Clin. exp. Immunol. (1979) 37, 247–258.

Predominance of immunoglobulin G sub-class 3 among the complement-fixing antibodies to streptococcal M-associated protein

G. E. MORTIMER & JEAN P. WIDDOWSON *Cross-Infection Reference Laboratory, Central Public Health Laboratory, Colindale Avenue, London*

(*Accepted for publication* 10 *January* 1979)

SUMMARY

During investigations of the absorption of group-A streptococcal antibodies from human sera by a protein A-positive *Staphylococcus aureus* strain, we found that the complement-fixing antibodies to M-associated protein (MAP) were only partially absorbed from the majority of sera tested, although they were shown to belong to the immunoglobulin G (IgG) class by density gradient centrifugation. In contrast, other streptococcal antibodies: anti-streptolysin O (ASO), anti-deoxyribonuclease B (anti-DNAase B), 'bactericidal' M antibody and anti-opacity factor (anti-OF), were completely absorbed from all but a minority of sera. We suggest that the complement-fixing antibodies to MAP may be of restricted heterogeneity and have an abnormal IgG sub-class distribution, with a marked predominance of IgG3 (the only sub-class that does not interact with protein A) over the IgG1 and IgG2 sub-classes; IgG4 does not participate in complement fixation. The concentration and relative porportions of IgG sub-classes are believed to be genetically influenced, so our findings may have some bearing on the immune responsiveness of different individuals to streptococcal infection, and possibly have important implications in the development of the secondary sequelae.

INTRODUCTION

The extracellular products of the group-A streptococcus, for example, streptolysin O and DNAase B, elicit a rapid antibody response that reaches a maximum 2–3 weeks after infection and then declines progressively during the next 4–6 months (Stollerman, 1975). Tests for these antibodies are thus useful in providing the evidence of recent infection that is needed for the diagnosis of acute rheumatic fever (ARF) and acute post-streptococcal glomerulonephritis (AGN). Antibodies to the cellular antigens of group-A streptococci, e.g., cell-wall carbohydrate, M protein, opacity factor and M-associated protein are slower to appear than antibodies to extracellular substances, but may persist in the serum for many months or even years after infection (Lancefield, 1959; Dudding & Ayoub, 1968; Widdowson *et al.*, 1974a). Thus, the diagnostic value of antibody tests to cellular antigens is severely limited in the individual patient, although such tests may be of value in epidemiological studies (Maxted, Widdowson & Fraser, 1973; Widdowson *et al.*, 1974b) and in certain groups of patients, in whom prolonged persistence of elevated levels of particular antibodies are characteristic of a particular disease, e.g. the marked persistence of antibodies to cell-wall carbohydrate in patients with valvulitis of streptococcal origin (Schulman *et al.*, 1974). Titres of antibody to M-associated protein (MAP) are invariably high ($\geqslant 80$) and persistent in patients with rheumatic fever (Widdowson, Maxted & Pinney, 1971; Widdowson *et al.*, 1974a,b), and a simple test to distinguish anti-MAP antibodies of recent origin belonging to the IgM

Correspondence: Dr J. P. Widdowson, Cross-Infection Reference Laboratory, Central Public Health Laboratory, Colindale Avenue, London NW9 5HT.

0099-9104/79/0800-0247$02.00

class, from IgG antibody, would greatly increase the diagnostic value of the test. Such a distinction between IgG and IgM antibodies might also be an aid to the interpretation of raised ASO and anti-DNAase B titres in patients with suspected acute glomerulonephritis (AGN) and acute rheumatic fever (ARF), especially when paired sera are not available.

Simple tests based on the ability of staphylococcal protein A (SPA) to interact with the Fc portion of IgG (Forsgren, 1968) and thus remove IgG but not IgM antibodies from human serum, have been used to determine whether patients possess recently acquired antibody to various agents (Ankerst *et al.*, 1974; Schmitz *et al.*, 1975). SPA reacts with the human IgG of sub-classes 1, 2 and 4, but not with sub-class 3 (Kronvall & Williams, 1969), but because IgG3 constitutes only about 5% of the total IgG in normal human serum (Morell *et al.*, 1972) it is assumed that absorption with SPA removes most of the IgG.

We proposed to use a SPA absorption method, similar to that of Ankerst *et al.* (1974), to investigate the antibody class of several streptococcal antibodies; ASO, anti-DNAase B, anti-MAP, anti-M and anti-OF (Maxted *et al.*, 1973). During these studies, we observed that complement-fixing antibodies to MAP were unusual in that, although they were confined to the 7S category in a density gradient, they were only partially absorbed by protein-A-positive staphylococci.

MATERIALS AND METHODS

Human sera. A total of twenty-four sera were absorbed with a protein A-positive *Staph. aureus* strain. Twenty-one of these were from patients aged between 5 and 60 years and all gave evidence of recent streptococcal infection (ASO titres $\geqslant 200$, anti-DNAase B titres $\geqslant 250$, or both). They included sera from patients with acute rheumatic fever (ARF) (eleven), acute glomerulonephritis (AGN) (six) and other suspected streptococcal diseases or sequelae: rheumatic heart disease (one), erythema nodosum (one), polyarthritis (one), streptococcal septicaemia (one). They were selected to cover a range of anti-MAP titres from 65 to 825. The remaining three sera were from laboratory workers, two of these (G.M. and C.N.) were from persons who had persistently raised titres (> 80) of anti-MAP and one (W.R.M.) had an anti-MAP titre of < 10; none of these sera gave evidence of recent streptococcal infection.

