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# Distinct IgG1 and IgG3 subclass responses to two streptococcal protein antigens in man: analysis of antibodies to streptolysin O and M protein using standardized subclass-specific enzyme-linked immunosorbent assays

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## SUMMARY

The IgG subclass composition of antibodies to two streptococcal protein antigens in sera following infection was analysed by enzyme-linked immunosorbent assays (ELISA). The assays were standardized using 5-iodo-4-hydroxy-nitrophenacetyl (NIP)-specific chimeric antibodies, to permit quantitative comparisons between subclasses. Antibodies to streptolysin O (SLO) were predominantly IgG1, with only minor contributions from the other subclasses. In contrast, antibodies to M protein were distributed between the IgG1 and IgG3 subclasses, and in approximately half the sera IgG3 predominated. The ratio of IgG1 : IgG3 was greater for SLO than for M protein in 22/23 sera. Little or no IgG4 antibody was detected to either antigen. Functional affinities of the IgG1 and IgG3 antibodies, determined by inhibition ELISA, were comparable for the two antigens. The demonstration that two protein antigens encountered during streptococcal infection elicit antibody responses with markedly different subclass profiles has implications for IgG subclass regulation and vaccine development.

## INTRODUCTION

In man there are four subclasses of IgG, which differ in the primary sequence of the constant region of their heavy chains. The four subclasses differ also with respect to their biological properties, including the ability to activate effector mechanisms.[1] Various factors are known to influence the IgG subclasses produced in an antibody response, e.g. route of immunization[2] and previous exposure to the antigen.[3,4] One factor which is clearly important is the ability of the antigen to recruit T-cell help. Thus, T-independent antigens, e.g. bacterial polysaccharides, tend to induce IgG2 antibodies, whereas antibodies to T-dependent protein antigens are frequently IgG1. T-dependent antibody responses may also have an IgG3 or IgG4 component: antibodies to viruses and red cell antigens are predominantly IgG1 and IgG3, whereas IgG4 antibodies are often found in secondary responses to injected protein antigens.[5–12]

In this study the IgG subclass composition of antibodies produced in response to two protein antigens of group A streptococci following infection has been analysed. M protein is a streptococcal cell surface antigen, the structure and properties of which have been well characterized.[13] More than 80 serotypes have been identified, and during an infection antibodies are produced against both type-specific and conserved determinants. M-like proteins are also present on streptococci of other groups, and some of these may show cross-reactions with M protein.[13] Streptolysin O (SLO) is a cytolytic protein exotoxin;[14] whilst cross-reactivity of SLO with sulphydryl-activated toxins of other species has been documented, antibodies to SLO in man are generally regarded as specific and diagnostic of streptococcal infection. An early report suggested that complement-fixing antibodies to M-associated protein (an antigen derived from streptococci which express M protein, and probably a fragment of the molecule) were predominantly IgG3, since they were not removed from sera by absorption on protein A.[15] This was in contrast to antibodies to other streptococcal antigens. In the present study the subclass composition of the antibodies to M protein and to SLO in a panel of sera was investigated, using quantitative ELISA based on subclass-specific monoclonal antibodies and highly purified recombinant antigens; the assays were standardized using a panel of NIP-specific chimeric antibodies.

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## MATERIALS AND METHODS

*Sera*

Sera were from patients with suspected recent streptococcal infections, and all had high titres of antibodies to SLO. They included patients with sore throats, erythema nodusum, cellulitis, arthropathy and glomerulonephritis.

*Antigens*

Recombinant M5 protein was purified to apparent homogeneity from *Escherichia coli* bearing a cloned M5 gene from the group A streptococcus strain Manfredo.[16,17] Recombinant SLO was purified from *E. coli* containing a cloned *slo* gene, giving the intact molecule.[18] The SLO truncate is encoded by the recombinant plasmid pMK307, which contains the 3′ third of the *slo* gene linked in frame to the sequence for the N-terminal 99 residues of the λN gene to encode a fused product termed 307 Ag, which was purified from *E. coli*.

