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# Influence of Gm allotype on the IgG subclass response to streptococcal M protein and outer membrane proteins of *Moraxella catarrhalis*

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## SUMMARY

The IgG antibody response to streptococcal M protein is distributed between the IgG1 and IgG3 subclasses, however individual sera vary with respect to the relative amounts of these two subclasses. The basis of this variation was investigated. Sera were also analysed for IgG subclass antibodies to the outer membrane proteins (OMP) of *Moraxella catarrhalis*, as these have also been reported to have a major IgG3 component. The mean percentage of IgG3 was higher in the antibody response to OMP and there was less variability between sera for this antigen than was seen for M protein. Non-specific binding of IgG3 in ELISA, which has been reported for some bacterial proteins (including M protein of some serotypes) was excluded as an explanation for the apparent IgG3 bias of these antibodies. The relative amount of IgG3 antibody to the two antigens showed a positive correlation, suggesting that some individuals tended to make a greater IgG3 response to unrelated antigens. Serial bleeds from two individuals maintained a relatively constant subclass profile over several months, suggesting that time since infection did not play a major role in determining the proportion of IgG1 and IgG3. Gm allotypes for the sera were determined, and found to correlate with both total serum IgG3 concentrations and with IgG subclass composition of specific antibodies. Mean serum IgG3 concentrations were highest in sera typed as Gm(fb/fb) homozygous and lowest in sera typed as Gm(ag/ag) homozygous. Similarly, in the M protein-specific antibodies, the mean percentage of IgG3 was much lower in the Gm(ag/ag) sera than in the Gm(fb/fb) homozygous sera. Sera which typed as Gm(fb/ag) heterozygous were not significantly different from the Gm(fb/fb) homozygous sera for either total serum IgG3 or for M protein-specific IgG3. Moreover, both Gm(fb/fb) homozygous and Gm(fb/ag) heterozygous sera included samples in which IgG1 was the predominant antibody subclass and the percentage of IgG3 was very low. In contrast to the M protein-specific antibodies, for the OMP-specific antibodies there was no correlation between Gm phenotype and the proportion of IgG3. The data suggest that Gm allotype may influence the IgG subclass composition of antibody responses to bacterial surface protein, but that other factors are also likely to be involved.

## INTRODUCTION

The heavy chain constant regions of the four human IgG subclasses share a remarkable degree of sequence homology, however the differences are sufficient to influence their biological properties.[1] Thus the IgG subclass composition of an antibody response will have consequences for the effector mechanisms which are activated and, in an infection, the efficiency with which the pathogen is eliminated. A variety of factors may affect IgG subclass production, e.g. age of an individual, atopy, route of exposure to antigen.[2-4] Different types of antigen will also lead to responses in which different IgG subclasses predominate. Bacterial polysaccharides tend to induce IgG2 antibody production whereas antibody responses to protein antigens are often IgG1, with contributions from IgG3 and IgG4.[5-12] The latter subclass, found in relatively low amounts in serum, tends to be associated with secondary responses to injected protein antigens,[4,6] whereas IgG3 has been found in responses to viral proteins and red cell antigens.[9,13] Antibody responses to protein antigens encountered in the same infection may also differ: analysis of antibodies to two proteins of group A streptococci demonstrated that, while IgG1 is the predominant subclass produced in response to the exotoxin streptolysin O (SLO), antibodies to the surface M protein have a major IgG3 component.[14] Antibodies to the outer membrane proteins of the Gram-negative diplococcus, *Moraxella catarrhalis*, have also been reported to have an IgG3 bias.[15]

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Although, on average, IgG3 accounted for approximately half of the antibody to M protein, the relative amounts of IgG1 and IgG3 anti-M protein showed a wide range between individual sera.[14] IgG3 is known to have the shortest biological half-life of all the subclasses, and one possibility was that the relative amount of IgG3 antibody was simply related to the time since exposure to the antigen. Another factor known to influence IgG subclass production is heavy chain allotype. The IgG3 constant region genes show polymorphism, and the variants are serologically distinguishable. Different allotypes of a particular IgG subclass appear to be similar in terms of function, although some differences have been noted,[16] but allotypes do appear to be linked with the levels of subclasses produced.[17–23] Sera from individuals who are homozygous for G1m(f) and G3m(b) (which are found to be in linkage disequilibrium) tend to have lower serum concentrations of IgG1 and higher concentrations of IgG3 than individuals who are negative for these allotypes, but express G1m(a) and G3m(g). For IgG2, high serum concentrations are reported to be associated with the G2m(n) allotype. Moreover, Gm allotype may influence the IgG subclass composition of specific antibody responses.[24–27]

In the present study the basis for the variation in the subclass composition of antibodies to M protein was investigated. Gm allotypic differences are shown to play a role in determining IgG subclass profiles, but other factors are also likely to be involved.

