IgA [Teale and Estes 1990].

(d) Isotype switching and V_H region usage

The absence of specific anti-*M. catarrhalis* IgG3 in the presence of specific IgG1 and IgG2 in young children, and the presumption that the isotypes have the same antigen specificity, raises the question of whether variable region usage for the different isotypes is the same. The OMP patterns obtained following immunoblotting suggest that while the OMP recognition of IgG1 and IgG2 is relatively restricted compared to that of IgG3, the isotypes still recognise the same OMPs. Underlying this question is the question of whether isotype switching *in vivo* occurs successively in linearly related B cells or selectively in different populations of B cells. Thus, if antigen specific antibodies of different isotypes have different V regions they are the products of B cells of different clonal origins. In contrast, if antigen specific antibodies of different isotypes always expressed identical V regions it is likely that they arose from a single pre-B cell as predicted by the linear switching model. Scott et al. [1989] have addressed this issue by analysing clonal antibodies specific for Hib purified from the serum of high responding adult vaccinees. 15 clonal antibodies (6 IgG1 and 9 IgG2) were examined and it was found that all 15 utilised heavy chain V regions of the $V_H III$ family. In contrast, the L chains of these antibodies were clearly derived from at least four different V_L families. Furthermore V_L expression correlated with the cross-reactivity of these antibodies with the capsular carbohydrate of *E. coli* K100. The light chain V regions may therefore be important contributors to the limited heterogeneity of the antibody repertoire. Nevertheless there was one individual in this study in whom different Vκ regions were found in association with IgG1 and IgG2 antibodies, suggesting

that the IgG1 and IgG2 anti-Hib-PS antibodies had arisen from different precursor B cells.

Should anti-*M. catarrhalis* antibodies of different isotypes similarly reflect identical V_H and V_L usage within an individual, the possibility that the V region determining anti-*M. catarrhalis* specificity is able to combine with IgG1 and IgG2 switch regions at an early age but not with the IgG3 switch region, is raised. In mice, the V_H region is organised into 9 large families on the basis of similarities at the nucleotide sequence level, and these families are organised into relatively discrete clusters. There is evidence from the study of murine neonatal liver derived lymphocytes that there is programmed use of the V regions, with those most 3', ie. closest to the constant region gene clusters, being utilised first [Alt et al., 1987]. This phenomenon therefore gives rise early on to a relatively restricted primary antibody repertoire. This might account for the inability of young infants to mount antibody responses to certain antigens when a variable region contributing to antibodies of that particular antigen specificity is relatively distant from the constant region. However several problems arise when one compares mouse with human. Firstly, the V_H region genes are organised differently in the two species. There are five V_H families in man and these families are not clustered but widely scattered. While there is some evidence that V region proximity to the DJ region results in preferential selection in the human neonate [Schroeder et al., 1988], more recent work has shown that Ig secreting clones from embryonic tissue, cord blood and adult peripheral blood all have similar polyspecific antibody frequencies and V_H family usage is not restricted in the embryonic or cord cells [Guigou et al., 1991]. Should V_H region usage be similar for all isotypes of *M. catarrhalis*, an inability to combine with the IgG3 switch region, may possibly underlie the observed phenomenon. DNA binding proteins have been postulated as possible switch factors, opening up or making available the switch region of the individual isotypes [Alt et al., 1986]. Recently Baltimore's group in Boston have

described two recombination activation genes (RAG-1 and RAG-2) which when transfected into a fibroblast cell line were able to activate V(D)J recombination [Oettinger et al., 1990]. The proteins encoded by these genes are presumably able in some way to mediate the complex recombination machinery required to produce functional immunoglobulin gene rearrangements.

(e) IgG subclasses and affinity.

The differences in antigen binding affinity of immunoglobulin isotypes with the same specificity also raises the question of V region usage. Affinity maturation occurs following contact between antigen and a B cell although the temporal relationship between affinity maturation and isotype switch is still unclear. Should B cells switch immediately following contact with the *M. catarrhalis* antigen, then it is possible that the IgG3 clone is favoured in some way for affinity maturation. In contrast if isotype switch occurs fairly late after antigen contact with the B cell, then affinity maturation will have taken place prior to switching and all the isotypes would then be expected to have identical affinity values. Manser [1989] has recently shown that somatic mutation and isotype switching are completely independent processes. In particular somatic mutation may occur after isotype switching and this would explain why different isotypes having the same specificity have different affinities. Devey et al. [1991] have recently investigated affinity maturation during primary, secondary and tertiary responses to vaccination with keyhole limpet haemocyanin. These authors found that high affinity IgG1 was produced early in the immune response and more rapidly following a secondary challenge. In contrast, high affinity IgG4 antibodies appeared late in the primary response and it is suggested that this may be due to direct isotype switching from high affinity IgG1 to IgG4 production. Alternatively, the IgG4 switch may be a late event possibly following prolonged contact between the antigen and the B cell in the lymph node germinal centre where antigen is known to localise [Bird and Lachmann,

1988]. Although it is assumed that the only determinants of affinity are to be found in the V regions, it is possible that affinity is also in some unknown way, influenced by the constant region of the isotype. The study of humanised monoclonal antibodies, such as rat variable regions combined with human constant regions, will hopefully facilitate the investigation of the contribution of isotype to affinity.

(f) Conclusions and Future Directions

Whilst we have made considerable progress in understanding infectious diseases since Jenner introduced scarification with cow pox, we are constantly reminded of how much there is still to discover in the field of the human immune responses to infection. The discovery of the IgG subclasses has opened up an area of exciting research and the emphasis that this has brought on the quality of antibody production is important. The measurements of such quality will hopefully facilitate more sophisticated analyses of the immune response following natural infection and vaccination. A comprehensive picture of the qualitative aspects of antibody responses may eventually help to illuminate some of the poorly understood manifestations of immunodeficiency disease. Furthermore, as the technology for the production of humanised monoclonal antibodies is developed and extended, the therapeutic use of such antibodies will become more widespread. An understanding of isotype function and the subtle differences between the IgG subclasses will undoubtedly aid the design of such molecules. The increasing sophistication of molecular techniques will also permit the exploration of differences between immunoglobulin subclasses at a molecular level and possibly explain the phenomenon of switching. The study of variable region usage particularly in relation to the ontogeny of antigen specific responses observed in-vivo may also shed some light on the mechanisms underlying switching.

With a better understanding of the complex responses to infectious agents, the ability to stimulate the immune response with recombinant cytokines or other factors may help us to boost the immune response in a highly specific way. Unresponsivenes to vaccines in individuals or groups of children may be modulated by the simultaneous administration of such factors and factors that stimulate the preferential production of isotype appropriate high affinity antibody responses may form part of an improved anti-microbial strategy in the future.

Appendix A

Monoclonal Antibody specificity

The specificity of the biotinylated mouse anti-Human IgG3 and IgG4

monoclonal antibodies HP 6047, HP6011 (Zymed) was established using the

following protocol. Rabbit anti-human IgG (heavy and light chain) was used as a

capture reagent and incubated in the wells of a microtitre plate overnight at 4°C.

Following blocking with normal mouse serum, preparations of human IgG1, IgG2,

IgG3, and IgG4 myeloma proteins (~20.000 ng/ml) were incubated for two hours at

room temperature. Following washing, the biotinylated Zymed monoclonals were

added and the plates incubated for a further two hours at room temperature. The

anti IgG3 and anti IgG4 antibodies were used at $^1/_{1000}$ and $^1/_{100}$, respectively. The

final steps were carried out as described for the general ELISA method outlined in

Chapter 2. The antibody binding data (expressed as optical density at 492 nm) is

shown in Table A1 and indicates that the monoclonal antibodies used were

essentially specific. Some cross reaction is seen with IgG2, but binding OD's are

one tenth that of the binding to a myeloma protein of the appropriate isotype.

Table A1: Specificity of anti IgG3 and IgG4 monoclonal antibodies HP 6047 and HP 6011. Binding of the monoclonals to preparations of human myeloma IgG subclass proteins are expressed as OD at 492nm. Control wells contained capture but no myeloma proteins.

|	Myeloma (~20.000 ng/ml) protein				
	IgG1	IgG2	IgG3	IgG4	Control
Anti IgG4	0.026	0.289	0.316	2.731	0.136
	0.019	0.256	0.304	2.702	0.134
	0.025	0.248	0.292	2.711	0.132
Anti IgG3	0.013	0.232	2.46	0.037	0.021
	0.036	0.256	2.54	0.032	0.040
	0.030	0.280	2.499	0.036	0.035

Appendix B

The use of Zymed monoclonal antibodies HP6069 (anti-IgG1), HP6002 (anti-IgG2), HP6047 (anti-IgG3) and HP6025 (anti-IgG4) in antigen specific assays.

Zymed monoclonal antibodies were used in a variety of antigen specific assays and showed no particular bias for detecting any given IgG subclass. ELISA's were developed as described in Chapter 2. Coating antigen concentrations and concentrations of monoclonal detector antibodies were adapted as shown in Table A2. Figure A1 shows the standard curves for two of the assays measuring anti-tetanus toxoid and anti-meningococcal polysaccharide capsule A/C/W/X/Y antibodies which were derived from the analysis of the binding of antibodies in the standard adult serum (meningococcal assay) or an immune adults serum (tetanus assay).

Table B1: Modifications to the antigen specific ELISA as described in Chapter 2 for the measurement of antigen specific antibodies

	Assay:	α-Tetanus Toxoid	α-Meningococcal Polysaccharide
Modification:			
Coating antigen conc: (O/N 4°C).		0.5 lf/ml	5 μcg/ml
Monoclonal antibody concentration:			
IgG1		1/6000	1/1000
IgG2		1/2000	1/1000
IgG3		1/500	1/1000
IgG4		1/1000	1/1000
IgG Total		1/8000	1/1000

Figure B1: Antigen specific IgG subclass assays using (a) meningococal and (b) tetanus toxoid IgG subclass specific assays. Standard curves obtained for binding of a standard serum (meningococcal assay) and an immune serum (tetanus assay) utilising Zymed monoclonal antibodies

(a)

■ IgG1   ▲ IgG2   ◆ IgG3   △ IgG4   ◇ IgGT

(b)
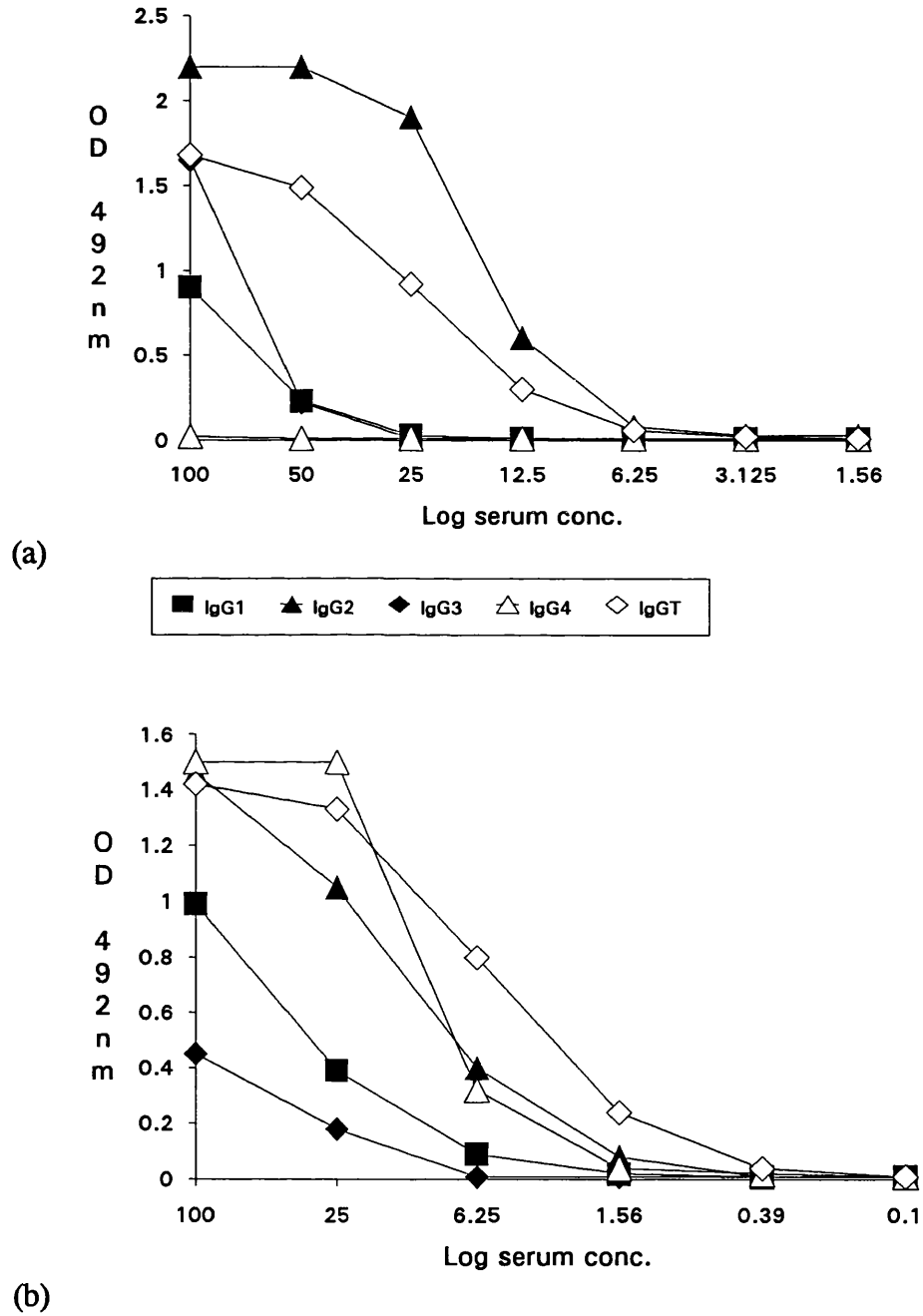
Appendix C

Total serum IgG3 and specific anti-*M. catarrhalis* IgG subclass levels in children undergoing elective surgery.