*Assays for anti-M protein and anti-SLO*

Antibodies of each IgG subclass were quantified by enzyme-linked immunosorbent assays (ELISA). Flexible Falcon 96-well plates were coated overnight at 4° with rM5 protein [2·5 μg/ml in borate-buffered saline (BBS) pH 8·4], SLO truncate (10 μg/ml) or whole rSLO (2 μg/ml). After washing in phosphate-buffered saline (PBS) containing 0·05% Tween 20 (PBS/Tween), 100 μl of PBS/Tween containing 5 mg/ml of bovine serum albumin (BSA; Sigma Chemical Co., Poole, U.K.) were 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, U.K.) 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, U.K.) 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, U.K.) 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. Interassay variability, calculated from values obtained for the same serum sample repeated on multiple occasions, ranged from 10 to 34%; intra-assay variability was negligible.

*Standardization of ELISA using chimeric anti-NIP antibodies*

The sensitivities of the four subclass assays were determined using chimeric 5-iodo-4-hydroxy-3-nitrophenacetyl (NIP)-specific antibodies of the four IgG subclasses:[19] these have identical antigen-binding sites and mouse λ light chains and differ only in their heavy chain constant regions. The cell lines producing the chimeric antibodies of each IgG subclass (transfectants of the mouse plasmacytoma J558) were obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, U.K.) and grown in RPMI-1640 supplemented with 10% heat-inactivated foetal calf serum (FCS; Northumbria Biologicals, Cramlington, U.K.), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin sulphate (Flow Laboratories, Irvine, U.K.). Culture supernatants of the cell lines were used as a source of the chimeric antibodies in ELISA for NIP-specific antibodies of each IgG subclass. These assays were essentially identical to the M protein and SLO subclass assays except that plates were coated with NIP–BSA (1 μg/ml in BBS), and, after washing and blocking with BSA, the culture supernatants were diluted down the plates. The antibody concentration in each supernatant was determined independently in IgG subclass ELISA,[20] and in this way the antibody concentration giving an OD equal to twice background in each assay could be calculated. As a check on the validity of the assay, duplicate plates coated with NIP–BSA and incubated with the chimeric antibodies were developed with an HRP-goat anti-mouse λ conjugate.

Titres of anti-M protein and anti-SLO antibodies were converted to μg/ml by multiplying by the concentration of anti-NIP giving an OD equal to twice background for each of the subclass assays.

*Inhibition ELISA*

Functional affinity of different subclasses of antibody to M protein and SLO was estimated using competition assays in which free antigen was added to inhibit binding of the antibodies to antigen-coated plates.[21] Sera were diluted to give a final OD of 1·0 for each subclass in the M protein and SLO-specific ELISA, and were added to antigen-coated plates containing a range of concentrations of the same antigen in 50 μl of diluent. After mixing the contents of each well, plates were incubated for 4 hr at 37°, washed, and the subclass ELISA was performed as described above. The concentration of free antigen was plotted against percentage inhibition, and from this the concentration needed to give 50% inhibition was calculated; this was taken as a measure of average functional antibody affinity, and was expressed as $\log_{10}$ pM free antigen ($I_{50}$). Thus, antibodies with a high average functional affinity were inhibited by low concentrations of free antigen and had a low $I_{50}$ value.

## RESULTS

### Standardization of antigen-specific ELISA

Using the NIP-specific chimeric antibodies, the IgG1, 3 and 4 subclass ELISA were shown to be of approximately similar sensitivity whilst the ELISA for IgG2 antibodies was considerably less sensitive (Fig. 1a). When the assay was repeated using HRP anti-mouse light chain instead of the anti-subclass monoclonal antibodies, the curves for the four chimeric antibodies were not significantly different (Fig. 1b), confirming that the difference between the assays was due to the properties of the individual subclass-specific reagents and not to a differential ability of the chimeric antibodies to bind to the antigen-coated plates.