## MATERIALS AND METHODS

*Human serum samples*

Two groups of human sera were obtained for analysis. Thirty-three sera from patients with suspected recent streptococcal infections or sequelae, and demonstrated to have high titres of antibodies to SLO, were gifts from Dr M. Barer (Department of Microbiology, University of Newcastle upon Tyne). They included patients with sore throats, erythema nodosum, cellulitis, arthropathy, and glomerulonephritis. A further 41 samples were collected from healthy adults. Samples were stored as aliquots at −80°.

*Antigens*

Recombinant M5 protein was purified to apparent homogeneity from *Escherichia coli* bearing a cloned M5 gene from the group A streptococcus strain Manfredo.[28] The antigens from *M. catarrhalis* were vesicles containing outer membrane proteins (OMP) from the bacteria, which were collected from culture supernatants as previously described.[15] Such preparations have been shown to be free from cytoplasmic contamination and contain approximately 10 to 20 proteins, of which six to eight proteins dominate.[29]

*Assays for antibodies to M protein and OMP*

Antibodies of each IgG subclass were measured by enzyme-linked immunosorbent assays (ELISA). Flexible Falcon 96-well plates were coated overnight at 4° with rM5 protein (2·5μg/ml) or OMP at an optimal dilution in borate-buffered saline (BBS) pH 8·4. After washing in phosphate-buffered saline containing 0·05% Tween-20 (PBS/Tween), 100 μl of PBS/Tween containing 5 mg/ml of bovine serum albumin (BSA; Sigma Chemical Co. Ltd, Poole, UK) was added to each well. Serum samples, appropriately diluted in PBS/Tween/BSA, were added to the top wells, and doubling dilutions were made down the plate. After incubation at 37° for 4 hr, plates were washed three times in PBS/Tween, and then optimal concentrations of subclass-specific monoclonal antibodies were applied overnight at 4°: NL16 for IgG1, ZG4 for IgG3, RJ4 for IgG4 (a gift from Drs R. Jefferis and N. Ling, University of Birmingham, UK) and HP6002 for IgG2 (a gift from Dr C. Reimer, CDC, Atlanta, Ga and Dr R. Hamilton, University of Texas, TX). NL16, ZG4 and RJ4 were IgG fractions of ascitic fluids, and were added at 2, 5 and 1 μg/ml respectively in PBS/Tween/BSA; HP6002 was added as a 1/20 dilution of culture supernatant. The following day plates were washed three times in PBS/Tween and then incubated with 100 μl of a 1/1000 dilution (in PBS/Tween/BSA) of horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (Dako Ltd, High Wycombe, UK) for 1 hr at 37°, washed, and then developed with *o*-phenylene diamine (OPD). After 30 min the reaction was stopped by adding 12·5% sulphuric acid, and the plates were read at 490 nm in a Dynatech MR5000 ELISA reader (Billingshurst, UK). For each subclass absorbance was plotted against serum dilution, and the titre was calculated for each serum as the reciprocal of the dilution giving an absorbance equal to twice the background absorbance of wells with no serum sample. Determination of antibody titre avoids problems arising from non-parallelism of curves, as is frequently seen in antigen-specific antibody assays, and minimizes differences in affinity, giving a result closer to the actual antibody concentration.[30] Interassay variability, calculated from values obtained for the same serum sample on multiple occasions, ranged from 10 to 34%. Intra-assay variability was negligible.