The twenty-one sera from patients were tested for their immunoglobulin levels, including the percentage IgG3 content. More detailed experiments were performed on: (1) the three sera from laboratory workers, and (2) three sera from patients (Nos. S76/111, S77/260 and S77/309) with clinical evidence of ARF and serological evidence of recent streptococcal infection.

As controls we used twenty-two sera from patients aged 5–60 years who, although they had various symptoms suggestive of recent streptococcal infection, e.g. joint pains, pyrexia, haematuria or skin rashes, had no serological evidence of recent streptococcal infection (both the ASO and the anti-DNAase B titre < 50). They were selected because they also had anti-MAP titres of < 10.

Staphylococcal strains and preparation of absorbing suspensions. The protein A-positive strain was *Staph. aureus* Cowan I (No. NCTC8530) and the protein A-negative strain used as a control in some experiments was *Staph. aureus* Wood 46 (No. NCTC7121). The strains were grown in Nutrient Broth No. 2. (Oxoid Ltd) in 15-l lots in a stirred fermentor vessel (LH Engineering Ltd) for 18 hr at 37°C. A 10% suspension of washed cells was prepared as described by Kronvall (1973) and could be stored at 4°C for periods of up to 1 month without loss of absorbing activity.

Absorption of sera. The sediment from 1 ml of 10% suspension was used to absorb 1 ml of a 1 in 10 dilution of serum. The standard procedure consisted of two absorptions at 37°C for 45 min (Ankerst *et al.*, 1974).

Serological tests. Anti-DNAase B titres. These were determined by the micromethod of Nelson, Ayoub & Wannamaker (1968).

Anti-streptolysin O titres. These were estimated by a micro-titration modification of the method of Gooder & Williams (1961). Streptolysin O and anti-streptolysin O (Wellcome Reagents Limited) were titrated against the International ASO Standard (State Serum Institute, Copenhagen, Denmark) before use.

Antibody to MAP. This was measured by a complement-fixation (CF) test with a purified MAP antigen from an M-type 30 strain (Widdowson *et al.*, 1971).

'Bactericidal' M antibody and anti-opacity factor. These were measured as described by Maxted *et al.* (1973).

Double gel diffusion tests. These tests (Ouchterlony, 1958) were performed with 1% w/v agarose (Koch Light Ltd) in veronal buffer, pH 8·6, I = 0·033.

Immunoelectrophoresis. This was performed according to Scheidegger (1965) with gels of 1% w/v agarose in veronal buffer, pH 8·6, I = 0·033, at 200 V and 2 mA per 7·5 × 2·5 cm slide for 120 min.

IgG concentrations. IgG concentrations in absorbed and unabsorbed sera were estimated by single radial immunodiffusion (Mancini, Carbonara & Heremans, 1965). Anti-IgG sera (Wellcome Reagents Limited) were incorporated in 1% w/v agarose gel buffered with 0·1 M glycine and 0·04 M EDTA, pH 7·0. The system was standardised with preparations from Nordic Laboratories. IgG3 concentrations were determined by the use of anti-IgG3 and standard sera obtained from the Department of Experimental Pathology, Birmingham University. After diffusion for 24 hr, the plates were washed, dried and stained with Coomassie Blue and the zones measured with vernier gauge calipers. The content (g/l) of immunoglobulin was estimated from a standard curve.

Density gradient centrifugation. Gradients containing from 12·5 to 37·5% w/v sucrose were prepared and the serum samples (0·25 ml) added and centrifuged at 147,000 *g* for 18 hr at 4°C. Fractions (0·2 ml) were collected from the bottom of the tube and analysed for IgG and IgM content by single radial immunodiffusion or double-gel diffusion in gels containing appropriate concentrations of anti-IgG and anti-IgM sera (Wellcome Reagents Limited) and for anti-MAP antibodies by the CF test.

Isoelectric focusing (IEF) of serum. Human serum (2–3 ml) was dialysed overnight against 1% w/v glycine and introduced in the light solution into a LKB 110 ml column, as described in the LKB Manual (1-8100-E02). Ampholines 1% v/v were used in the pH range 3·5–10. The sample was focused for a minimum of 24 hr at 600 V × 2 mA after the gradient was established to give a total running time of 44–48 hr. The temperature was maintained at 14°C by means of cooled water circulated through the column jacket. Fractions (2 ml) were pumped from the bottom of the column at a rate of 1 ml/min and the pH of each fraction measured at 14°C. Fractions were monitored for the presence of total IgG, IgG1 and IgG3 by double-gel diffusion; anti-DNAase B and anti-MAP antibody were tested for as described above. The electrophoretic mobility of some of the IgG fractions was tested by immunoelectrophoresis against anti-IgG serum.