Table C1: Anti-*M. catarrhalis* IgG subclass antibodies and total serum IgG3 levels in 53 children of different ages undergoing elective surgical procedures with no evidence of any immunologically related abnormalities. Levels of specific antibody are expresssed as the percentage of a standard serum (see Chapter 2 for details) and levels of serum IgG3 are in mg/dl. Specific IgG4 antibodies are marked with a plus if they were detected.

	Anti-*M. catarrhalis* specific antibodies (% standard)					Serum (mg/dl)
Age (years)	IgG1	IgG2	IgG3	IgG4	IgG Total	IgG3
0.11	72	49.2	5.1		35.5	60
0.11	60	40	4.5		35.1	66
0.125	40.7	70.3	11.6		33.3	
0.21	37	59.2	0.5		13.8	
0.3	29.2	29.2	3		23.1	48
0.7	26.6	44.02	1.2		16.2	
0.8	27.2	44.7	1.6		15.4	68
0.99	10.4	40.8	0.6		18.4	88
0.99	22	50	0.7		38.3	48
0.99	27.7	43.2	1.3		15.3	48
1.6	35.7	47.5	2.4		7	18
2	92	53.9	4.8		41.8	
2.11	64	43.6	1.3		32.6	28
2.4	57.8	50.8	2.8		70.2	42
2.5	115	38.8	1.4		61.7	48
2.9	38.8	56.7	9		18.7	20
2.99	111.1	52.2	9		40.7	
3	92	56.3	1.7		55.2	56
3.11	48.6	49.2	3.7		31.5	6
3.3	75	52.6	0		37.8	28
3.8	196	56	47		159	66
3.98	51.1	44.7	15.8		21.6	38
3.99	138	40.2	20.2		81.4	26
3.99	58.3	52.2	4.9		40	34
4.6	163.1	65.5	127.2		180.8	18
5	15.7	120.6	2.9		14.8	76

Age (years)	IgG1	IgG2	IgG3	IgG4	IgG Total	IgG3
5.4	61.2	50.7	71.1		78.9	76
5.7	205	178	171		252.6	72
5.9	97.2	62.2	20		77.6	28
6.25	192	296	33.9		142	
6.3	168	100	191		181.5	62
6.99	55.5	208	47	+	36.9	26
7.33	59.2	68.5	8.7		38	
7.9	75	51.1	2.5		6.8	70
9.33	55	118	7.6		66.6	
9.58	382	75.9	64		214	
9.7	333	95	11.8	+	17	20
9.99	106	84.4	45.4		97.8	66
10	155	96.2	248		178.5	
10.11	168.4	89.6	71.9		28.2	26
10.25	81	55.5	29.1		46.4	34
11.08	85	79.2	27.1		47.6	
11.2	205.5	159.7	190		153.8	56
11.99	184	62	164.5	+	160	48
12.1	118	63	41.8	+	85.5	56
12.2	53	53.3	181		153.2	
12.6	68.4	122.4	78.9		70.2	76
13.99	34.2	124.1	16.3		35.7	42
15.11	136	81.6	117.7		107.8	56
15.8	86.1	300	24.4	+	46.3	34
15.9	178.9	75	92.1	+	148.9	42
16.1	66	57.7	76.3		68	80
16.3	252.6	68.7	116.14		195.7	36

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The clinical manifestations of IgG subclass deficiency

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Introduction

Many immunological mechanisms have been clarified by the study of patients with inborn or acquired errors of immunological function. Bruton's classic paper (1) described patients with agammaglobulinaemia, correctly linked their clinical presentation of frequent infection to their immune deficiency, and finally showed that treating them with replacement immunoglobulin protected them from recurrent infection.

When IgG subclasses were first discovered (2) and further characterized (3) in the early 1960s, it was not unreasonable to expect that patients with an inborn error of IgG subclass production would provide the clues to the biological consequences of a deficiency of one or more of the subclasses. The paper by Schur et al. (4) was one of the first to link a clinical syndrome with selective IgG subclass deficiency, and described three patients with recurrent bacterial infection. Since then many groups have attempted to characterize the clinical manifestation of this group of disorders and despite the 18 years that have elapsed, the clinical presentation of this group of patients remains ill defined. The field has been further complicated by the description of individuals lacking IgG subclasses who remain entirely asymptomatic (5).

We have analysed retrospectively the case notes of patients attending the Hospital for Sick Children, Great Ormond Street, London, who have been investigated and found to have an IgG subclass deficiency.

Methods

Patient selection

The Hospital for Sick Children, Great Ormond Street is a tertiary paediatric referral centre and thus evaluates a highly selected population of children. The Department

of Immunology consults on patients in the Hospital as well as having an outpatient department where 150–200 new patients are seen each year. These are patients generally referred from other paediatricians and most have suspected immuno-deficiency or allergy. IgG subclasses have been measured in the department since May 1985 and included in this study were all patients over the age of six months whose IgG subclasses were found to be low at presentation in the period up to and including December 1987, and whose clinical case notes were available for analysis. Patients with a known immunodeficiency syndrome, e.g. Wiskott Aldrich, X-linked agamma-globulinaemia and severe combined immunodeficiency have all been excluded. Deficiency of a subclass was defined as a level more than two standard deviations below the age matched mean. The normal ranges used were developed in the Department using sera from age matched elective surgical cases and healthy schoolchildren, with at least 20 individuals in each two year age band.

Measurement of immunoglobulins

Serum IgG subclasses were determined by a single radial immunodiffusion technique using monoclonal antibodies (Unipath, Bedford, UK): JL512 (anti IgG1), GOM1 (anti IgG2), ZG4 (anti IgG3) and combined RJ44 and GB7B (anti IgG4). The monoclonal antibodies were used at concentrations of $7 \mu l/ml$, $10 \mu l/ml$, $1 \mu l/ml$ and $6 \mu l/ml$ respectively in 0.1 M barbitone buffer pH 8.6 containing 7% PEG 4000 and 1% agarose. Test samples were applied into 2 mm wells and allowed to diffuse at 4°C for 72 h and the precipitation zones then read. A reference serum calibrated against a commercial standard was used with each assay. The inter-assay variation was a maximum of 2.5% and intra-assay variation was a maximum of 6%. Total IgG, IgA and IgM were measured on a centrifugal fast analyser employing monoclonal antibodies (Atlantic, Scarborough, Maine, USA). An age matched normal range developed as described above was used, and a deficiency was described as a level more than two standard deviations below the age matched mean.

Results

Patterns of IgG subclass deficiency

Fig. 1 shows the distribution of subclass deficiency in the patients studied. The most common deficiency encountered was an isolated IgG4 deficiency (23%) followed by isolated IgG3 (12%) and isolated IgG1 (11%). The most common combination of deficiencies was the association between IgG2 and IgG4 seen in 11% of the patients. The overall ratio of males to females in the total patient population was 1.5:1 and this held true for each individual subclass deficiency as well. There was no significant change in this ratio in the older compared to the younger age groups.

There were similar numbers of children in each age group yet differences were found in the frequency of subclass deficiency encountered in the different age groups (Fig. 2). The proportion of patients presenting under the age of six with an IgG3 deficiency was much lower than those in the same age group with an IgG2, IgG1 and especially an IgG4 deficiency. Over the age of six, IgG3 deficiency assumed a greater importance which lasted until adolescence.
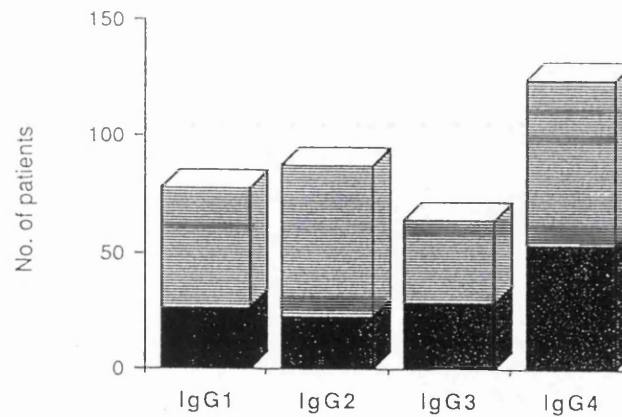
Figure 1. Distribution of IgG subclass deficiencies in 232 selected paediatric patients. Patients presented with either isolated deficiencies (dark stipple) or combined deficiencies (striped).
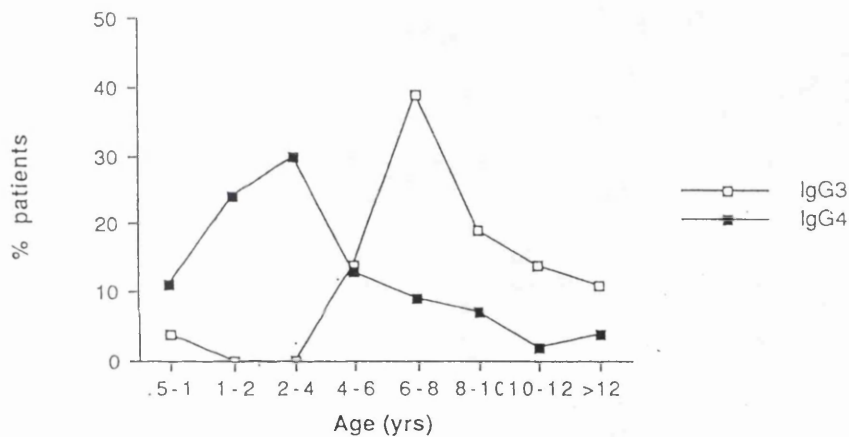

Figure 2. Age at presentation of patients with isolated deficiencies of IgG3 and IgG4, plotted as a percentage of the total number of subclass deficient (isolated or combined) patients in each group.

Of 209 patients who had their total IgG, IgA and IgM measured, 49 (24%) had an associated IgA deficiency. In 21 of these patients there was an associated IgG2/IgG4 deficiency (either alone or in combination). Table 1 shows a further breakdown of this group according to subclass deficiency. IgG deficiency was identified in less than 10% of the total group of patients.

Clinical characteristics

The most common clinical presentation of the group as a whole, was that of recurrent or severe infection (Fig. 3). Forty two percent of all the patients had such infections as their presenting problem. A further 20% had allergy as a presenting complaint and a further 12% presented with a combination of allergy and recurrent infection.

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Table 1

Subclass deficient groups associated with
IgA deficiency (n = 49)

	No.
Isolated IgG4	9
Isolated IgG3	7
Combined IgG2 and IgG4	7
Isolated IgG2	5
Combined IgG1 and IgG4	4

The remaining group of patients (26%) was heterogeneous with a wide range of diagnoses. The two largest groups of patients with a diagnosis other than infection or allergy, were those with a seizure disorder ($n = 22$) and patients with autoimmune disease ($n = 8$).
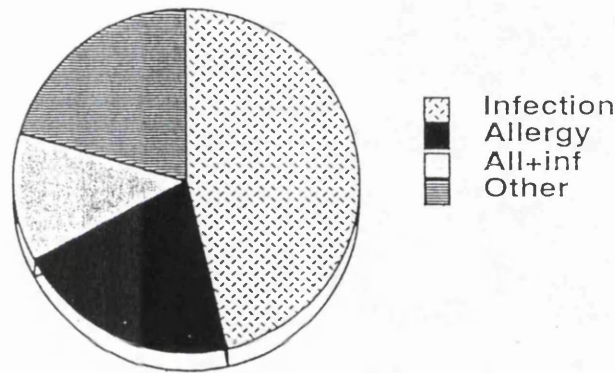
Figure 3. Clinical presentation of 232 patients with IgG subclass deficiencies, illustrating the preponderance of cases presenting with infection and/or allergy.
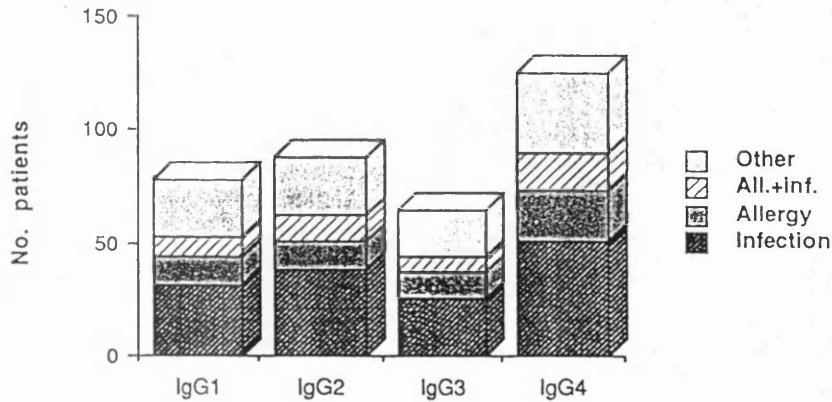
Figure 4. Breakdown of clinical presentation of 232 subclass deficient patients by subclass, illustrating similar proportions of patients with infection and/or allergy within each subclass deficient group.