The relative sensitivities of the four subclass assays using the above clones under these conditions have been determined using NIP-specific chimeric antibodies of each subclass and plates coated with NIP-BSA, as detailed previously.[14] By this protocol the assays for IgG1, IgG3 and IgG4 antibodies were shown to be of approximately similar sensitivity, while the ELISA for IgG2 antibodies was approximately eightfold less sensitive. The standardization procedure was conducted on several occasions with similar results. Titres of anti-M protein and anti-OMP antibodies were converted to μg/ml as described previously.[14]

*Determination of Gm allotypes by haemagglutination inhibition*

Sera were typed for G1m(a), G1m(f), G3m(b) and G3m(g) by haemagglutination inhibition. Rhesus-positive human erythrocytes were sensitized with anti-Rhesus sera of known Gm allotypes, and agglutinated with anti-Gm allotype antisera. All reagents, including human reference sera of known allotype were obtained from Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, the Netherlands).

Human erythrocytes, blood group O R2R2, washed five times in PBS, were coated with each of four human anti-Rh sera, of known Gm allotype, by adding 6·25 μl of packed erythrocytes to tubes containing 25 μl of anti-Rh serum with 25 μl of PBS. The contents were incubated at 37° for 60 min, washed four times in PBS and resuspended to a total volume of 3125 μl in PBS. This gave sufficient coated erythrocytes at a 0·2% dilution for an entire 96-well plate. Test sera or plasma samples, diluted 1/20 with PBS, were placed in a water-bath at

65° for 10 min and then further diluted to 1/60. Each test sample (25 μl) was put into three wells of a 96-well V-bottom microtitre plate (Nunc, Roskilde, Denmark), two wells contained test sample at 1/20, and one well at 1/60 dilution. To one of the wells containing a 1/20 dilution of each test sample was added 25 μl of PBS. Anti-Gm-allotype antiserum (25 μl of an optimal dilution) was added to the other 1/20 dilution and the 1/60 dilution of each test sample, the two dilutions of each reference serum, and the two wells with PBS. The contents of the wells were mixed by shaking the plate. Finally 25 μl of the appropriate Gm-coated erythrocytes in a 0·2% suspension in PBS were added to all the wells, and mixed again. The plates were closed by stacking and incubated overnight in a humidified box at 4°.

Plates were read the next day. Each plate contained three controls which were: anti-Gm-allotype antiserum together with Gm-coated erythrocytes, to demonstrate haemagglutination; 1/20 test sample with Gm-coated erythrocytes, to ensure that the test samples were not causing haemagglutination themselves; and reference sera, anti-Gm-allotype antisera, and Gm-coated erythrocytes, as positive and negative controls to demonstrate Gm-allotype specificity. Samples were scored as positive or negative for an allotype only if both dilutions of the sample showed concordant haemagglutination inhibition or haemagglutination.

*Statistics*

Non-parametric tests were used to compare data between groups. The Mann–Whitney test was used for comparing the medians of two groups of data, while the Spearman rank test was used for testing correlations between paired sets of data.

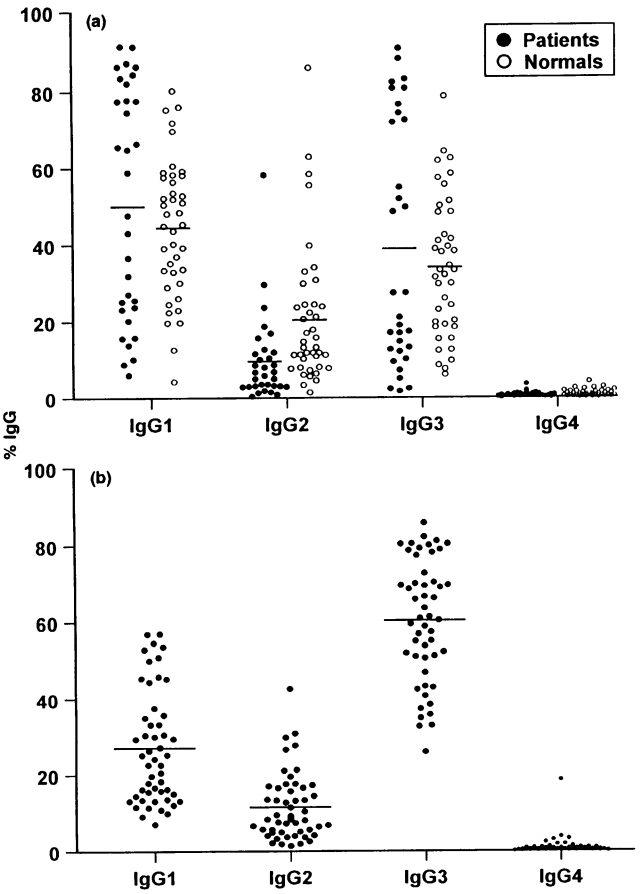


**Figure 1.** IgG subclass composition of antibodies to bacterial proteins. (a) Streptococcal M protein (rM5); (b) *M. catarrhalis* OMP. Each point represents a single serum sample, and the horizontal bars indicate arithmetic means.