RESULTS

Absorption of streptococcal antibodies from human sera by Staph. aureus strain Cowan I

Twenty-one sera from patients with elevated titres of one or more streptococcal antibodies (see the Materials and Methods section) were absorbed with the washed cells of the protein-A-positive strain. The absorbed and unabsorbed sera were titrated in the ASO, anti-DNAase B and anti-MAP CF tests, and the percentage reduction in titre was calculated for each serum. The results are represented dia-

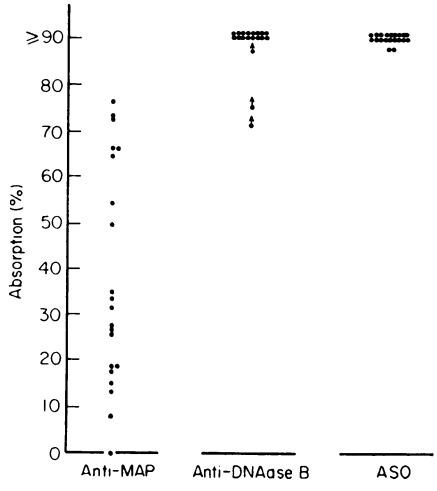

FIG. 1. Percentage absorption by protein A-positive staphylococci of antibodies to MAP, streptolysin O and desoxyribonuclease B, from the sera of patients with recent streptococcal infection. ↑ = Greater than the indicated percentage absorption, the actual value could not be calculated.

grammatically in Fig. 1; the partial absorption of anti-MAP antibodies for most of the sera contrasts sharply with the almost total removal of ASO and anti-DNAase B antibodies from nearly all of the sera. The percentage absorption of MAP antibodies ranged from 0 to over 70%, but more than half (thirteen out of twenty-one) of the sera showed less than 50% absorption. The amount of IgG in the absorbed and unabsorbed sera was measured by single radial immunodiffusion against the anti-IgG and this showed that in nineteen out of twenty-one sera over 90% of the IgG had been removed by two absorptions.

A serum of a laboratory worker (W.R.M.) with bactericidal (anti-M) antibody to types 3, 12 and 22 and persistently high titres of antibody to type-2 and type-22 opacity factor (OF) was absorbed with the Cowan I strain. An indirect bactericidal test on the same serum samples showed that anti-M antibodies to all three M-types had been absorbed as the sera absorbed with Cowan I had no bactericidal activity against the appropriate test strains. An anti-OF test (Maxted *et al.*, 1973), on the absorbed and unabsorbed sera, demonstrated that all OF-antibody had been absorbed by the staphylococcal suspension.

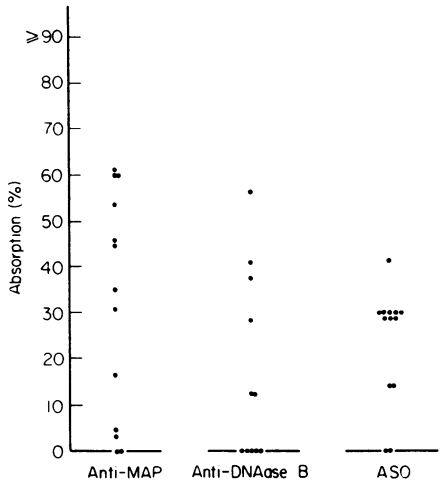

FIG. 2. Percentage absorption by protein A-negative staphylococci of antibodies to MAP, streptolysin O and desoxyribonuclease B, from the sera of patients with recent streptococcal infection.

Absorption of streptococcal antibodies by Staph. aureus strain Wood 46

Thirteen of the twenty-one patients' sera were also absorbed with the protein-A-negative strain Wood 46, and the percentage absorption of ASO, anti-DNAase B and complement-fixing MAP antibodies was estimated. Most of the sera exhibited small ($\leqslant 30\%$) reductions in ASO and anti-DNAaseB titre, and only one serum showed a reduction of above 50% for either antibody (in this case, anti-DNAase B:56%). On the other hand, the absorption of MAP antibodies by the Wood 46 strain varied from 0 to around 60% and approximately half of the sera showed absorptions of between 40% and 60% (see Fig. 2). A small reduction in titre is to be expected due merely to the physical handling of the serum, but absorptions of 40% or more by the protein A-negative strain are difficult to explain. This phenomenon of 'non-specific' uptake of IgG by the Wood 46 strain has also been observed by workers studying the absorption of toxoplasmosis and anti-nuclear antibodies (Chantler *et al.*, 1976). The number of sera (thirteen) used to compare the extent of absorption by the Cowan I and Wood 46 strain was too small to draw any definite conclusions, but it appears that the sera from which anti-MAP was most readily absorbed by the Cowan I strain showed the greatest reduction in titre after Wood 46 absorption. It is therefore possible that some of the absorption of anti-MAP by the Cowan I strain is also 'non-specific', and that the extent of 'true' absorption of anti-MAP by staphylococcal protein A is even lower than the results in Fig. 1 would suggest. Absorption with the Wood strain had no effect in the anti-OF and anti-M antibody content of serum W.R.M.

Investigation of antibody class of MAP antibodies by density gradient ultracentrifugation

The possibility that MAP antibodies were not absorbed by the Cowan I strain because they belong to the IgM and not the IgG class was investigated. Six sera, in which the anti-MAP titre was reduced by less than 30% by Cowan I absorption, were subjected to density gradient ultracentrifugation. The fractions were analysed for IgG, IgM and complement-fixing antibodies to MAP. In all six samples the anti-MAP activity was associated with the 7S peak and no activity could be detected in the 19S peak (Fig. 3).

It has been suggested (Hällgren & Wide, 1976; Kekomäki, 1977) that the absorption by staphylococcal protein-A of IgG present as immune complexes or as aggregates is inhibited in the presence of a greater concentration of monomeric IgG. It seemed unlikely that antibody to MAP existed as a complex because none of the sera examined exhibited any obvious high molecular weight IgG in a density gradient. Moreover, the anti-MAP titre could not be decreased further by additional absorptions. In sera containing aggregates, this procedure would favour further absorption because of the prior removal of monomeric IgG, leaving any complexes or aggregates free to react with the staphylococci.