The clinical manifestations of IgG subclass deficiency

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Table 2

Diagnoses in patients presenting with
recurrent or severe infection (n = 128)

	No.
URTI/LRTI	63
Severe chest infection	17
Septicaemia/Severe infection	15
Skin infection	11
Otitis media	10
Mouth ulcers	7
Sinopulmonary disease	5

Analysis of each individual subclass deficient group revealed no particular deficiency associated with any diagnosis, and the proportion of patients with recurrent infection or allergy in each subclass group reflected the overall trend (Fig. 4).

Of all those patients presenting with recurrent infection the spectrum of disease was broad (Table 2). The majority of patients in the group with recurrent upper and lower respiratory tract infection were children with an average of one infection per month who often required frequent courses of antibiotics. The patients with recurrent severe chest infection usually had significant sputum production and abnormal chest X rays. The patients with septicaemia or severe infection included a group with meningitis or overwhelming sepsis. No specific subclass deficiency was associated with any particular form of recurrent infection, although in the group with overwhelming sepsis, a low level of IgG1 was more common than in the recurrent infection group as a whole.

A total of 22 children had seizures as a major clinical problem. Fourteen of these had an associated IgG2/IgG4 deficiency (alone or in combination) and seven had an associated IgA deficiency.

Discussion

We have observed that the IgG subclass most frequently found to be low is IgG4 and have confirmed the frequent association between IgG2 and IgG4 deficiency. The ratio of males to females remained consistently 1.5:1 and we found no tendency for this to reverse in adolescence. While we had similar numbers of patients in each age group, the distribution of subclass deficiencies did not remain constant. Our finding that IgG4 deficiency presents more commonly below the age of six and that the incidence falls in the older age groups would be consistent with what we understand of the maturation of the immune system, and in particular, the maturation of IgG subclass production (6). However we found that IgG3 deficiency is apparently unimportant below the age of six and much more common in older children. The maturation of IgG3 is thought to occur earlier than IgG4 and hence a different explanation needs to be sought for this observation. It is possible that this group of children represents those who when entering school at the age of 5 or 6, encounter for the first time a spectrum of infectious agents which unmasks this pre-existing immunological defect.

It has been established that the genes encoding the heavy chain of human IgG and IgA are closely linked on chromosome 14 (7), and deficiencies involving both IgA and IgG subclasses have been reported. We have confirmed this association but unlike previous reports selectively linking IgG2/IgG4 deficiency with IgA deficiency (8), we have found that all four subclasses may be involved.

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In common with others (9,10), we have observed the association between IgG subclass deficiency and frequent or severe infection. However, because of the highly selected nature of our patient group we can conclude little about the frequency of IgG subclass deficiency in association with recurrent infection. Others (5) have described patients with absent or deficient subclasses who are apparently healthy and it remains for population based studies to define the frequency of IgG subclass deficiency in the community and the frequency of patients with recurrent infection who have normal IgG subclasses. The group of patients described under the recurrent or severe infection heading, represented a group with a broad spectrum of clinical severity. We have not identified a quantifiable difference in IgG subclasses between those who have severe infection and those with mild, though frequent, infection. Our group with undetectable deficiency were not more severely affected than those who were simply low in a subclass. We believe that more work needs to be done in the area of subclass specific antibody responses to determine why patients with a comparable subclass deficiency and no other abnormality should be so differently affected and what influences compensation for any one subclass deficiency.

Many of our patients with recurrent infection were treated with prophylactic antibiotics (cotrimoxazole once daily) as a first line treatment and appeared to improve. A minority of patients were identified who did not respond to antibiotic prophylaxis and who had, in addition, evidence of end organ damage (especially lung). This group required intravenous immunoglobulin replacement and in general showed a good response to treatment with a reduced frequency of infection.

The association between epilepsy and subclass deficiency has been previously described (11) and intravenous immunoglobulin has been suggested as a form of therapy. We found that 10% of our total group had seizures although three of these had recurrent infection and a further seven had a diagnosis of food allergy. The role of IgG subclass deficiency in epilepsy is not clear and needs further investigation.

In conclusion, we feel that for the data presented here to be viewed in proper perspective, more information will be required on the incidence of IgG subclass deficiencies in the paediatric population as a whole. What proportion of healthy children have low levels of IgG subclasses and what proportion of children presenting with the clinical manifestations we have described have normal levels of IgG subclasses? We have observed the variation of subclass levels in an individual with time and believe that for a meaningful assessment of a subclass deficiency no single measurement should suffice, and a subclass should be documented to be low on at least two occasions three months apart. Furthermore we have shown that in 90% of cases an IgG subclass deficiency is associated with a normal total level of IgG and hence total immunoglobulin levels are inadequate as a screening procedure for subclass deficiencies. In an effort to explain why patients with a similar quantitative defect of single or combined subclasses are so differently affected, assessment of subclass specific antibody produced in response to a specific protein or carbohydrate antigen needs to be explored. Finally we believe that prophylactic antibiotics are an important form of therapy for patients with recurrent infection and can be used profitably. However a small group of patients do not respond to this form of therapy and need intravenous immunoglobulin. This group of patients needs further characterization.

Acknowledgments

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Discussion

Dr Ferrante *(Adelaide):* Did the IgA deficient patients have undetectable levels of IgA?

Dr Goldblatt: All patients who had a level more than 2 SD below the mean were included.

Dr Ferrante: Is there a difference in IgA subclass deficiency in patients with values close to -2 SD of the range and those in whom it was undetectable? From our own experience there appears to be some association between IgG subclass deficiency and IgA which is related more to levels closer to -2SD.

Dr Goldblatt: We do not have enough patients in the IgA group to judge but we do have enough patients in the undetectable IgG subclass group. Again however, we could not correlate with severity of infection or with a different pattern of clinical presentation, so those patients who presented with a persistently undetectable level represented the overall characteristics of the group.

Dr Mitchell *(Dublin):* Do you recommend antibiotic treatment for those patients with subclass deficiency who present with allergic symptoms?

Dr Goldblatt: Prophylactic antibiotics are reserved for patients who have recurrent infection. However, if you suspect that a patient's recurrent attacks of bronchospasm are being triggered by an infection, and the patient also has a subclass deficiency, then there is no reason why prophylactic antibiotics should not be tried.

An enzyme-linked immunosorbent assay for the determination of human IgG subclass antibodies directed against *Branhamella catarrhalis*

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An ELISA procedure to determine the distribution of human IgG subclass antibodies directed against the gram-negative bacterium *Branhamella catarrhalis* has been developed using commercially available monoclonal anti-IgG subclass antibodies. Using whole bacteria as coating antigen the specificity of the assay was determined and showed minimal cross-reactivity with a range of other bacteria.

Estimations of IgG1, IgG2, IgG3, IgG4 and total IgG antibodies directed against this antigen were performed. All normal adult sera tested had measurable antibody levels of specific IgG1, IgG2, IgG3 and total IgG. Specific IgG4 was undetectable in the majority of adult sera. These assays will be of value for investigation of both children and adults with suspected immunodeficiency and recurrent upper respiratory tract infection.

Introduction

Branhamella catarrhalis is a ubiquitous, gram-negative organism that lives in the mouth and is part of the normal oral flora. For many years it has been considered a harmless commensal despite reports dating back to 1927 implicating it in the aetiology of otitis media (Hart, 1927). Sporadic reports over the years have, however, documented it as a cause of meningitis (Pfister et al., 1965), septicaemia (Feigin et al., 1969; Baron et al., 1985), endocarditis (Douer et al., 1977), tracheitis (Wong et al., 1987) and conjunctivitis (Righter et al., 1983). More recently the organism has received attention as an increasingly important cause of otitis media in childhood (Van Hare et al., 1987), implicated in lower respiratory tract infections in patients with underlying lung abnormalities (Srinivasan et al., 1981; Mcleod et al., 1983) and as a pathogen in immunocompromised patients (Diamond et al., 1984). Despite isolation of the organism in clinical settings suggestive of infection, its presence as a commensal in the mouth has always complicated the interpretation of a positive identification in cultures from the upper respiratory tract (Editorial Lancet, 1982). In order to overcome this problem several groups have attempted to analyse the serology of patients thought to be infected with *Branhamella catarrhalis*. To this end complement fixation (Brorson et al., 1976), immunodiffusion (Eliasson, 1980), ELISA (Leionen et al., 1981), bactericidal assays (Chapman et al., 1985), and immunofluorescent anti-

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body techniques (Black et al., 1988) have all been used to determine total specific IgG. However, the reports disagree as to the presence of antibodies in the general population. In addition, most studies have not adequately demonstrated the specificity and the degree of cross-reaction in their techniques. We report here the development of assays for both total IgG and IgG subclass antibodies directed against *Branhamella catarrhalis*. We envisage that the assays will be of value in the investigation of patients, particularly children, with recurrent respiratory tract infections.

Materials and methods

Reagents

The sources of materials used in this work were as follows: bovine serum albumin (BSA), methyl glyoxal and poly-L-lysine were obtained from the Sigma Chemical Company (St. Louis, MO), and *o*-phenylenediamine dihydrochloride (OPD) was obtained from Sigma Chemical Company (Dorset, U.K.). Biotinylated monoclonal mouse anti-human IgG subclass and anti-total IgG antibodies were obtained from Zymed Laboratories (San Francisco, CA) and were of the following clones: IgG1 clone HP 6069, IgG2 clone HP6002, IgG3 clone HP6047, IgG4 clone HP6025 and total IgG clone HP6045. Peroxidase-conjugated streptavidin was also obtained from Zymed Laboratories (San Francisco, CA).

Antigen

A whole cell suspension of *Branhamella catarrhalis* was used as coating antigen and was prepared as follows: a reference isolate (code no. NC 11020) was obtained from the National Collection of Type Cultures, Colindale, London. The organism was grown on nutrient agar and subsequently stored on horse blood agar slopes. Prior to overnight culture the organism was plated out on horse blood agar and colony purity was checked after 18 h. A colony scraping was then emulsified in 2 ml of broth and then this suspension was inoculated into Bactec aerobic culture vials (Becton Dickinson, Maryland, U.S.A.). Following an 18 h culture at 37°C with agitation the culture medium was centrifuged at $10,000 \times g$ for 15 min

at 4°C. The bacterial pellet was recovered and washed twice by spinning at 2500 rpm and resuspended in phosphate-buffered saline pH 7.4 (PBS). After washing, the bacterial pellet was finally resuspended to an optical density of 0.9 at 540 nm. This suspension was then referred to as whole cell antigen.

Buffers for ELISA

The buffers used in the ELISA were prepared as follows: the coating buffer was 100 mM sodium carbonate-bicarbonate buffer (pH 9.6); the wash buffer was phosphate-buffered saline (pH 7.4) containing 0.05% Tween 20 (PBS-T); the diluent buffer for serum antibodies and peroxidase conjugated streptavidin was PBS-T containing 1% BSA.

Serum standard

Clotted blood from 60 apparently healthy adults was collected at a single sitting, then centrifuged and the serum pooled in equal amounts, aliquoted and stored at −70°C. This pool was referred to as standard serum and was included in each assay. The levels of antibody in an unknown serum were extrapolated from a standard curve derived from this pooled serum, and expressed as a percentage of the bound standard. An adult range was obtained by analysis of the serum of forty apparently healthy laboratory and hospital workers.