The concentration of anti-NIP (ng/ml) giving an OD equal to twice background in Fig. 1a is shown in Table 1. The end-point titres for IgG1, 3 and 4 were very similar, corresponding to 12·8, 10·2 and 9·5 ng/ml respectively, whilst for IgG2 this value was much higher at 80 ng/ml. These values were used to convert titres obtained in the M protein and SLO antibody assays to ng/ml for each subclass, enabling quantitative comparisons.

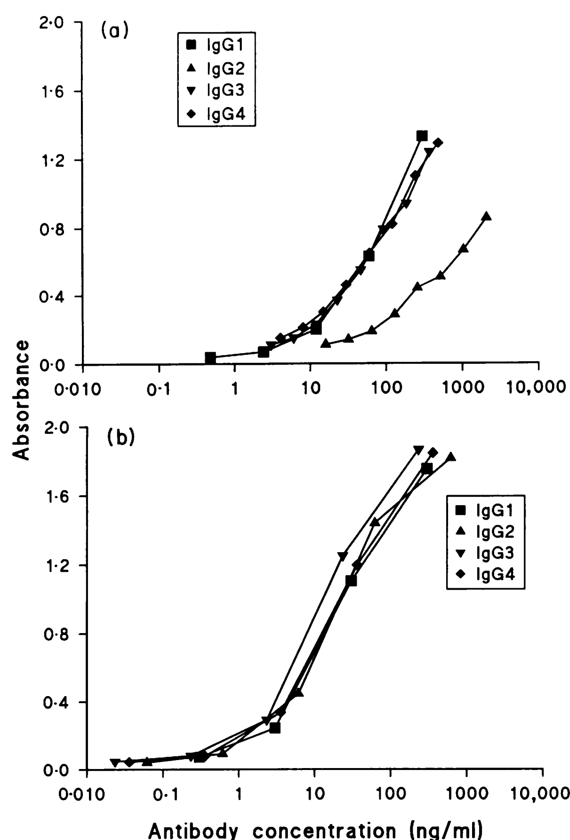


**Figure 1.** Titration curves for NIP-specific chimeric antibodies on plates coated with NIP–BSA. Assays developed with monoclonal subclass-specific antibody followed by HRP anti-mouse IgG (a), or with HRP anti-mouse λ (b).

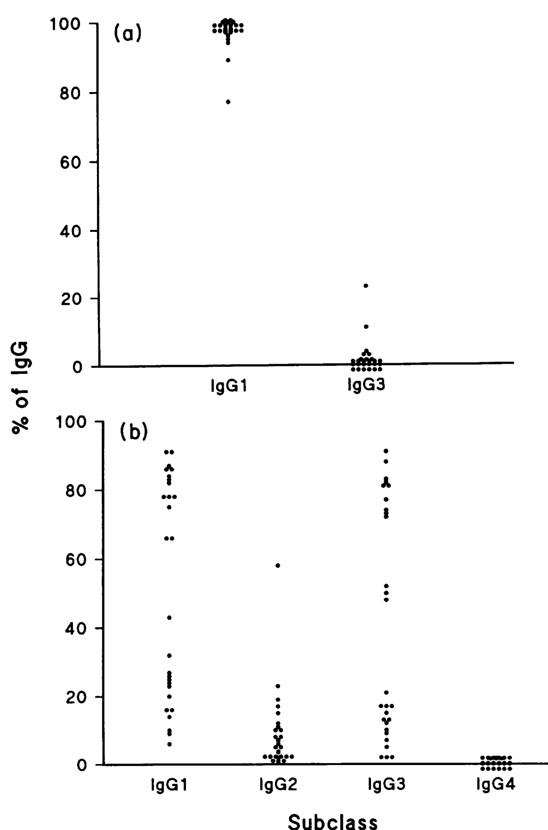


**Figure 2.** Subclass composition of the IgG antibodies to (a) streptolysin O (truncate) and (b) M5 protein. Each point represents one serum. IgG2 and IgG4 antibodies to SLO were undetectable.