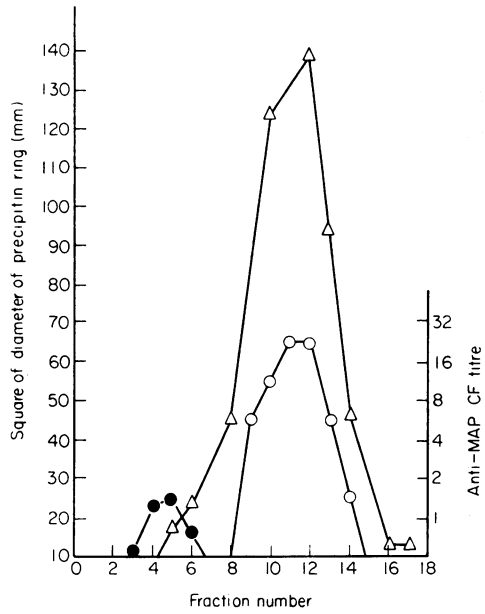

FIG. 3. Sucrose density gradient determination of antibody class of complement-fixing anti-MAP antibodies in human sera. (●—●) Radial diffusion against anti-IgM serum, (△——△) radial diffusion against anti-IgG serum, and (○—○) complement fixation titre of fractions with MAP antigen.

TABLE 1. Effect of absorption with a protein A-positive *Staph. aureus* strain (Cowan I) on the anti-MAP titre, the levels of IgG1 and IgG3, and the total IgG content of six human sera

			IgG content*		Anti-MAP		Precipitin reaction* in gel against:	
Serum no.	Diagnosis	No. of absorptions*	(g/l)	Percentage remaining†	Titre	Percentage After absorption‡	Anti-IgG1	Anti-IgG3
S76/111	?ARF, joint	None	50·0		825		++	++
	pains	2	0·64	12·8	600	72·7	−	++
S77/260	ARF	None	4·35		240		++	++
		2	0·27	6·1	160	67·7	−	++
S76/280	ARF	None	3·38		130		++	+
		2	0·17	5·1	30	23·0	−	+
S76/282	ARF	None	2·50		90		++	±
		2	0·05	2·0	30	33·3	−	±
G.M.	Normal	None	2·00		110		++	±
		2	0·08	4·0	50	45·5	−	±
W.R.M.	Normal	None	1·75		< 10		++	−
		2	0·05	2·6	< 10	—	−	−

* All sera were diluted 1 in 10 before absorption. The IgG content and the precipitin reactions against the sub-class sera were determined for the 1 in 10 dilution.

† Residual IgG of absorbed serum expressed as a percentage of the IgG content of the unabsorbed sample.

‡ Anti-MAP titre of absorbed serum expressed as a percentage of the original titre.

— = Not calculable.

Precipitin reactions: ++ = strong reaction, + = moderate reaction, ± = weak reaction, − = no reaction.

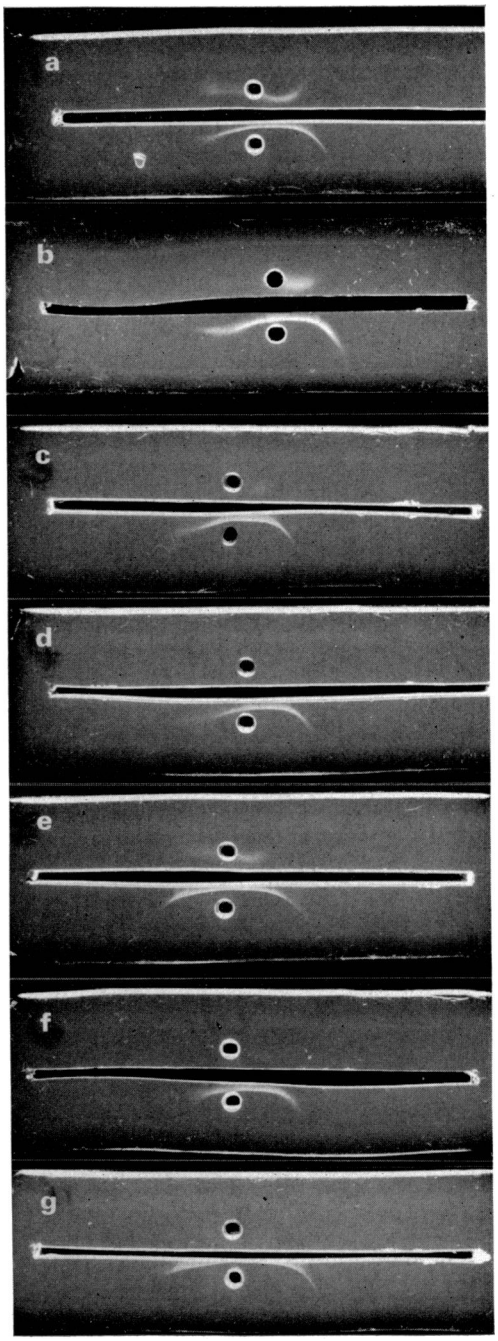

FIG. 4. Immunoelectrophoresis of human sera absorbed with protein A-positive staphylococci. (a) S76/111, (b) S77/260, (c) S76/280, (d) S76/282, (e) G.M. (undiluted), (f) G.M., and (g) W.R.M. (undiluted). Uppermost well contains the absorbed aliquot and the lower well the unabsorbed aliquot. All sera were tested at a dilution of 1 in 10 unless otherwise indicated. All troughs were filled with anti-IgG serum; the cathode is to the right of the figure.