Solid-phase ELISA for the estimation of specific antibodies to Branhamella catarrhalis

Immulon no. 2 flat bottomed ELISA plates (Dynatech, Virginia, U.S.A.) were coated with 100 μl/well of a 1/4 dilution of whole cell antigen in carbonate buffer and left for 24 h at room temperature (or 48 h at 4°C) before the coating mixture was discarded and the plates allowed to air dry. The plates were then used immediately or stored wrapped in polythene and aluminium foil at 4°C for a period of up to 3 months. Aliquots of the test sera and standard serum diluted in 1% BSA in PBS-T were added to the wells (80 μl/well) and the plate incubated for 2 h at room temperature (18–25°C). The serum was then discarded and the plate washed four times in wash buffer. Biotinylated antibodies were then diluted as follows in 1% BSA PBS-T: IgG1 1/500; IgG2 1/1000; IgG3

1/5000; IgG4 1/500 and IgG total 1/2000. The wells were filled with 80 μl of the antibody solution and the plates incubated for a further 2 h at room temperature. The plates were then washed four times in wash buffer following which 80 μl of a 1/50,000 solution of peroxidase-conjugated streptavidin were added. After standing at room temperature for 1 h plates were washed four times in wash buffer before the addition of 80 μl/well of substrate solution containing 0.5 mg/ml of OPD in a solution of 0.05% H_2O_2/0.1 M citric acid/0.2 M Na_2HPO_4. The colour reaction was stopped after 10 min with 4 N H_2SO_4 (40 μl/well) following which the optical densities were measured at 492 nm using an ELISA reader (Titertek multiskan, Flow). A range of dilutions of the standard serum (1/40–1/20,480 depending on the assay) was included on each plate and the results of the unknown sera expressed as a percentage of this internal standard.

Results

Antigen binding to solid phase

Several methods were compared for the binding of whole *Branhamella catarrhalis* to the solid phase. These included diluting the antigen suspension in methyl glyoxal or poly-L-lysine prior to coating the plates as described by Czerkinsky et al. (1983). Neither method showed any advantage over the use of carbonate-bicarbonate buffer. Both live and formaldehyde treated bacterial preparations were used to coat the plates and the use of live bacteria showed no advantage over killed bacteria (results not shown).

Detector antibody binding

Optimal dilutions of the detector monoclonal antibodies were achieved when binding resulted in optical densities within a similar range. Within each isotype specific ELISA the dilution curves for test sera were all approximately parallel both to each other and to the standard curve (data not shown). No attempt was made to quantify gravimetrically the amounts of specific antibody binding to the solid phase. Values for unknown sera were expressed as a percentage of the standard serum bound at the same dilution. Values for
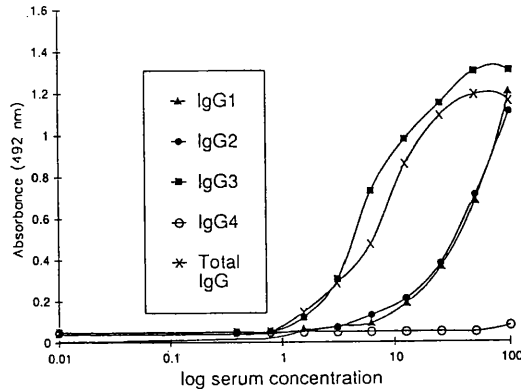

Fig. 1. ELISA procedure for the detection of anti-*Branhamella catarrhalis* antibodies. Standard curves obtained from the binding of standard serum (doubling dilutions ranging from 1/40 to 1/10,240). Specific antibodies were detected using mouse anti-human IgG subclass monoclonals (see key).

unknown sera were rejected if their absorbance was less than four times the background absorbance. Background absorbance was generally low except for the IgG2 assay. The standard curves for IgG1, IgG2, IgG3, IgG4 and total IgG are shown in Fig. 1. It was not possible to detect bound IgG4 in the standard serum.

Assay specificity

To determine the specificity of binding of IgG to antigen on the solid phase a suspension of live *Branhamella catarrhalis* was added in increasing concentrations to a constant dilution of standard serum (40 μl) in each well. As shown in Fig. 2 there was complete inhibition of binding of specific IgG3 to the solid phase and partial inhibition of IgG1, IgG2 and total IgG.

In order to determine whether binding to the solid phase was due to cross-reacting antibodies, aliquots of standard serum were adsorbed individually with a variety of bacteria by incubating the serum and bacteria together at 37°C for 2 h and then spinning at 10,000 × g to remove the bacteria. The bacteria used for adsorption included *Neisseriae meningitidis* group B, a nontypable *Haemophilus influenza*, *Pasteurella multocida*, *Escherichia coli*, *Staphylococcus aureus* and *Branhamella catarrhalis*. As shown in Fig. 3 there was little inhibition of IgG3 and total IgG binding to the solid
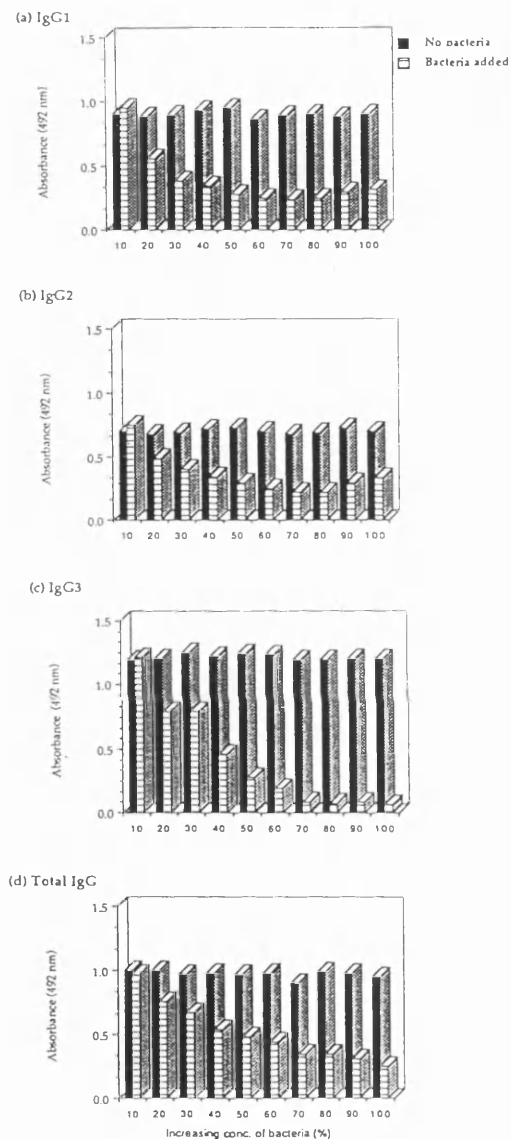
Fig. 2. Inhibition of binding of specific anti-*Branhamella catarrhalis* antibody to the solid phase. A suspension of live *Branhamella catarrhalis* was added in increasing concentrations to 40 μl of a pooled standard serum at dilutions of 1/40 (IgG1, IgG2) or 1/80 (IgG3 and total IgG). Cross-hatched bars represent the absorbance readings from wells to which bacteria were added and solid bars represent values for wells with serum alone.

TABLE I

INTERASSAY VARIATION FOR IgG1, IgG2, IgG3 AND TOTAL IgG ANTIBODIES TO *BRANHAMELLA CATARRHALIS* AS MEASURED BY ELISA (SEE TEXT FOR DETAILS)

	Coefficient of variation	
	High level serum	Low level serum
IgG1	6.28	10.08
IgG2	5.73	8.78
IgG3	5.59	6.7
Total IgG	6.63	14.7

phase in sera adsorbed with bacteria other than *Branhamella catarrhalis*. The IgG3 assay again showed maximal inhibition exclusively by *Branhamella catarrhalis*. The IgG2 assay (Fig. 3*b*) showed some degree of cross-reaction with *Pasteurella multocida* and *Neisseriae meningitidis* group B.

Assay variation

Interassay variation was measured by assaying a known high titre and a known low titre serum in addition to the standard serum on the same batch of antigen coated plates on four different days. Results are expressed as the coefficient of variation for both the high and the low titre serum and results are shown in Table I

Intra-assay variation was measured by assaying a known high titre and a known low titre serum in multiple replicates on the same plate together with a dilution curve for the standard serum. This same experiment was repeated on three consecutive days and the results are expressed as the mean and standard deviation of the coefficient of variation obtained from the replicates over the three days.

TABLE II

INTRA-ASSAY VARIATION FOR IgG1, IgG2, IgG3 AND TOTAL IgG ANTIBODIES TO *BRANHAMELLA CATARRHALIS* AS MEASURED BY ELISA (SEE TEXT FOR DETAILS)

	Coefficient of variation			
	High level serum		Low level serum	
	Mean	SD	Mean	SD
IgG1	10.78	3.12	12.14	2.79
IgG2	11.72	2.08	14.35	4.25
IgG3	3.35	1.99	5.28	0.94
Total IgG	3.64	0.55	6.63	2.6

(a) IgG1

(b) IgG2

(c) IgG3

(d) Total IgG

Legend:
— Std   ▲ Past.   ✶ HI non b   ▲ Staph
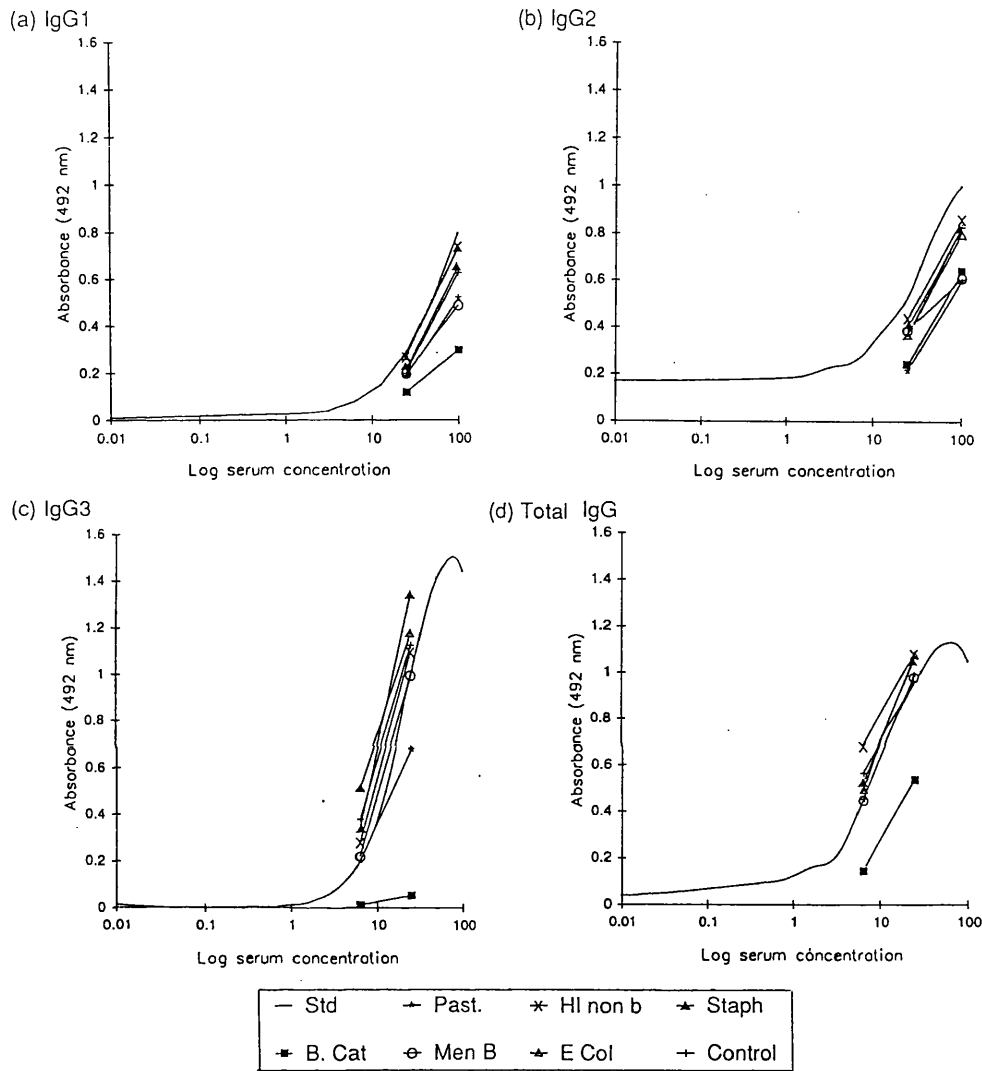▪ B. Cat   ⊕ Men B   ▲ E Col   ✛ Control

Fig. 3. *a – d*: specificity of the binding of anti-*Branhamella catarrhalis* antibody to the solid phase. Standard serum was adsorbed with live *Branhamella catarrhalis* or a variety of other live bacteria (Std: standard curve; B. Cat: *Branhamella catarrhalis*; Past.: *Pasteurella multocida*; Men B.: *Neisseria meningitidis* group B; HI non b: *Haemophilus influenza* non type b; E Col: *Escherichia coli*; Staph: *Staphylococcus aureus*). Curves obtained for the binding of specific anti-*Branhamella catarrhalis* antibody of the IgG1, IgG2 and IgG3 subclasses and total IgG were then detected by ELISA.