## RESULTS

### Subclass composition of IgG antibodies to streptococcal M protein and *M. catarrhalis* OMP

The IgG subclass composition of antibodies to the two bacterial proteins was determined in quantitative ELISA (Fig. 1). Sera analysed were either from healthy individuals or from patients who had suspected recent streptococcal infections. The results for the two types of sera are shown separately for M protein antibodies, but are treated as a single group for *M. catarrhalis* OMP antibodies, as *M. catarrhalis* is unrelated to *Streptococcus pyogenes*. Consistent with an earlier study, antibodies to M protein were predominantly of the IgG1 and IgG3 subclasses. This was true for both the patients and the healthy individuals. For both groups of sera there was a large variation between individuals, ranging from 4 to 91% IgG1 and 2 to 90% IgG3. Antibodies to *M. catarrhalis* OMP showed an even higher percentage of IgG3, and the results, although still variable between individuals, were more tightly clustered around the means than the M protein results. In most sera IgG3 was the predominant antibody subclass, followed by IgG1 and IgG2. Little or no IgG4 was detected to either antigen. A positive correlation was observed between the percentage of IgG3 in the M protein and OMP-specific antibodies ($P < 0{\cdot}02$), suggesting that some individuals tended to make antibody responses with a greater IgG3 component to both antigens.

### Binding of IgG subclasses to M protein and OMP is antigen specific

As some bacterial proteins, including some serotypes of M protein, are known to bind to human immunoglobulin constant regions, the ability of rM5 and OMP to bind IgG paraproteins was investigated. Myeloma proteins of different subclass, light chain, and Gm allotype were investigated for binding in ELISA to antigen-coated plates. Some binding of the paraproteins to plates coated with rM5 was observed for all four IgG subclasses, but only at IgG concentrations in excess of 20 μg/ml (data not shown). Titres of IgG1 and IgG3 in most sera tested were such that this non-specific binding would not affect the assays. However, titres of IgG2 and IgG4, which tended to be much lower, might have been over-estimated owing to non-specific binding of these subclasses. Only IgG3 subclass proteins were investigated for binding to *M. catarrhalis* OMP, as this was the predominant subclass in this response. As for the M protein ELISA, some binding of the paraproteins was observed but only at IgG3 concentrations which were high relative to the IgG3 antibody titres in the sera.

### IgG subclass composition of M protein-specific antibodies in different sera is relatively constant

Although the mean percentages of IgG1 and IgG3 in the M protein-specific antibodies were similar, large variations were seen between individuals, with IgG1 as the predominant subclass in some sera, and IgG3 in others. One possibility is that the relative amount of IgG3 relates to the time which has elapsed since exposure to the antigen: as IgG3 has a much shorter half-life than IgG1 those individuals who have more recently encountered M protein might be expected to have a greater percentage of IgG3. To investigate this possibility, serial bleeds from two healthy individuals were analysed for IgG subclass antibodies to rM5 (Fig. 2). Bleeds were taken over a period of several months. It can be seen that in one individual IgG1 is the predominant subclass, accounting for > 60% of the IgG antibody in all of the sera. In the other individual IgG3 is the predominant subclass in all of the sera tested, with a much smaller contribution from IgG1. This suggests that the IgG subclass composition of M protein-specific antibodies in an individual is unlikely to be related simply to the period of time since the last exposure to antigen, but may be determined by other factors.

### Influence of Gm allotype of IgG subclass responses to M protein and OMP

The influence of Gm allotype on IgG subclass composition of antibodies to M protein and OMP was investigated. G1m(f), G1m(a), G3m(b) and G3m(g) allotypes were determined for 67 sera, from both patients with suspected recent streptococcal infections and healthy individuals. Three serotypes were expected: Gm(f,b), representing Gm(f,b)/Gm(f,b) homozygotes, Gm(a,g) representing Gm(a,g)/Gm(a,g) homozygotes and Gm(f,a,b,g) representing Gm(f,b)/Gm(a,g) heterozygotes. Only one serum sample did not fit this pattern, and typed reproducibly as Gm(f,a,b), with no detectable G3m(g). This serum was plotted as a Gm(f,b)/Gm(a,g) heterozygote, assuming a non-functional G3m(g) gene, but omission of the data from this serum makes no significant difference to the statistical analyses. Of the remaining 66 sera, 33 were typed as Gm(f,b) homozygotes, 30 as Gm(f,b)/(a,g) heterozygotes and four as Gm(a,g) homozygotes.