Characterization and measurement of IgG remaining after absorption with Staph. aureus strain Cowan I

Having established that the complement-fixing antibodies to MAP belonged to the IgG class but were not absorbed by a protein A-positive *Staph. aureus* strain, we investigated the nature and quantity of the residual IgG that apparently contained most, if not all, of the anti-MAP activity in absorbed sera.

Selected sera were tested, before and after absorption, against anti-human IgG serum by immunoelectrophoresis and single radial immunodiffusion. In addition, several absorbed sera were tested against sub-class-specific antisera to IgG1 and IgG3 by double gel diffusion. The IgG2 and IgG4 contents of the absorbed sera were not tested since it is known that IgG4 cannot fix complement and IgG2 is only weakly active in this respect (Natvig & Kunkel, 1973). It has been shown by Kronvall & Williams (1969) that myeloma proteins of sub-class 3, unlike those belonging to sub-classes 1, 2 or 4, do not react with staphylococcal protein-A and it seemed possible that complement-fixing anti-MAP antibodies were not absorbed because they belonged predominantly to sub-class 3.

Table 1 shows the results of double gel diffusion analysis of six sera against anti-IgG1 and anti-IgG3 sera. All the sera tested gave a strong precipitin line with antisera to sub-class 1 at a dilution of 1 in 10, but after absorption they failed to react even when undiluted. On the other hand, the reactions of the absorbed sera with anti-IgG3 were virtually undiminished in comparison with those of the corresponding unabsorbed sera. Serum W.R.M. diluted 1 in 10, gave no reaction with the anti-IgG3 serum and only a moderate to weak reaction when tested undiluted. These results support those of Kronvall & Williams (1969), and show that two absorptions with a protein-A-positive strain remove all detectable IgG1 but leave the IgG3 content of the sera unreduced. Table 1 also gives the values for the total IgG content of the absorbed and unabsorbed sera, and it can be seen that the sera with the greater concentration of unabsorbed IgG (Nos S76/111 and S77/260) gave the strongest precipitin lines with antisera to sub-class 3. In contrast, sera S76/282 and W.R.M. both with very much lower levels of IgG in the absorbed samples, gave either weak or negative results with the anti-IgG3 serum.

The sera described in Table 1 were also subjected to immunoelectrophoresis against anti-IgG serum. Insufficient IgG3 serum of suitable quality was available for use in this test. The results (Fig. 4) show that all six unabsorbed sera exhibited the wide precipitin arc characteristic of IgG, but after absorption, only 1 in 10 dilutions of the sera S77/260 and S76/111 showed any reaction, although a faint line was seen with serum S76/280. The reaction given by the absorbed samples of these sera, both of which had high anti-MAP titres before and after absorption with the Cowan I strain (see Table 1), differed qualitatively from the reactions of the corresponding unabsorbed samples in that the precipitin arcs obtained were much shorter and very hazy. Such precipitin arcs resemble those described by Yount, Kunkel & Litwin (1967) for IgG3 proteins from both normal and myeloma sera which demonstrated a partial absorption at the origin.

The 1 in 10 dilutions of the other four sera tested had no detectable IgG in the absorbed samples by this method; serum G.M. in the undiluted, absorbed state gave a short hazy line similar to the diluted samples of sera S76/111 and S77/260.

That the precipitin arc demonstrated by certain absorbed sera was not merely a feature of those with very high total serum IgG (as in Nos S76/111 and S77/260) and thus only reflecting a small residue of total IgG left unabsorbed, could be shown by the lack of any precipitating line in the absorbed portions of sera S76/574 and S76/270. The IgG concentrations of the latter two sera were respectively 50 g/l and 47 g/l before, and 2·00 g/l and 2·10 g/l after absorption.

Investigation of the sub-class distribution of streptococcal antibodies by isoelectric focusing

Five sera were examined by this technique: G.M., C.N. and W.R.M. from laboratory workers and S77/260 and S77/309 from patients with rheumatic fever.

After focusing, fractions were tested by immunoelectrophoresis against anti-IgG serum. The results (Fig. 5) show that as its pI increased the IgG became more slow moving towards the anode and a gradation of electrophoretic mobilities could be seen similar to that obtained by Howard & Virella (1969). When tested by gel diffusion with sub-class specific antisera, only fractions from sera G.M.