**Table 1.** Relative sensitivities of the NIP-specific subclass ELISA. Concentration of antibody giving an OD equal to twice the background OD (obtained when anti-NIP was replaced by diluent): this value was approximately 0·1 for all four assays. Results shown are means of three (IgG2, 3 and 4) or five (IgG1) experiments

| | Antibody concentration (ng/ml) at twice background OD | | | |
|---|---|---|---|---|
| | IgG1 | IgG2 | IgG3 | IgG4 |
| Mean | 12·8 | 80 | 10·2 | 9·5 |
| SEM | 1·4 | 8·4 | 1·4 | 2·8 |

### Subclasses of IgG antibodies to SLO and M5

Twenty-three sera were analysed for antibodies of each IgG subclass to the two streptococcal antigens M protein and SLO (truncate). Titres were converted to ng/ml as described above, and these values used to calculate the percentage of each IgG subclass in the individual serum samples (Fig. 2).

For SLO only IgG1 and IgG3 antibodies were detectable, and in all sera IgG1 was the major subclass accounting, on average, for 97% of the IgG antibody. For M protein the pattern was quite different, with similar amounts of IgG1 and IgG3 antibodies, these subclasses accounting, on average, for 51% and 40% of the IgG antibody respectively, and in approximately half of the sera IgG3 was the predominant antibody subclass. Some sera also had detectable IgG2 and IgG4 antibodies, although the latter were present in very small quantities.

Ten sera were also assayed against the whole SLO molecule. Although the titres were frequently higher, the subclass distribution of the antibodies was similar to that seen for the truncated form of the antigen, with IgG1 forming the predominant subclass (data not shown).

### Relative amounts of IgG1 and IgG3 antibodies to SLO and M protein

The most marked difference between the antibodies to M protein and to SLO was seen in the relative amounts of IgG1 and IgG3 antibodies to the two proteins. Whilst, on average, the amounts of IgG1 and IgG3 antibodies to M protein were similar, for SLO the amount of IgG1 antibody greatly exceeded that of IgG3 antibody. This is emphasized by comparing the ratio of IgG1 : IgG3 antibodies to M protein and to SLO in each serum (Fig. 3). For anti-M5 the geometric mean ratio of IgG1 : IgG3 is 1·4 whereas for SLO the mean ratio is 208, and in 22 of the 23 sera the IgG1 : IgG3 ratio is higher for SLO than for M protein ($P < 0{\cdot}001$, Sign test).