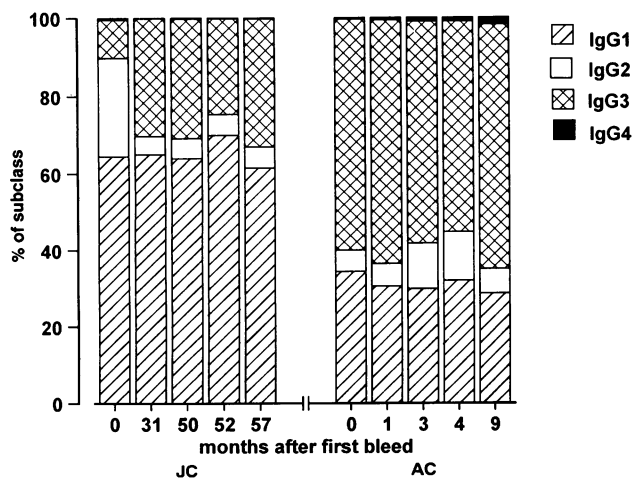


**Figure 2.** IgG subclass composition of rM5-specific antibodies in two individuals (JC and AC) at different times.

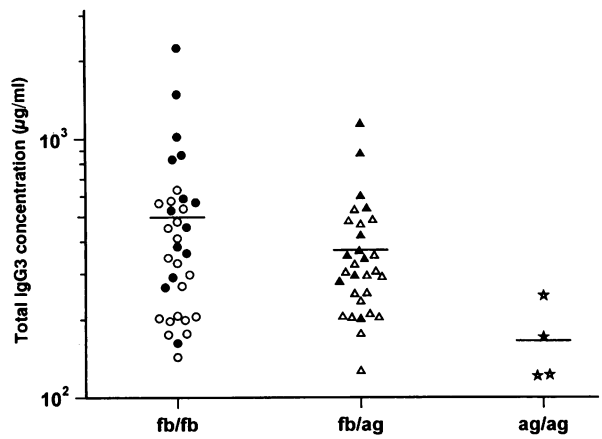


**Figure 3.** Relationship of serum IgG3 concentration to Gm allotype. Open symbols represent healthy adults, closed symbols represent patients with suspected recent streptococcal infections.

Total serum IgG3 concentration was determined for each of the sera, and results are shown in Fig. 3. The mean IgG3 concentrations for Gm(fb/fb) homozygotes, Gm(fb/ag) heterozygotes, and Gm(ag/ag) homozygotes were 381, 313 and 146 μg/ml respectively. Both the Gm(fb/fb) homozygotes and the heterozygotes were significantly higher than the Gm(ag/ag) homozygotes ($P < 0{\cdot}01$; Mann–Whitney), but were not significantly different from each other.

When the percentage contribution of the IgG subclasses to M protein was compared for the allotype groups, some interesting differences were observed (Fig. 4a). Most notably, the percentage of M protein-specific IgG3 was very low in the four Gm(a,g) homozygotes (mean 11%), in comparison to the Gm(f,b) homozygotes or the heterozygotes (mean 37 and 38% respectively). In three of the Gm(a,g) sera the major M protein-specific subclass was IgG1; unusually, in the other serum, IgG2 was the predominant antibody subclass. The difference between the IgG3 percentages in the Gm(a,g) homozygotes and the other two groups was significant ($P < 0{\cdot}02$ for both), but the IgG3 percentages in the Gm(f,b) homozygotes were statistically indistinguishable from the heterozygotes. Thus, the G3m(g) allotype is associated with low IgG3 production in the response to M protein, but this does not account for all the sera with a low percentage of IgG3, as both the heterozygotes and the Gm(f,b) homozygous groups include sera with high antibody titres and a low percentage of IgG3.