Table II shows that while the intra-assay variation differed for the four assays it was acceptably low overall.

Adult antibody range

Sera from 40 healthy adults were assayed to establish the range of naturally occurring specific ·
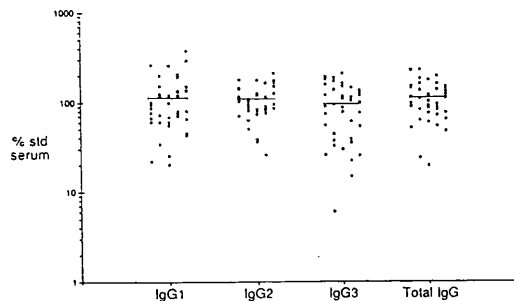
Fig. 4. Range for anti-*Branhamella catarrhalis* antibodies detected in the sera of 40 apparently healthy adults. The mean value for each subclass and for total IgG is indicated by a horizontal bar.

anti-*Branhamella catarrhalis* antibodies present in adult sera. All sera had detectable IgG specific for *Branhamella catarrhalis* although the subclass pattern differed from individual to individual. The binding activity for apparently healthy adults is shown in Fig. 4. IgG4 antibodies could only be detected in a small minority (17%) of the sera tested.

Discussion

Branhamella catarrhalis, previously thought to be a harmless commensal, has recently emerged as an important pathogen in childhood and in adults and infants with underlying lung disease. The clinical association between *Branhamella catarrhalis* pneumonia in adults and underlying immunoglobulin abnormalities (Diamond et al., 1984) suggests that humoral immunity is important in preventing infection with this organism. The immunology of *Branhamella catarrhalis* infection has, however, not been well elucidated. Paired sera from patients with maxillary sinusitis of unknown cause were examined by Brorson et al. (1976) who found precipitating antibodies to *Branhamella catarrhalis* in both patients and uninfected controls. Complement fixing antibodies were demonstrated in 25% of patients but in only one control ($n = 20$). No bacteriological data was given for any of the patients and hence a relationship between positive serology and *Branhamella catarrhalis* infection could not be made. More-

over, no data was provided regarding the specificity of the antibodies detected for *Branhamella catarrhalis*. In a later study Leinonen et al. (1981) used a whole cell ELISA to investigate children with otitis media. They found an increase in IgG between acute and convalescent sera in ten of 19 children with *Branhamella catarrhalis* isolated from the middle ear. A significant proportion of children with otitis media, but in whom no *Branhamella catarrhalis* was isolated, also demonstrated specific IgG, although in this group of patients there was no rise in titre in the convalescent sera. In addition they noted that antibody titres were not detected in patients below the age of 10 months but thereafter increased with age. *Branhamella catarrhalis* antigen was prepared by combining ten different strains. Hyperimmune rabbit sera were used as a positive control and no cross-reaction was shown with serum from a rabbit immunised with a mixture of non-typable *Haemophilus influenza*. More recently Chapman et al. (1985) used a bactericidal assay to investigate patients with pneumonia or tracheobronchitis. No bactericidal activity was found in pooled normal human sera against 20 of the 21 bacterial isolates. The patients in whom *Branhamella catarrhalis* was isolated demonstrated bactericidal activity in seven of 19 and 18 of 20 convalescent sera. This bactericidal effect appears to be mediated by IgG and the classical complement pathway. Black and Wilson (1988) used an immunofluorescence antibody test to evaluate IgG antibodies in patients with acute bronchopulmonary infection due to *Branhamella catarrhalis*. 45 adults were studied and convalescent sera showed a higher titre of antibody than acute sera, which in turn showed a higher titre of antibodies than the sera of age- and sex-matched controls, although these two groups overlapped considerably.

Published studies therefore disagree as to the existence of antibody in uninfected individuals and no studies convincingly exhibit specificity and a lack of cross-reaction in their methodology. In addition, no studies have examined the IgG subclass distribution of specific anti-*Branhamella catarrhalis* antibodies.

Reliable binding of *Branhamella catarrhalis* antigen was achieved using a standard carbonate buffer procedure and it was found that the anti-

gen, once bound, was stable for several months when stored in the dark at 4°C. There was parallelism in the binding of the detector antibody used in the IgG1, IgG2 and IgG3 subclass assays and the optical densities obtained were in roughly the same range. The assays described were shown to be specific for *Branhamella catarrhalis* and antibody binding was not due to cross-reactive antibodies. Inter- and intra-assay variation was found to be acceptably low for all the assays. All adult sera tested had demonstrable levels of *Branhamella catarrhalis*-specific IgG1, IgG2, IgG3 and total IgG although only a minority had detectable IgG4.

Since *Branhamella catarrhalis* is an organism found in the upper respiratory tract of humans it is likely that its presence provides ongoing antigenic stimulation to the immune system, and this explains the presence of antibodies in all of the adults tested.

Because of the emergence of this organism as an increasingly recognised pathogen, it is important to achieve an understanding of the immune response associated with it. The IgG subclass-specific response to infection is important since certain antigens induce a subclass restricted response (Yount et al., 1968). The measurement of total IgG may be insufficient in assessing the immune response to this organism since the ability to mount a particular subclass response may be necessary for protection.

Acknowledgements

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Branhamella cattarhalis: Antigenic Determinants and the Development of the IgG Subclass Response in Childhood

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A recently developed whole cell ELISA was used to investigate the development of IgG subclass antibodies to *Branhamella catarrhalis* in childhood. In addition, SDS-PAGE and immunoblotting were used to study the interaction between the outer membrane proteins (OMPs) of *B. catarrhalis* and IgG subclass antibodies. Specific IgG3 antibodies were undetectable or present only in low amounts in children <4 years old but were an important constituent of the response of older children. OMPs prepared from different isolates had similar molecular masses and bound IgG with identical immunoblotting patterns. Binding appeared subclass-specific, with IgG3 binding to the broadest range of OMPs. These findings should provide a better understanding of the pathogenic role of this organism and suggest possible strategies for the development of a vaccine.

Branhamella catarrhalis is a gram-negative diplococcus commonly found in the upper respiratory tract. Over the past 10 years it has been increasingly recognized as a major pathogen in childhood otitis media, and it is the third most commonly isolated bacterium in both acute and chronic otitis media [1]. In addition, there are isolated reports of a causal role for *B. catarrhalis* in a variety of diseases including septicemia [2], endocarditis [3], meningitis [4], urethritis [5], mastoiditis [6], and tracheitis [7]. While generally not causing disease in healthy adults, it may be a significant pathogen in adults who are immunocompromised [8] or who have underlying lung disease [9]. The occurrence of *B. catarrhalis* infection in children and immunocompromised adults suggests that an acquired immune response may be necessary to protect healthy adults from infection with this organism and that children are susceptible to serious infections before the development of such immunity.

To define the humoral immune response to *B. catarrhalis*, we developed an ELISA to measure specific IgG antibody responses to the whole organism. Previously we have shown that all healthy adults have demonstrable levels of specific anti-*B. catarrhalis* antibody of the IgG1, IgG2, and IgG3 isotypes and that IgG3 antibody appears to be of high affinity [10]. For this report, we investigated the age-related development of the IgG subclass response and, by SDS-PAGE and immunoblotting, examined the interaction between the outer membrane proteins (OMPs) of *B. catarrhalis* and naturally occurring human IgG subclass antibodies.

Materials and Methods

Bacteria

A reference isolate of *B. catarrhalis* (NC 11020) was obtained from the National Collection of Type Cultures (London). Two clinical isolates were obtained from the microbiology laboratory at the Hospitals for Sick Children, Great Ormond Street (London). The identity of the clinical isolates was confirmed by morphology, Gram's staining, catalase production, oxidase reduction, the ability to reduce nitrate, and the inability to produce acid from glucose, maltose, sucrose, or lactose. The organisms were stored on blood agar slopes and before use were plated out on horse blood agar and incubated at 37°C. After an 18-h incubation, colony purity was checked. A colony scraping was emulsified in 2 ml of broth, and this suspension was inoculated into Bactec aerobic culture vials (Becton Dickinson, Oxnard, CA). After an 18-h incubation at 37°C with agitation, the culture medium was centrifuged at 10,000 g for 15 min at 4°C.

The bacterial pellet was recovered and washed twice by resuspending in PBS, pH 7.4, and centrifuging at 1000 g. After washing, the pellet was resuspended to an optical density of 0.9 at 540 nm. This suspension, referred to as whole-cell antigen, was used in both ELISA and immunoblotting.

The supernatant was subjected to two further centrifugations at 10,000 g for 20 min at 4°C, after each of which the supernatant was recovered and the pellet discarded, followed by a final centrifugation at 100,000 g for 2 h at 4°C. The supernatant was discarded; the recovered pellet contained vesicles of OMPs. This method of OMP isolation has been evaluated by Murphy and Loeb [11] and shown to produce OMPs free of cytoplasmic contamination and comparable to those isolated by sucrose gradient. The pellet was resuspended in 100 μl of PBS and used in the ELISA or subjected to SDS-PAGE and Western blot analysis.

Sera

Standard serum. Serum derived from the clotted venous blood of 60 apparently healthy medical personnel was pooled, divided into aliquots, and stored at −70°C. This serum was used to construct a standard curve on each ELISA plate. In addition, standard serum

for adsorption experiments was prepared by incubating an equal volume of serum and a bacterial pellet at 37°C for 3 h, after which serum was centrifuged at 10,000 g for 10 min. Recovered serum (adsorbed standard serum) was stored at −70°C.

Pediatric test sera. Clotted venous blood was obtained from 53 children of various ages admitted to the Hospitals for Sick Children, Great Ormond Street, for elective surgical procedures. For all of them, case notes revealed no family history or clinical condition that would affect their immunity. Serum obtained was stored at −70°C until analyzed.

Miscellaneous sera. Serum from a 2½-year-old patient with hypogammaglobulinemia had 0.075 g/l IgG, 0.05 g/l IgA, and elevated IgM of 1.0 g/l.

Detector Antibodies

Biotinylated monoclonal mouse anti-human IgG subclass and antitotal IgG antibodies were used in the ELISA and for immunoblotting (Zymed Laboratories, San Francisco) and were of the following clones: IgG1, HP 6069; IgG2, HP6002; IgG3, HP6047; IgG4, HP6025; and total IgG, HP6045. Additional monoclonal mouse anti-human IgG subclass antibodies used for immunoblotting (Oxoid/Unipath, Basingstoke, UK) were of the following clones: IgG1, JL 512; IgG2, GOM1; IgG3, ZG4; and IgG4, RJ4. Iodinated sheep anti-mouse IgG, sheep anti-human IgG, and streptavidin were obtained from Amersham International (Aylesbury, UK). A 3% (wt/vol) solution of skim milk in PBS containing 0.02% sodium azide (milk-PBS) was used to dilute all antibodies and for all washing steps. Nitrocellulose was autoradiographed using Kodak XAR-5 film and Cronex (Du Pont, Poole, UK) intensifying screens.

ELISA

The ELISA procedure used to measure antibodies to *B. catarrhalis* was developed in our laboratory and has been described in detail elsewhere [10]. Briefly, flat-bottomed ELISA plates (Immulon 2; Dynatech, Alexandria, VA) were coated with 100 μl of a 1:4 dilution of whole-cell antigen or a 1:60 dilution of OMPs in PBS in carbonate-bicarbonate buffer (pH 9.6) for 24 h at room temperature. Serial dilutions of the pooled standard and test sera were diluted in 1% bovine serum albumin (BSA) in PBS containing 0.05% Tween 20 (PBS-T) and added to the wells (80 μl/well) in duplicate. After a 2-h incubation at 37°C, the serum was discarded and the plates washed four times in wash buffer.

Biotinylated antibodies diluted in 1% BSA with PBS-T (IgG1, 1:500; IgG2, 1:1000; IgG3, 1:5000; IgG4, 1:500; and total IgG, 1:2000) were then added to the wells and the plates incubated for a further 2 h at room temperature (18–25°C). The plates were washed four times in wash buffer, a 1:50,000 dilution of peroxidase-conjugated streptavidin (Zymed) was added, and the plates were allowed to stand at room temperature for 1 h and washed four times in wash buffer. Substrate solution containing 0.5 mg/ml *o*-phenylenediamine dihydrochloride (Sigma Chemical, Poole, UK) in a solution of 0.05% H_2O_2, 0.1 M citric acid, and 0.2 M Na_2HPO_4 was then added. The color reaction was stopped after 10 min with 4 N H_2SO_4, after which the optical densities were measured at 492 nm using an ELISA reader (Titertek Multiskan; Flow ICN, Rickmansworth, UK).