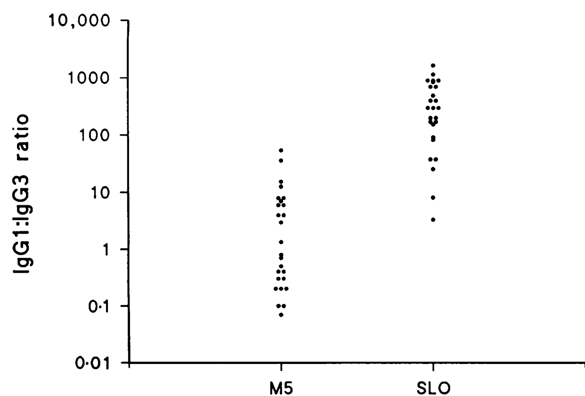


**Figure 3.** Ratio of IgG1:IgG3 concentration of individual sera for antibodies to M protein and streptolysin O.

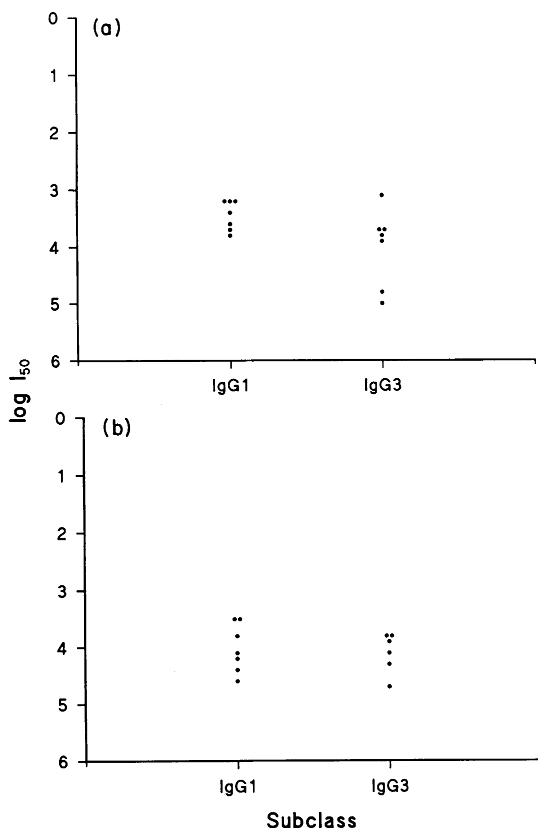


**Figure 4.** Functional affinity of IgG1 and IgG3 antibodies to (a) streptolysin O and (b) M protein in seven sera. Results are plotted as the concentration of free antigen ($log_{10}$ pM) giving 50% inhibition (log $I_{50}$).

### Affinity of IgG subclass antibodies to M5 and SLO

The functional affinities of IgG1 and IgG3 antibodies to M protein and SLO were determined in seven sera, and are shown in Fig. 4. The sera which were selected were those having a sufficiently high concentration of IgG3 antibodies to SLO to enable analysis. For both antigens IgG1 and IgG3 antibodies were of comparable functional affinities, with the exception of two out of seven sera in which the IgG3 antibodies to SLO had lower affinities than IgG1.

## DISCUSSION

The data presented in this report demonstrate a distinct difference in the IgG subclass profile of antibodies to two protein antigens of group A streptococci. Quantification of the subclass antibodies was achieved in ELISA which were standardized using NIP-specific chimeric antibodies. Previous studies have adopted alternative approaches to compare the sensitivity of isotype-specific antibody assays: these have included the use of purified myeloma proteins on the solid phase, preparation of isotype-enriched fractions from polyclonal antisera, and measurement of IgG subclass concentrations in affinity-purified antibody preparations.[7,22,23] The advantage of using the NIP-specific chimeric antibodies is that these differ only in their heavy chain constant region and have the same binding site and the same affinity for hapten.[24] Nonetheless, the possibility was considered that these antibodies might differ in their ability to bind to NIP in the solid phase perhaps, for example, because of differences in charge or binding site availability: the finding that, when equal amounts of each subclass were applied to the plates, equivalent binding of anti-mouse light chain was achieved demonstrated that this was not a problem, and that differences between sensitivities of the assays were due to the individual subclass-specific monoclonal antibodies. It should be noted, however, that the physicochemical properties of the antigen itself may affect antibody estimations in this type of ELISA assay. The low sensitivity of the IgG2 assay demonstrates the necessity of standardization for each assay system and set of reagents employed, and that studies in which a comparable sensitivity for all isotypes is assumed may be misleading. Indeed, another IgG2-specific monoclonal antibody which has been used in some studies of this nature (clone GOM2) was found to be much less sensitive than the clone used here. Whilst the sensitivity of assays using such antibodies may sometimes be improved by increasing the concentration of the monoclonal anti-subclass reagent, the concentrations used in this report were optimal and it was not possible to overcome the problem of the low sensitivity of the IgG2-specific antibody in this way.