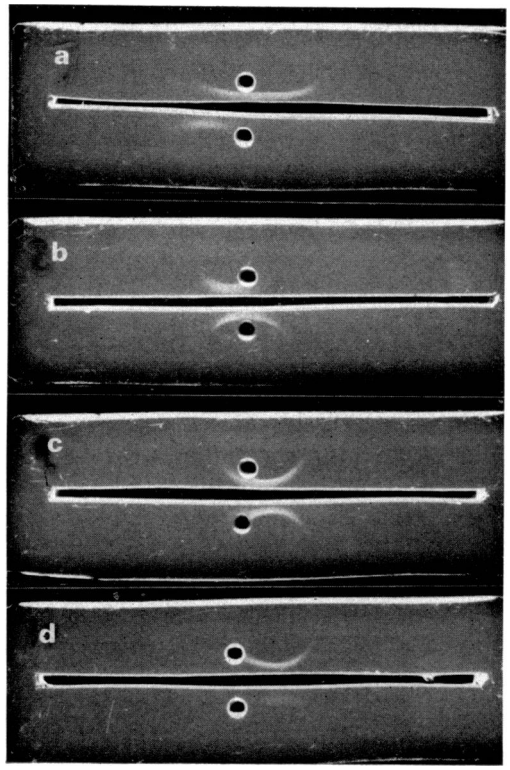

FIG. 5. Immunoelectrophoresis of isoelectric focusing fractions against anti-IgG serum. Results of double gel diffusion precipitation are indicated in parentheses. (a) Top half: whole normal human serum, bottom half: pI = 6·06; (IgG+, IgG3−); (b) top: pI = 6·80; (IgG++, IgG3−), bottom: pI = 7·40; (IgG+++, IgG3−); (c) top: pI = 8·06; (IgG+++, IgG3+) bottom: pI = 8·65; (IgG+++, IgG3+); (d) top: pI = 9·14; (IgG+++, IgG3++) bottom: pI = 10·35; (IgG+, IgG3−).

S77/260 and S77/309, in the pH range 8·59–9·36, contained IgG3. Fractions from serum W.R.M. contained no detectable IgG3 and serum C.N. was not tested. In contrast, IgG1 could be detected over a very wide range (6·07–10·35) in all sera tested. Antisera to whole IgG gave precipitin lines over the range 5·65–11·68. The rather narrow range of pI covered by IgG3 resulted in a more restricted electrophoretic mobility on immunoelectrophoresis in comparison with whole IgG, and the peak fractions of IgG3 activity (pI = 9·14) gave a short, rather hazy precipitin line directed towards the cathode. This line was very similar to that obtained on immunoelectrophoresis of the IgG remaining after absorption of certain sera with the Cowan I strain (see Fig. 4). Fig. 6 shows the elution profile and precipitation results for serum S77/309.

The fractions were also monitored for anti-DNAase B and complement-fixing antibodies to MAP. Anti-DNAase B activity was detected over a wide pH range (5·65–9·56), with peaks of activity at 6·66 and 8·72. The main peak (pI 8·72) corresponded with the peak of total IgG. Thus, the anti-DNAase B activity of this serum, which could be completely absorbed by protein A-positive staphylococci, appeared to be unrestricted in its distribution into different IgG sub-classes. The detection of complement-fixing antibodies to MAP after isoelectric focusing presented serious technical problems. No activity could be detected in the fractions of IgG with pIs of 5·65–8·25. This was to be expected if the anti-MAP antibodies were indeed of restricted heterogeneity as our absorption results suggested. However, the IgG3-containing fractions showed non-specific binding of complement in all five sera tested, so we were unable to detect any specific fixation with our MAP antigen in this pI range (8·26–9·56). The treatment of fractions with excess complement, titration in the presence of 8 M urea (Capra & Kunkel, 1970), or incorporation of 8 M urea into the column gradient failed to eliminate the complement-binding effect. Serum S77/260 was absorbed with the Cowan I strain before application to the column and again the

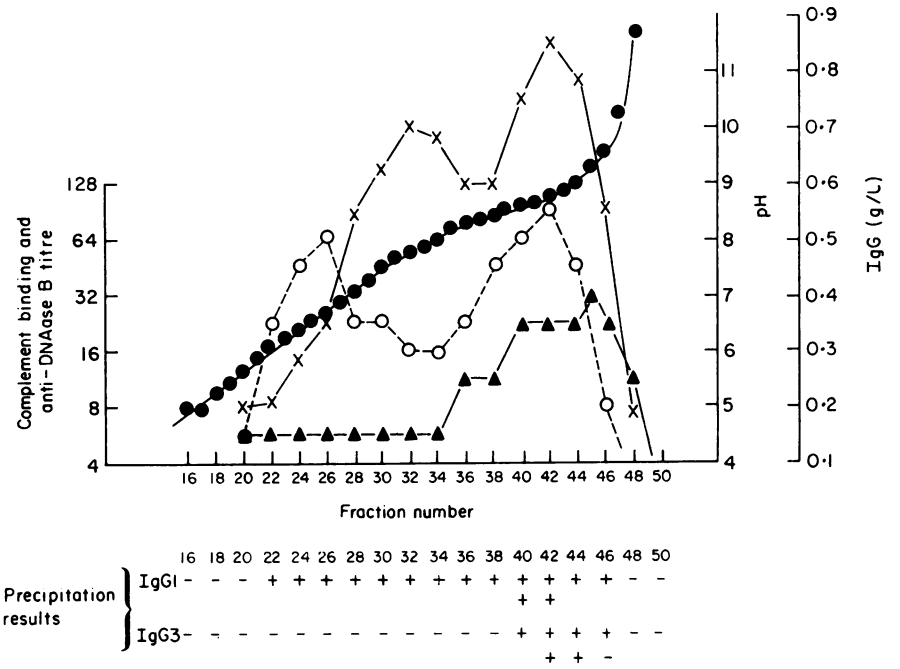

FIG. 6. Isoelectric focusing of human serum—distribution of complement-binding and anti-DNAase B antibodies. (●——●) pH gradient, (▲——▲) complement-binding titre (see text), (○ — — ○) anti-DNAase B titre and (×——×) IgG concentration.

same peak of anti-complementary activity was detected. Fractions from this peak gave positive precipitin reactions with anti-IgG3 but not with anti-IgG1, which suggests that this effect is probably the result of concentrating IgG3 at its isoelectric point. The avid attraction and binding of complement is a known property of IgG3 (Capra & Kunkel, 1970; Natvig & Kunkel, 1973), and IgG3 has been shown to have a marked tendency to form aggregates especially if present in high concentration (Capra & Kunkel, 1970).