A range of dilutions of the standard serum (1:40–1:20,480 depending

on the assay) was included in duplicate on each plate, and the mean absorbance of the dilutions was used to construct a standard curve. The results of the unknown sera were determined from this standard curve and expressed as a percentage of the internal standard. Within each isotype-specific assay the dilution curves for the test sera were all about parallel, both to each other and to the standard curve.

Assay specificity was determined by comparing the binding of serum to the solid phase after adsorption of the sera with a variety of bacteria for 3 h at 37°C. The bacteria studied included *Pasteurella multocida*, *Neisseria meningitidis* group B, *Escherichia coli*, *Staphylococcus aureus*, and a nontypable *Haemophilus influenzae*. Inter- and intraassay variation was determined by the estimation of antibody levels in both a high- and a low-level serum. The variation between plates over 3 consecutive days and between several plates on the same day was evaluated. In addition, levels of specific antibody determined in assays using the reference isolate of *B. catarrhalis* were compared using different isolates of *B. catarrhalis* or an OMP preparation as capture.

SDS-PAGE and Western Blotting

SDS-PAGE was done essentially as described by Laemmli [12]. Samples of both bacterial whole cell antigen and supernatant pellet were applied after boiling for 5 min in SDS-PAGE reducing sample buffer. Gels were calibrated with the following Coomassie brilliant blue-stained molecular mass standards (Sigma; subunit molecular masses in kilodaltons are in parentheses): β-galactosidase (116), phosphorylase B (97.4), transferrin (78), BSA (68), ovalbumin (45), glucose-3-phosphate dehydrogenase (36), carbonic anhydrase (29), trypsinogen (24), and lysozyme (14.3). After electrophoresis the gels were stained with Coomassie brilliant blue or silver stain as described by Morrissey [13].

Western blotting on nitrocellulose after electrotransfer was done as described by Towbin et al. [14]. Briefly, after transfer nonspecific binding sites on the nitrocellulose strips were blocked by incubation for 1 h at room temperature in milk-PBS. The blocking solution was then discarded and the strips incubated in serum diluted in milk-PBS for 3 h with agitation at room temperature. After washing with milk-PBS, the strips were incubated with mouse anti-human IgG (total or subclass-specific) antibodies diluted in milk-PBS with continuous agitation for 2 h at room temperature. The strips were then washed and incubated with either ^{125}I-labeled sheep anti-mouse IgG or ^{125}I-labeled streptavidin (for biotinylated detector antibodies) for 2 h at room temperature. Finally the strips were washed in PBS containing 0.02% sodium azide and autoradiographed. The molecular masses of the radioactive bands were determined by comparison with Coomassie brilliant blue-stained markers run on the same gel.

Results

ELISA. Evaluation of our ELISA procedure revealed no cross-reaction with a variety of bacteria in the IgG3 and total IgG assays, although some cross-reaction was noted in the IgG1 and IgG2 assays. Prior incubation of the serum with live *B. catarrhalis* completely abolished specific IgG3 binding to the solid phase and partly abolished total IgG, IgG1, and IgG2

binding [10]. Inter- and intraassay variations as determined by estimation of the antibody levels in high- and low-level sera within the same plate and between plates on successive days were <15% for all assays described [10]. Binding of the standard serum to the solid phase was similar irrespective of the source of antigen used on the solid phase.

Figure 1 shows standard curves obtained for the binding of specific IgG1, IgG2, and IgG3 and total IgG binding to a reference isolate of *B. catarrhalis*, two clinical isolates of *B. catarrhalis*, and an OMP preparation obtained from the culture supernatant of the reference isolate. Different amounts of coating antigen resulted in minor variability in the standard curves, although overall the curves obtained showed parallelism and gave similar IgG subclass profiles. In addition, specific antibody levels in any given test sera were similar regardless of the source of capture antigen. Furthermore, serum adsorbed with the reference organism reduced (IgG1, IgG2, and total IgG) or completely abrogated (IgG3) the binding of the antibody to the solid phase irrespective of the source of capture antigen and the type of antigen (whole cell vs. OMP). The unique nature of the monoclonal reagents used

in assays of this type precludes comparisons between subclasses as regards gravimetric quantitation.

Figure 2 shows the age range for specific anti-*B. catarrhalis* total IgG and IgG1, IgG2, and IgG3. IgG4 could be detected in only a few children (13%). Antibodies of the IgG1 and IgG2 isotype were detected in most younger infants, and those of the IgG1 isotype rose to adult levels (i.e., close to 100% of the standard serum) between the ages of 2 and 3. In contrast, levels of IgG3 antibodies were <5% of that of the pooled standard in patients <4 years old but rose rapidly thereafter.

SDS-PAGE and Western blotting. Figure 3 shows the bands obtained on SDS-PAGE from the OMPs of the reference isolate and two clinical isolates after Coomassie brilliant blue staining, and figure 4 shows the OMPs of the reference organism after silver staining. The patterns of OMPs obtained after Coomassie brilliant blue staining were similar for the three isolates tested. Silver staining revealed bands that were invisible or only poorly seen on Coomassie brilliant blue staining, and these were subsequently observed to bind antibodies on immunoblotting.

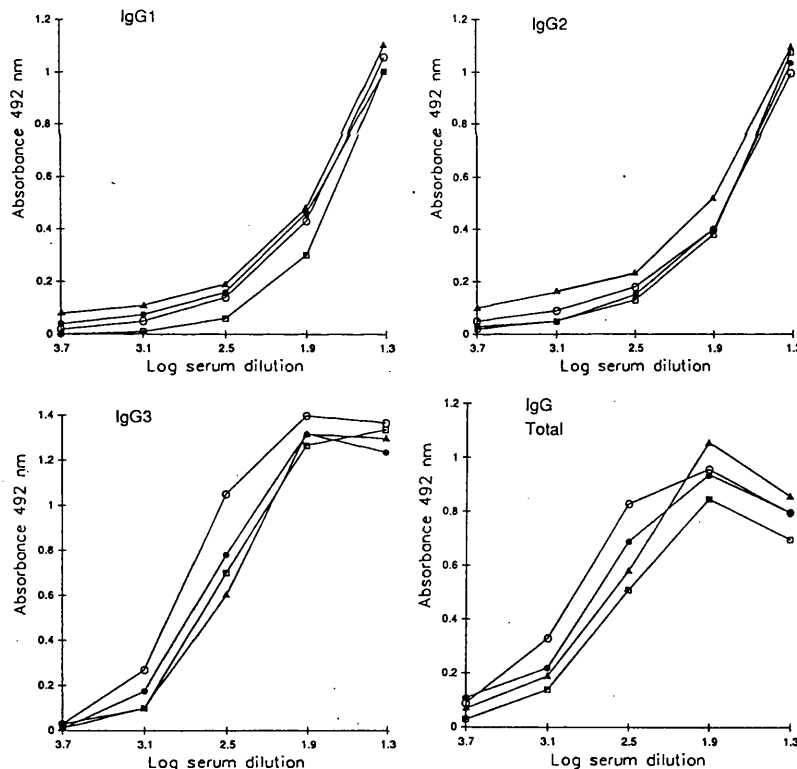As illustrated in figure 5, IgG subclass binding differed for

Figure 1. Comparison of different coating antigens for anti-*Branhamella catarrhalis* isotype-specific ELISA: whole-cell *B. catarrhalis* antigen derived from reference organism (O) or two clinical isolates (▲, ●) or outer membrane protein preparation (□) derived from culture supernatant of reference organism. Binding of standard serum was detected using mouse anti-human IgG monoclonal antibodies.
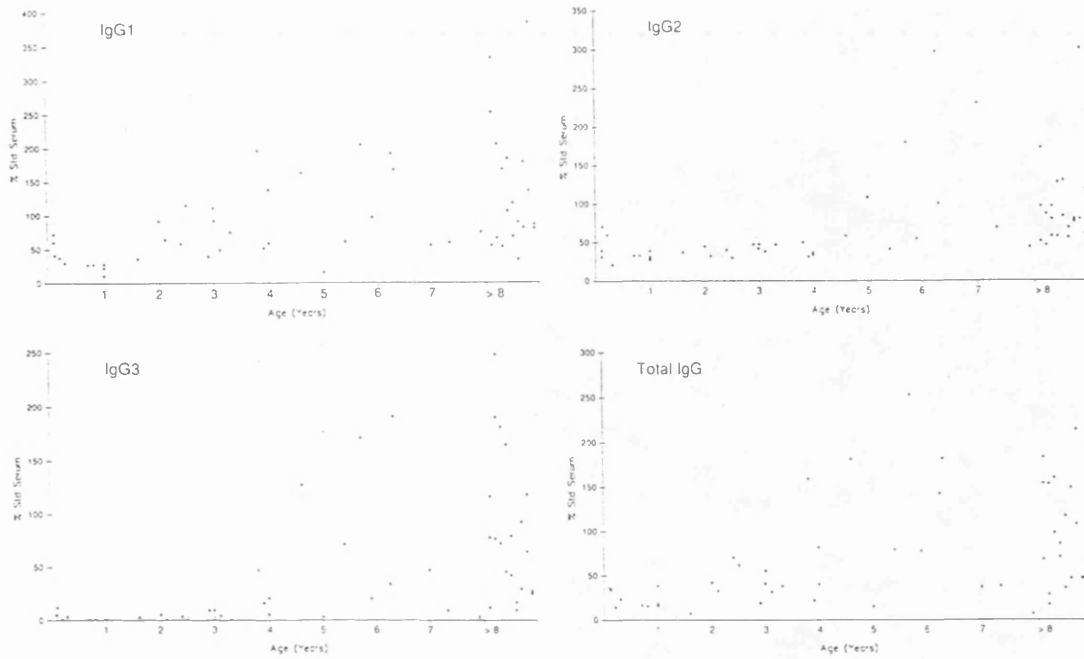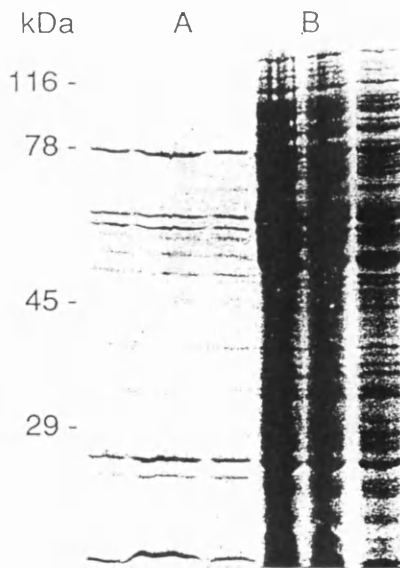
Figure 2. Range of age-related naturally occurring anti-*Branhamella catarrhalis* antibodies in children undergoing elective surgical procedures. Each star represents individual child ($n = 53$) and levels, determined by ELISA using whole-cell antigen as capture, are expressed as percentage of level of antibody found in pooled standard prepared from 60 apparently healthy adults.

Figure 3. Coomassie brilliant blue–stained 10% SDS-polyacrylamide gel after electrophoresis of outer membrane protein preparations (A) and whole bacterial antigen (B) of reference isolate and two clinical isolates of *Branhamella catarrhalis*. Molecular mass standards are indicated at left.

OMPs of differing molecular masses, although the recognition of any given protein appeared to be consistent regardless of the source of the bacterial isolate. This binding was shown to be specific for most OMPs by its disappearance on prior adsorption of the serum with live *B. catarrhalis*. However, in the case of IgG2, the ability of antibody to bind to a 50-kDa OMP was only partly abrogated after adsorption although cross-reactivity with other organisms (previously noted in ELISA) could account for this. Moreover, serum adsorbed with a reference organism inhibited binding on immunoblotting to OMPs derived from the two clinical isolates, suggesting that antibodies to *B. catarrhalis* may not be strain-specific (see figure 6).

Serum from a patient with hypogammaglobulinemia or from patients with low or absent levels of specific antibodies on

Figure 4. Silver-stained 10% SDS-polyacrylamide gel showing outer membrane proteins of reference isolate of *Branhamella catarrhalis*. Molecular mass standards indicated at left.

ELISA showed no binding to OMPs on immunoblotting (data not shown), while serum from different adults known to be immune showed minor variations in binding patterns. IgG1, IgG2, and IgG4 binding was not prominent at the concentration of detector antibodies used on immunoblotting and generally appeared limited to a few high-molecular-mass proteins, in particular a protein of ~82 kDa that bound all the subclasses. Although this observation could be due to an affinity effect of the detector antibodies, the phenomenon was true for different clones of the detector antibodies.