In the OMP subclass response, the percentage of IgG3 in the Gm(ag) homozygotes is also lower than in the other two allotype groups, but this difference is not significant (Fig. 4b). However, the Gm(ag) homozygous sera do have a significantly higher percentage of IgG1 anti-OMP than the other two groups ($P < 0{\cdot}02$), suggesting that allotype-related subclass differences occur in this antibody response also.

## DISCUSSION

It has previously been noted that, following streptococcal infection, the IgG antibodies to M protein tended to show a

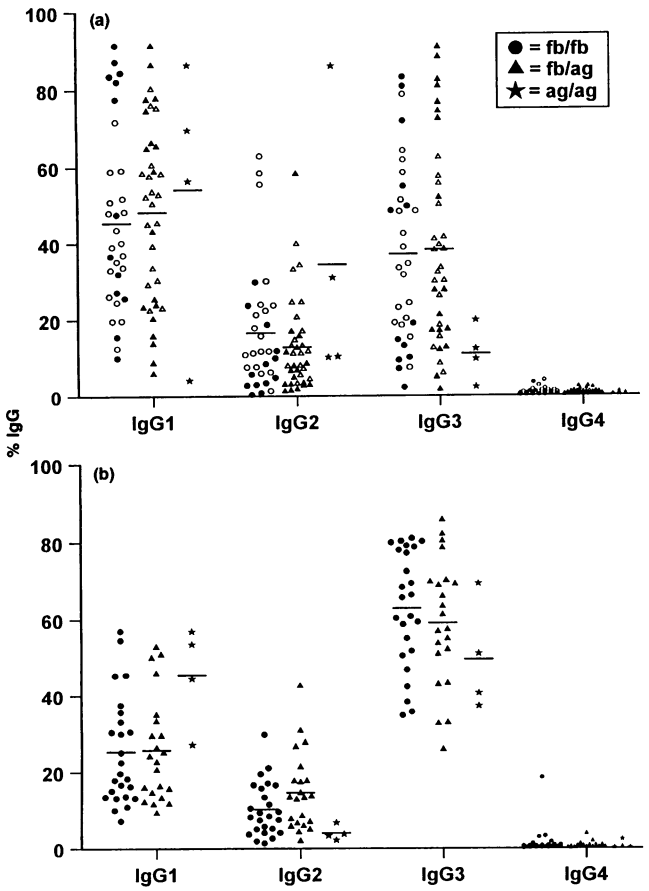


**Figure 4.** Influence of Gm allotype on IgG subclass composition of antibodies to: (a) rM5; (b) OMP. Open symbols represent healthy adults, closed symbols represent patients with suspected recent streptococcal infections.

marked IgG3 bias in comparison to antibodies to streptolysin O. In this report the subclass composition of antibodies to M protein in sera from healthy adults was shown to be similar to that seen in patients suspected of having had recent streptococcal infections. In both groups there was considerable variation between individuals in the relative amount of IgG3. This paper investigates the basis for this variation and concludes that multiple factors are likely to be involved.

As a recent report demonstrated that M protein of another serotype (M12) bound in a non-antigen-specific manner to IgG3,[31] the ability of paraproteins of the four subclasses to bind to M5 in ELISA was investigated. While some binding was observed, this was seen only at relatively high concentrations of IgG, and was observed with all subclasses, and would not account for the IgG3 binding from sera at the dilutions used in the assays. Non-specific binding of IgG3 by *M.catarrhalis* OMP was also excluded as an explanation for the apparent IgG3 bias of this antibody response.

One factor known to influence IgG subclass production in individuals is Gm allotype. Sera were therefore typed for expression of IgG1- and IgG3-associated allotypic markers. Consistent with previous reports, the total serum IgG3 concentration was related to Gm allotype, the mean concentration being highest in Gm(fb) homozygotes and lowest in Gm(ag) homozygotes. Percentages of IgG3 in the antibody response to M protein also appeared to relate to allotype, in that the four sera which typed as Gm(ag) homozygous all had a very low percentage of IgG3 antibody in comparison with the means for the Gm(fb) homozygotes and the heterozygotes. Interestingly, there was no significant difference between the Gm(fb) homozygotes and the heterozygotes in respect of either total serum IgG3 or the percentage of IgG3 anti-M protein. This finding is in contrast to some previous reports.[22,23] One possible explanation is that the number of sera studied was too small to detect a difference between these two groups: this could certainly be the case for the total serum IgG3, where the mean value for the heterozygous group was somewhat lower than for the Gm(fb) homozygotes, but is less likely to be the case in the M protein response, where the mean percentage of IgG3 was actually slightly higher in the heterozygotes.