Levels of IgG3 in patients with evidence of recent streptococcal infection

Very little is known about the levels of IgG3 in patients with various diseases. To investigate the possibility that patients with high anti-MAP titres have higher levels of IgG3 (as a percentage of total IgG) than individuals with no evidence of recent streptococcal infection we measured, by radial immunodiffusion against the appropriate antisera, the total IgG and IgG3 content of twenty-one sera from patients with evidence of recent streptococcal infection (ASO $\geqslant 200$, anti-DNAase B $\geqslant 250$, anti-MAP $\geqslant 40$) and twenty-two sera from persons with no evidence of recent streptococcal infection (ASO < 50, anti-DNAase B < 50, anti-MAP < 10). The difference in total IgG and IgG3 content between the two groups was highly significant ($P = < 0{\cdot}001$). The sera from patients with evidence of recent streptococcal infection had an average IgG content of 30·9 g/l (range$\pm$1s.d., 22·3–42·9 g/l) in comparison with 18·4 g/l (range 11·5–29·2 g/l) for the control group. The levels of IgG3 were 0·69 g/l (range 0·33–1·44 g/l) in the patients in comparison with 0·30 g/l (range 0·16–0·58 g/l) in the controls. However, the difference in IgG3 levels expressed as a percentage of the total IgG level in each group (2·2% in the patients in comparison with 1·6% in the controls) was not significant at the $P=0{\cdot}05$ level.

DISCUSSION

Antibodies against streptolysin O and anti-DNAase B were almost completely removed from the sera of twenty-one patients with evidence of recent streptococcal infection by absorption with protein A-positive staphylococci, and none of the sera contained demonstrable amounts of ASO or anti-DNAase

belonging to the IgM class. This is in agreement with the findings of Sonazaki, Takizowa & Torisu (1970) who detected ASO of the IgM class only in the sera of very young children (< 2 years old) suspected of experiencing their first streptococcal infection. Thus, absorption with protein-A does not appear to be a useful adjunct to the conventional serological tests when applied to the detection of recent streptococcal infection, because patients with suspected ARF or AGN are very likely to have experienced more than one previous streptococcal attack.

In our studies, the ease of removal of ASO, anti-DNAase B, anti-M and anti-opacity factor antibodies contrasted sharply with the incomplete absorption of the complement-fixing antibodies to MAP by the protein A-positive staphylococcus. Absorption of the same sera by a protein A-negative strain (Wood 46) resulted unexpectedly in a greater reduction of anti-MAP titre than of the ASO or anti-DNAase B titres. This raised the possibility that the extent of the 'true' interaction of anti-MAP with protein A on the Cowan I strain may have been even less than was suggested by the results in Fig. 1. Although the strain Wood 46 is considered to be devoid of protein A, one group of workers (Maxim, Mathews & Mengoli, 1976) have suggested that it may contain small amounts. Even if this is so, it does not explain why the anti-MAP titres were more affected than the ASO or anti-DNAase B titres; possibly both strains contain substances other than protein A that interact with MAP antibodies.

Density gradient ultracentrifugation showed that the complement-fixing anti-MAP antibodies belonged to the IgG and not the IgM class. Of the four human subclasses of IgG, only sub-class 3 is unreactive with protein A (Kronvall & Williams, 1969). We have shown that two absorptions with the Cowan I strain removed all detectable IgG1 but left the IgG3 content of the sera virtually undiminished. This suggested that the complement-fixing antibodies to MAP antigen were of restricted heterogeneity and may belong disproportionately to the IgG3 subclass.

The alternative explanation that IgG3 antibodies are much more active than IgG1 in complement fixation reactions, so that removal of all the IgG1 does little to reduce the CF titre of a serum, seems unlikely from the evidence presented here. Although it has been shown that IgG3 is somewhat more efficient than IgG1 in complement fixation (Müller-Eberhard 1968,), in order to explain our results on this basis IgG3 would have to be at least twenty times more active than IgG1, because of the low serum concentration of IgG3. Moreover, complement-fixing IgG antibodies directed against other bacterial and viral antigens are almost completely absorbed by protein A. Thus, Christensen *et al.* (1976) demonstrated protein A absorption of CF antibodies to surface antigens of *N. gonorrhoeae*. Serum levels of CF antibodies to cytomegalovirus have been seen to be reduced by at least 80% by absorption with the Cowan I strain (G. E. Mortimer, unpublished observations).

We have used only one immunological method to measure MAP antibodies and, in future experiments, it will be necessary to use other methods not involving complement fixation and also to measure antibody in terms of μg of IgG. The mass of immunoglobulin represented by an antibody titre may vary widely and depends both on the nature of the antibody and the method used to detect it.

IgG3 proteins have a number of special properties (Natvig & Kunkel, 1973), including a striking concentration-dependent aggregation, which probably affects their affinity for complement. This property hindered our attempts to demonstrate the restriction of CF anti-MAP antibodies to the IgG3-containing fractions in our isoelectric focusing experiments, because all fractions containing IgG3 bound complement in the absence of antigen. Significantly, however, none of the fractions that contained IgG1 in the absence of IgG3 fixed complement with MAP antigen, demonstrating that IgG1 molecules are not concerned in the reactions observed with whole serum.