Discussion

Despite the recognition of *B. catarrhalis* as a pathogen by Sir William Osler in 1919 [15] and its implication in otitis media in 1927 [16], only in the past decade has its role as a pathogen become better defined. After *Streptococcus pneumoniae* and *H. influenzae* it is the third most common organism isolated from the middle ear of children in both acute and chronic otitis media and has been described as pathogenic in a number of other clinical pediatric settings [2, 4, 6, 7, 17, 18]. In contrast, despite its ubiquity in the moist mucosa of the upper respiratory tract, adults are rarely infected unless they have underlying lung disease or are immunocompromised, for example, patients with multiple myelomatosis or leukemia or those receiving immunosuppressive therapy [8]. This suggests that immunity to this organism is acquired
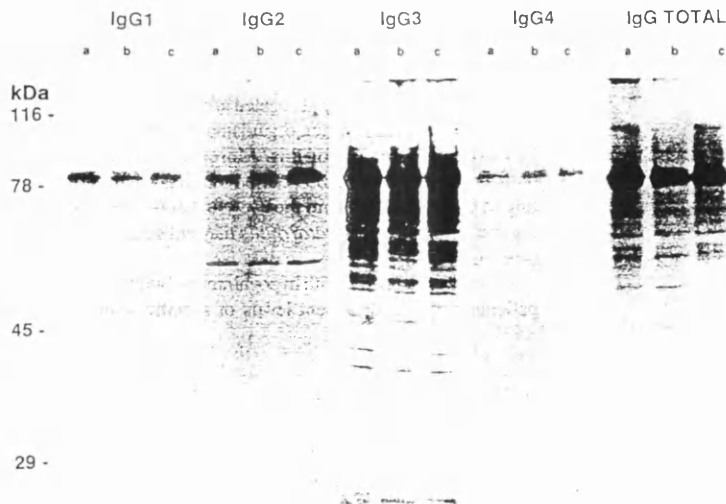

Figure 5. Immunoblots in which outer membrane proteins of (a) reference strain and (b and c) two clinical strains of *Branhamella catarrhalis* were incubated with serum known to contain specific antibodies and then probed. Molecular mass standards are indicated at left.
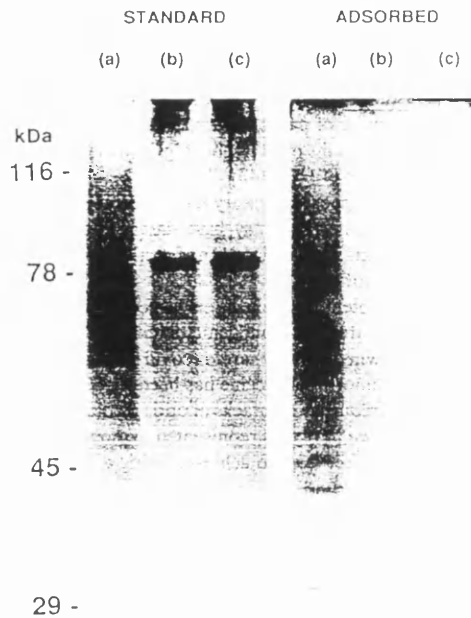
IgG Subclass Responses to *B. catarrhalis*

STANDARD   ADSORBED

(a)   (b)   (c)   (a)   (b)   (c)

Figure 6. Immunoblots in which (a) irrelevant bacterial protein (*Pseudomonas* antigen) and (b and c) OMPs of two clinical isolates were incubated with standard pooled serum or standard pooled serum that had been adsorbed with reference isolate of *Branhamella catarrhalis* and then probed for presence of IgG. Molecular mass standards are indicated at left.

during childhood and that most adults have developed protective immunity.

While potentially important, the immunology of *B. catarrhalis* infection has not been well elucidated. Various methods have been used to assess host responses to *B. catarrhalis*, including complement fixation [19], immunodiffusion [20], ELISA [21], bactericidal assays [22], and immunofluorescent antibody techniques [23]. Leinonen et al. [21] showed an increase in specific IgG between acute and convalescent sera in 9 of 19 children in whom *B. catarrhalis* was isolated from the middle ear. Chapman et al. [22] showed that sera from patients with pneumonia or tracheobronchitis secondary to *B. catarrhalis* was bactericidal for the organism in vitro and that this effect was mediated via complement-fixing antibodies. While establishing that an IgG response is mounted to infection with this organism, studies have disagreed as to the existence of antibody in uninfected individuals and have failed

to demonstrate convincingly either specificity or a lack of cross-reaction in their chosen methodology.

To date, no studies have examined the IgG subclass response induced by exposure to this organism. An understanding of this may be important since the ability to mount an appropriate immune response to a given antigen may be subclass-restricted and age-dependent [24, 25]. Anti-carbohydrate antibodies are characteristic of the IgG2 isotype [26], and an inability to mount an appropriate immune response to carbohydrate antigens in early childhood (<2 years of age) limits the use of unconjugated polysaccharide vaccines (e.g., *H. influenzae* type b) in this age group [27]. In contrast, protein antigens such as tetanus toxoid induce predominantly an IgG1 response with some IgG3 and occasionally IgG4 [28], and this response does not appear to be age-restricted.

While all adults have antibodies to *B. catarrhalis*, the acquisition of antibodies in childhood was found to be age-related. IgG1 and IgG2 antibodies are produced at a younger age than are IgG3 antibodies, which are absent in children <4. The reason for the absence of specific IgG3 antibody during the first few years of life and the apparent "switch" to significant levels of synthesis during the fourth year is unclear, since synthesis of IgG3 begins in the first year of life and by age 2 is already approaching adult levels [29]. Nonexposure to the organism is an unlikely explanation, as nasopharyngeal carriage rates for this organism have been reported in as many as 46% of children (age range, 2 months to 18 years) attending the well-child care and pediatric outpatient department of the Cleveland Metropolitan General Hospital [30]. Furthermore, our demonstration that total specific IgG levels increase from the age of 1 year onward and data showing that all adults have specific anti-*B. catarrhalis* antibody [10] suggest that the constant presence of the organism in the upper respiratory tract provides an ongoing stimulus to antibody production.

The relevance of the specific IgG3 response may well be clarified after further elucidation of the antigenic determinants of this bacterium. While IgG3 antibodies are usually elicited by viral antigens, bacterial proteins, such as the OMPs of nontypable *H. influenzae*, may nevertheless be potently immunogenic for this subclass. Antibodies to the OMPs of nontypable *H. influenzae* have been shown to be protective [31] and belong to the IgG1 and IgG3 subclasses [32]. Our data show that part of the immune response to *B. catarrhalis* is mounted against the OMPs present in the cell wall, and it is possible that the importance of IgG3 in response to this organism is analogous to that described for nontypable *H. influenzae*.

It has been noted that in vitro *B. catarrhalis* acts as a B cell mitogen [33] and appears to stimulate preferentially an IgG3 response as shown by increased intracytoplasmic IgG3 expression [34] and secretion of IgG3 into the culture supernatants [35]. This phenomenon is being investigated using both polyclonal and antigen-specific models.

In common with other gram-negative bacteria [36, 37], *B.*

catarrhalis has OMPs present in the cell wall and secretes vesicles containing OMPs into the culture medium during growth. Murphy and Loeb [11] found that collection of these OMPs from the culture medium yields a preparation representative of the surface proteins that is free of cytoplasmic contamination. In addition, a striking degree of homology was seen in the patterns of OMPs obtained from 50 diverse isolates [38], and after immunization of rabbits, antisera tested against a variety of strains appear to recognize conserved antigenic determinants common to various isolates [39]. The observed homology of OMPs derived from diverse isolates of *B. catarrhalis* contrasts with the heterogeneity of OMPs derived from organisms such as nontypable *H. influenzae*. Furthermore, antibodies directed against the latter have been shown to be bactericidal. It follows that antibodies that recognize strain-specific proteins will provide protective immunity on a strain-specific basis [31].

Our results suggest that the OMPs of *B. catarrhalis* are antigenic for human antibodies and that most not only are conserved between strains, as evidenced by similar patterns after SDS-PAGE and Coomassie brilliant blue staining, but also bind antibodies on immunoblotting in a similar fashion. In addition we have shown similar ELISA antibody binding independent of the source of *B. catarrhalis* used on the solid phase. Also, serum adsorbed with live reference *B. catarrhalis* was able to reduce the binding of antibody both to clinical isolates of *B. catarrhalis* and to OMPs used as capture in the ELISA system or on Western blotting. This suggests that antibodies directed against whole organisms and OMPs may not be strain-specific and that OMPs may be important constituents of the antigenic determinants on the surface of *B. catarrhalis*. Nevertheless, the recognition of antigens appears to be subclass-restricted. IgG3, previously noted to be highly specific in this ELISA assay system [10], binds to a broad range of OMPs, whereas the binding of IgG1, IgG2, and IgG4 appears to be restricted to the higher-molecular-mass proteins. In particular, an 82-kDa OMP appears to bind all four subclasses. Prominent OMPs have been described for other organisms (cf. P2 protein of nontypable *H. influenzae* [40]), and further work is required to elucidate the significance of this 82-kDa protein in *B. catarrhalis* infections.

Because of the frequency with which this organism is isolated from the upper respiratory tract and the difficulty presented by the interpretation of such isolation (pathogen vs. innocent bystander) [41], the analysis of IgG responses to this bacteria in convalescent sera may help clarify its role as a true pathogen. While it is unclear at present which of the specific anti-*B. catarrhalis* isotypes are protective, the ability to mount an appropriate subclass response to this organism may be crucial in protection from infection; hence the measurement of total IgG responses alone may be inadequate to assess immunity to this organism. In addition, the ability to measure subclass-specific responses may be a useful ad-

junct to the assessment of humoral immunity in patients suspected of having minor immune deficiencies, especially those characterized by normal levels of IgG subclasses but an inability to respond appropriately to antigen-specific challenges [42]. This may be of particular importance in children >6 years old, in whom the most frequent IgG subclass deficiency appears to be that of IgG3 [43]. This may be a factor in the pathogenesis of upper respiratory tract infections in children in whom viral infections may have damaged the mucosa, thereby compromising local immune function and permitting bacterial superinfection.

Finally, if a potential vaccine is envisaged [44], it is important to identify the major antigenic determinants of the organism and whether they are recognized by protective antibody. Pneumococcal vaccine has been shown previously to reduce the frequency of attacks of otitis media attributable to *S. pneumoniae* serotypes represented in the vaccine [45], and a similar effect may be achieved using a *Branhamella catarrhalis* vaccine.

Acknowledgment

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Delayed maturation of antigen-specific IgG3: Another variant of paediatric immunodeficiency?

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INTRODUCTION

The description of healthy individuals with IgG subclass deficiencies [1] and the difficulty in correlating quantitative IgG subclass deficiencies with clinical syndromes [2] has increasingly led investigators to evaluate qualitative aspects of IgG subclass function. The age- and antigen-restricted nature of the IgG subclass response has been well described though the clinical significance is less certain [1,2]. In adults IgG1 and IgG3 are thought to be the subclasses produced in response to protein antigens while IgG2 has predominantly been associated with responses to carbohydrate antigens [3]. Nevertheless, a role for IgG1 in the anti-carbohydrate response of adults is now recognized [4]. The role of IgG4 is unclear although it appears to be an important part of the response to repeated stimulation with the same antigen [5]. In children the relative contribution of individual IgG subclasses are even less clear. While IgG1 is an important part of the antibody response to proteins it also appears to have an anti-carbohydrate role in younger children. This may be an important compensatory mechanism for the age-restricted IgG2 anti-carbohydrate response in children, particularly those under the age of two [6]. The inability to mount an isotype-appropriate IgG subclass response to carbohydrate antigens in childhood is thought to explain the susceptibility of young children to infection with encapsulated bacteria and limits the usefulness of vaccines containing unconjugated carbohydrate antigens in this age group. Much of the work on the antigen-restricted nature of the IgG subclass response has focused on carbohydrate antigens (T-independent antigens) and the IgG2 response and there is little information on the age-restricted nature of the anti-protein isotype response in childhood. The successful immunization of infants with tetanus and diphtheria toxoid, proteins which predominantly induce an IgG1 response, has suggested that antibody responses to protein antigens are not age-restricted.

We have previously described the recognition of the outer membrane proteins (OMP's) of a Gram-negative organism *Moraxella (Branhamella) catarrhalis (M. catarrhalis)* by serum antibody [7] and demonstrated the importance of IgG3 in OMP recognition. This organism is ubiquitous and lives in the moist mucous membranes of the nasopharynx. Previously thought to be a harmless commensal

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it is now recognized as an important cause of paediatric ear and sinus disease and may cause disease in adults with immunodeficiency or lung damage [8,9]. This pattern of infection suggests that immunity is acquired during childhood and subsequently protects the healthy adult from infection. Prior to the development of such immunity in childhood, or following the loss of such immunity in adult life, the individual is susceptible to infection. Having previously shown that healthy adults have demonstrable levels of anti-*M. catarrhalis* antibody [10] we have undertaken this study in order to analyse the IgG subclass nature of the response in healthy children and adults susceptible to recurrent infection. To characterize further the nature of the IgG subclass response we have measured the affinity of the antigen-specific antibodies directed against this organism.