The reason for the higher IgG3 production in Gm(fb) homozygotes is not clear, and could relate to frequency of class switching to IgG3 in IgH loci of this allotype, or differential production of the different allotypes. Some evidence suggests that the former explanation is more likely.[32] If this were the case then the similar proportions of IgG3 anti-M protein in the Gm(fb) and Gm(fb/ag) sera could be accounted for by postulating the existence of a trans-acting factor, encoded by the chromosome bearing the Gm(fb) alleles which could promote class switching to IgG3 on the homologous chromosome. This would be consistent with the observation that isotype switching appears to occur similarly on both the functional and non-functional IgH alleles in several systems,[33-37] and a candidate for such a trans-acting factor might be the sterile transcripts which are detected prior to switch recombination.[38,39] It was also apparent that the effect of allotype on the IgG3 antibody response was more marked for M protein than for the OMP antibodies. In general the percentage contribution of IgG3 was higher in the latter response, and it is possible that this antigen, being highly efficient at inducing switching to IgG3, obscures allotype-related differences to some extent, although the percentage of IgG1 antibody was significantly higher for the Gm(ag) homozygotes than for the other two groups. A similar effect of Gm allotype on the IgG subclass composition of the antibody response to *Haemophilus influenzae* polysaccharide has previously been reported.[24] In this case the ratio of IgG1 to IgG2 antibodies was much higher for individuals who were negative for Gm(f,n,b) than for those who were positive for these allotypes, and it was suggested that IgH-linked regulatory elements might determine whether switching to IgG1 or IgG2 occurs preferentially in response to this antigen. A similar situation could be occurring in the M protein response, in which B cells may have the option of switching to IgG1 or IgG3. In the current study G2m status was not investigated, and it is possible that presence or absence of the G2m(n) marker might have a modifying effect on the IgG1 and IgG3 responses. However, in a study investigating the influence of allotype on serum IgG subclasses, no significant differences in IgG1 or IgG3 concentrations were found between Gm(fb/fb) individuals subdivided according to G2m allotype.[22]

Although allotype accounts for some of the sera in which the percentage of IgG3 anti-M protein is low, both the Gm(fb) homozygous and the heterozygous groups also included sera with a very low proportion of IgG3 antibody. Another possibility was that the relative amount of IgG3 related to the time which

had elapsed since exposure to the antigen: as IgG3 has a relatively short half-life in comparison to other subclasses, the percentage of IgG3 antibody might decrease with time since infection. This may contribute to the observed variations, but was considered unlikely to be a major factor, as sequential sera from two healthy adults remained relatively constant for the percentage of IgG3, despite fluctuations in titre over time. Moreover, approximately half the patients' sera analysed had an excess of IgG1 over IgG3, and this pattern was similar to the healthy adult sera.

The precise nature of the stimuli required to induce switching to IgG3 has not been defined, but protein antigens are generally thought to be T dependent, and by analogy with other isotype switches it is likely that the switch to IgG3 is influenced by interactions of the B cell with T-cell surface molecules and/or cytokines. Polymorphism of any of these molecular components or qualitative differences in T-cell help could therefore contribute to the isotype differences observed between sera. One factor worthy of consideration is HLA. Interaction between HLA and Gm has been noted in several situations.[40-42] Both are linked independently to immune response genes and implicated in disease susceptibility, and an effect of HLA in modifying IgG subclass responses cannot be excluded. The nature of the T-cell response to a protein antigen is probably dictated by the antigen-presenting cell, and antigens occurring on the surface of bacteria may be predominantly presented by phagocytic antigen-presenting cells, such as macrophages. In this context it may be significant that other antigens which tend to induce an IgG3 response may also be expressed on particles such as erythrocytes or virus-infected cells. If the protein can be cleaved from the bacterial surface it could then behave like a soluble protein and be presented by non-phagocytic antigen-presenting cells, such as dendritic cells, with consequences for the type of T-cell help and ultimately the isotype of antibody produced. If individuals differed in their ability to cleave M protein from the surface of streptococci, this might provide another source of variation. Alternatively, serotypes of M protein might be processed differently. Currently experiments are underway to investigate *in vitro* the nature of the T-cell response to M protein.

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