The demonstration that IgG1 predominated in antibodies to SLO whilst IgG3 formed a higher proportion of anti-M protein antibodies confirmed and extended an earlier report of isotype differences between these two responses.[15] In some sera IgG3 was the major subclass of M protein antibody: this is particularly remarkable in view of the fact that this forms a minor subclass accounting for less than 10% of normal serum IgG.[25] It was also of interest that the percentage of IgG3 anti-M protein was highly variable, and the sera appeared to segregate into two groups on this basis. The reason for this is not clear, but one possibility is that IgG3 production to this antigen is influenced by IgG allotypes, as reported for total serum concentrations of this subclass.[26] Alternatively, the relative amount of IgG3 may reflect the time elapsed since exposure to the antigen, since this subclass has a shorter half-life than the other three.

Despite this variability, however, the ratio of IgG1:IgG3 was higher for SLO than for M protein-specific antibodies in virtually all sera tested. The mechanisms underlying the distinct

subclass responses to M protein and SLO are of interest. One possibility is that the subclasses of the antibodies relate to the mode of presentation of the two antigens. M protein is a cell-surface component of streptococci and, as such, might be presented preferentially by professional phagocytes following ingestion of bacteria. In contrast, SLO is a secreted protein and so might be available for presentation by non-phagocytic antigen-presenting cells. It is of interest in this context that IgG antibodies to the outer membrane protein of *Branhamella catarrhalis* may also have an IgG3 bias.[27] Differences in antigen presentation could have consequences for the quality of the T-cell response to the two antigens (e.g. the nature and amounts of the cytokines produced), which might in turn affect IgG subclass production. Currently the role of cytokines in regulating human IgG subclass production is not clear, but evidence from murine systems suggests that differential cytokine production by T-helper cells is centrally important in determining the isotype profile of an antibody response.[28,29] Analysis of the T-cell response to M protein and SLO *in vitro* may provide an answer to these questions. It was of interest to note that IgG1 and IgG3 antibodies to both M protein and SLO are of comparable functional affinity: this differs from results reported for antibodies to hepatitis B surface antigen and for keyhole limpet haemocyanin,[21,30] where IgG1 antibodies tended to be of higher affinity than IgG3.

The failure to detect significant levels of IgG4 to either antigen was of interest in view of the association of this subclass with secondary responses to protein antigens and the likelihood that at least some of the patients would have had multiple streptococcal infections. Factors required for IgG4 antibody production are not completely understood, but may include localization of antigen on follicular dendritic cells in the form of immune complexes,[31] and the production of interleukin-4 (IL-4) by Th cells.[32]

The distinct subclass responses to the two antigens are likely to be of biological significance. Both IgG1 and IgG3 are efficient at activating complement through the classical pathway,[19,24] both are active in antibody-dependent cellular cytotoxicity[19] and bind well to Fc receptors on phagocytes.[1] This is particularly important in the response to M protein, since opsonic antibodies against serotype-specific determinants of this antigen confer protection. Since the sera analysed were from infections of unknown serotype, it is likely that the majority of antibodies detected against rM5 would be to epitopes which are not serotype specific, and it would be of interest to determine the subclass distribution of antibodies to serotype-specific epitopes. The most notable functional difference between IgG1 and IgG3 relates to the much shorter half-life of IgG3 (only 7 days, compared to 21 days for IgG1). It is interesting to note that the response to M protein, with a major IgG3 component, is similar to the response to several viral antigens, and it is possible that the production of relatively short-lived antibodies might be advantageous in viral infections where there is a strong potential for immunopathological consequences. In relation to this, the cross-reactivity between M proteins of certain serotypes and some autoantigens may be significant.[13]

The finding that antibody responses to protein antigens encountered by infection may differ markedly in isotype from the response to immunization with soluble proteins has important implications for the vaccine development. The regulation of IgG subclass responses in man is poorly understood, and the distinct IgG subclass responses to M protein and SLO provide a model for dissecting isotype regulation to protein antigens.

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