METHODS

Enzyme linked immunoassays previously developed [10] were used to determine the levels and IgG subclass distribution of antibodies to both the whole bacteria and outer membrane protein antigens. Serum from 53 healthy children undergoing elective surgical procedures were used to assess the levels of naturally-occurring antibodies in childhood. Antibodies to *M. catarrhalis* were also assessed in a group of 25 adults with a diagnosis of non-allergic recurrent rhino-sinusitis (kindly provided by Dr G. Scadding, Royal Ear, Nose and Throat Hospital, London) and their antibody levels were compared to those in a group of 40 healthy adults. Statistical analysis was performed using Student's t test.

Functional affinity (avidity) of the specific anti-*M. catarrhalis* antibodies (IgG1, IgG2 and IgG3) was measured by an inhibition assay and a modification of the ELISA technique [11]. The results obtained were confirmed by analysing all sera in the presence or absence of diethylamine (DEA), a mildly chaotropic agent which inhibits low-affinity but not high-affinity antibody-antigen binding.
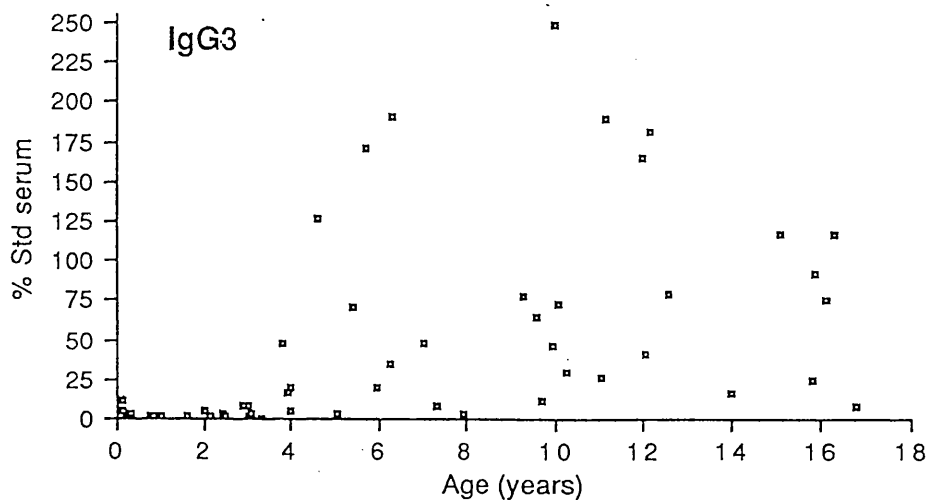

Figure 1 *Range of age-related naturally-occurring anti*-Moraxella (Branhamella) catarrhalis *IgG3 antibodies in children undergoing elective surgical procedures. Each symbol represents an individual child* (n = 53) *and levels (determined by ELISA [10]), are expressed as percentage of the level of antibody found in a pooled standard prepared from 60 apparently healthy adults.*
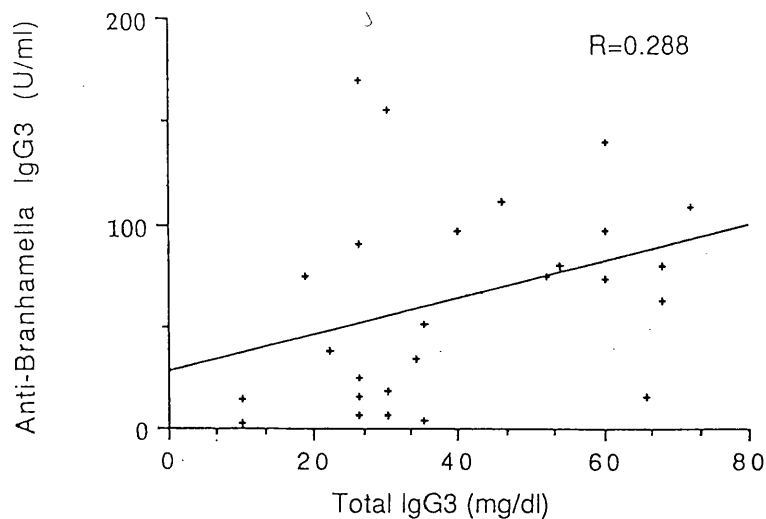
Figure 2   *Correlation between the specific anti-*Moraxella *(*Branhamella*) catarrhalis IgG3 antibody levels in a group of adult patients with chronic rhinosinusitis and their total serum IgG3. Levels of specific IgG3 are expressed as a percentage of a pooled adult standard and levels of total IgG3 are in mg/dl. (Patients kindly provided by Dr G. Scadding, Royal Ear, Nose and Throat Hospital, London.)*

RESULTS

Most healthy adults have antibodies to *M. catarrhalis*. Levels of specific IgG1 in children begin to rise during the first year of life, yet specific IgG3 antibodies cannot be measured under the age of four (Fig. 1).

The levels of specific anti-*M. catarrhalis* IgG1 and IgG2 antibodies were measured in a group of adults with recurrent URTI and compared to an adult control group; there were no significant differences. In contrast the levels of specific IgG3 antibody were significantly lower ($p = 0.016$) in the patient group; as shown in Fig. 2, these lower levels of specific IgG3 were not related to the total serum IgG3 levels.

The affinity of the specific antibodies as measured by both the inhibition assay and the DEA method ranked the affinity of the specific antibodies in the following order: IgG3 > IgG1 > IgG2 (Figs. 3 and 4).

DISCUSSION

Structurally IgG3 is the most unusual of the four subclasses. Possessing a long hinge region it is probably more flexible than the other subclasses although it is also more susceptible to enzymatic degradation which probably accounts for its shorter half life. This extended hinge region is also thought to potentiate the binding of C1q to the C_H2 domain leading to the efficient activation of the complement cascade [12]. IgG3 is also known to bind to Fc receptors [13].

IgG3 antigen-specific responses have mostly been reported in association with anti-viral antibodies and appear to be important in the responses to various viral infections including herpes simplex, polio, rotavirus and some vaccinations [14]. The isotype distribution of anti-bacterial antibodies is dependent on the antigenic
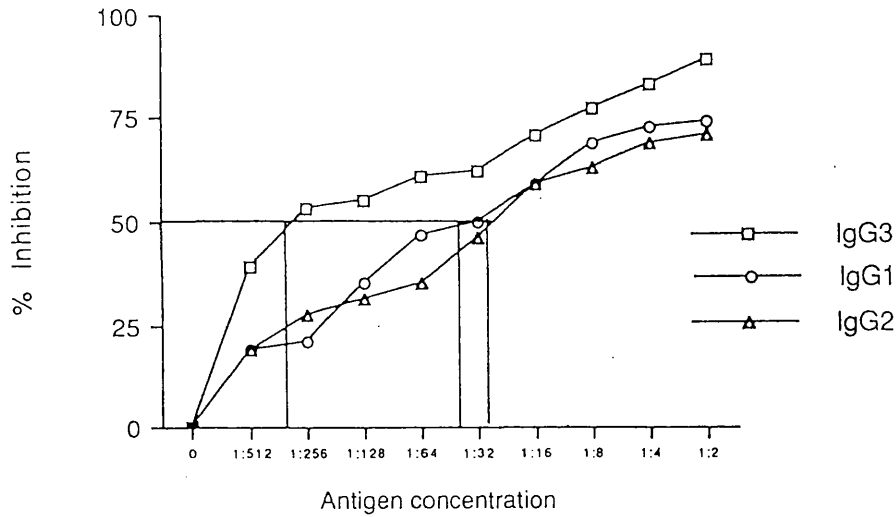
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Figure 3 *Functional affinity (avidity) of anti-*Moraxella *(Branhamella) catarrhalis antibodies as measured by inhibition assay. Free antigen (live* M. catarrhalis*) in increasing concentration was added to serum of fixed concentration. The amount of antigen required to produce 50% inhibition was compared for the three subclasses. Less free antigen is required to inhibit the binding of high affinity than low affinity antibodies, and for high affinity antibodies inhibition approaches 100%.*
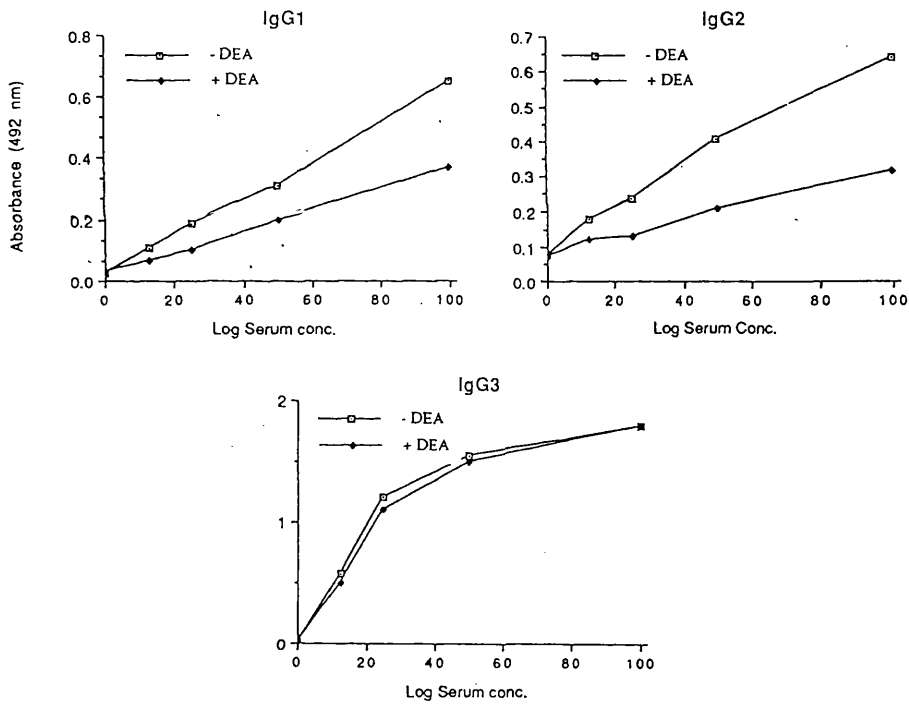

Figure 4 *Functional affinity (avidity) of anti-*Moraxella *(Branhamella) catarrhalis antibodies as measured by ELISA in the absence or presence of a chaotropic ion, diethylamine (20 mM). Low affinity antibody binding is more easily disrupted than high affinity antigen-antibody interactions [11]. The absence of any significant deviation in the case of IgG3 suggests higher functional affinity.*

determinants of any given bacteria and the OMP's of nontypable *Haemophilus influenzae* have previously been shown to be one of the few important bacterial targets for IgG3 [15]. It has also been shown that antibodies directed against these antigenic determinants are bactericidal and protect individuals from recurrent infection [16].

The OMP's of *M. catarrhalis* appear to be targets for serum antibodies and a rise in antibody levels to these determinants has previously been shown in patients recovering from infections with *M. catarrhalis* [17]. The importance of IgG3 in this recognition is supported by our observation that specific IgG3 antibodies are of higher affinity than IgG1 and IgG2 antibodies to this organism. High affinity responses are an essential part of the protective immune response and it is for this reason that it is likely that IgG3 has an important role.

If IgG3 does have such a role in anti-bacterial immunity the absence of specific IgG3 antibodies in the sera of children under the age of four is interesting. The mechanism for this is unclear although non-exposure to the organism is unlikely since specific IgG1 rises in the second year of life and studies have shown 50% nasal carriage in both adult and paediatric populations [18]. The epidemiological data showing that younger paediatric patients are particularly susceptible to otitis media with this organism suggests that this clinical susceptibility may be related to the lack of antibody.

The finding of lower levels of anti-Moraxella IgG3 in adults with frequent infection may also reflect an inability in some individuals to mount an adequate immune response to this organism. Convalescent rises in anti-*M. catarrhalis* antibodies have been observed in a group of children with proven upper respiratory tract viral infections and the authors have postulated that the damaged nasal mucosa subsequently became permissive to the normal bacterial flora [19]. Microbiological data from adult patients such as those described above will provide further insight into the relevance of the reduced specific IgG3 noted in this group.

Mechanisms for IgG3 production *in vivo* are poorly understood although antigen-specific IgG3 responses have not previously been shown to be age-restricted. It is possible that the V-region sequences which confer anti-*M. catarrhalis* specificity are restricted in some way from combining with the γ-3 heavy chain constant region in younger children although lower affinity IgG1 responses are not age-restricted. Alternatively, antigen presentation and accessory cell involvement may be crucial for the isotype appropriate response, perhaps through the involvement of particular cytokines (*cf* the role of IL-4 in IgE and IgG4 synthesis [20]). The fact that *M. catarrhalis* has been shown to be a B cell mitogen *in vitro* where it appears to stimulate a preferential IgG3 response [21] may provide a clue to the importance of the IgG3 contribution seen *in vivo*. Whether naive B cells are stimulated to switch to IgG3 production or whether pre-committed IgG3 cells are preferentially stimulated remains unclear and is the subject of further investigations in our laboratory.

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