Relatively few quantitative observations have been made on the class and sub-class distribution of antibodies to specific antigens (Speigleberg, 1974), but IgG sub-class antibodies to most protein antigens investigated, e.g. diphtheria and tetanus toxin, seem to be formed in quantities relatively similar to the sub-class distribution in normal sera. The immune response to carbohydrate and teichoic acid antigens, on the other hand, often appears to be restricted to sub-class 2. Anti-rheumatoid factor antibodies are found predominantly in sub-classes 1 and 3 and anti-DNA antibodies are often restricted, but may belong predominantly to any one of the four sub-classes.

The exact nature of MAP antigen is not known, although its sensitivity to proteolytic enzymes suggests

that it is largely protein in nature (Widdowson *et al.*, 1971). Other workers (Vosti, Johnson & Dillon, 1971; Beachey & Stollerman, 1971) have also described non-type-specific M-associated protein antigens similar to MAP, but more recently Beachey, Ofek & Bisno (1973) have suggested that a teichoic acid component may be important. There are also certain similarities between MAP antigen and the streptococcal membrane antigen that elicits heart-reactive antibody (HRA) in patients with rheumatic fever (Zabriskie, Hsu & Seegal, 1970; Widdowson *et al.*, 1971). It is therefore interesting to note a recent suggestion that HRA isolated from the sera of rheumatic fever patients may also be of restricted heterogeneity (van de Rijn & Zabriskie, 1977).

The concentration and relative proportions of IgG sub-classes are believed to be genetically influenced, so our findings may have some bearing on the immune responsiveness of different individuals to streptococcal infection with possible implications for the development of secondary sequelae.

REFERENCES

Ankerst, J., Christensen, P., Kjellen, L. & Kronvall, G. (1974) A routine diagnostic test for IgA and IgM antibodies to Rubella virus. Absorption of IgG with *Staphylococcus aureus J. infect. Dis.* **130**, 268.

Beachey, E.H., Ofek, I. & Bisno, A.L. (1973) Studies of antibodies to non-type specific antigens associated with streptococcal M protein in the sera of patients with rheumatic fever. *J. Immunol.* **111**, 1361.

Beachey, E.H. & Stollerman, G.H. (1971). Toxic effects of streptococcal M protein on platelets and polymorphonuclear leukocytes in human blood. *J. exp. Med.* **134**, 351.

Capra, J.D. & Kunkel, H.G. (1970) Aggregation of γ G_3 proteins. Relevance to the hyperviscosity syndrome. *J. clin. Invest.* **49**, 610.

Chantler, S., Devries, E., Allen, P.R. & Hurn, B.A.L. (1976) A rapid immunofluorescent procedure for the detection of specific IgG and IgM antibody in sera using *Staphylococcus aureus* and latex-IgG as absorbents. *J. Immunol. Methods*, **13**, 367.

Christensen, K.K., Christensen, P., Mardh, P. & Weström, L. (1976) Quantitation of serum antibodies to surface antigens of *Neisseria gonorrhoeae* with radiolabelled protein A of *Staphylococcus aureus*. *J. infect. Dis.* **134**, 317.

Dudding, B.A. & Ayoub, E.M. (1968) Persistence of streptococcal group A antibody in patients with rheumatic valvular disease. *J. exp. Med.* **128**, 1081.

Forsgren, A. (1968) Protein A from *Staphylococcus aureus*. V. Reaction with guinea pig γ G-globulins. *J. Immunol.* **100**, 921.

Gooder, H. & Williams, R.E.O. (1961) *Titration of antistreptolysin O. Ass. clin. path.*, Broadsheet No. 34 (new series). London.

Hällgren, R. & Wide, L. (1976) Detection of circulating IgG aggregates and immune complexes using ^{125}I protein A from *Staphylococcus aureus*. *Ann. rheum. Dis.* **35**, 306.

Howard, A. & Virella, G. (1969) The separation of pooled human IgG into fractions by isoelectric focusing; and their electrophoretic and immunological properties. *Protides biol. Fluids*, **17**, 449.

Kekomäki, R. (1977) Detection of platelet bound IgG with ^{125}-labelled staphylococcal protein A. *Med. Biol.* **55**, 112.

Kronvall, G. (1973) A rapid slide agglutination method for typing pneumococci by means of specific antibody absorbed to protein A-containing-staphylococci. *J. med. Microbiol.* **6**, 187.

Kronvall, G. & Williams, R.C. (1969) Differences in anti-protein A activity among IgG subgroups. *J. Immunol.* **103**, 828.

Lancefield, R.C. (1959) Persistence of type-specific antibodies in man following infection with group A streptococci. *J. exp. Med.* **110**, 271.

Mancini, G., Carbonara, A.O. & Heremans, J.F. (1965) Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry*, **2**, 235.

Maxim, P.E., Mathews, H.L. & Mengoli, H.F. (1976) Single tube mixed agglutination test for detection of staphylococcal protein A. *J. clin. Microbiol.* **4**, 418.

Maxted, W.R., Widdowson, J.P. & Fraser, C.A. (1973) Antibody to streptococcal opacity factor in human sera. *J. Hyg. Camb.* **71**, 35.

Morell, A., Skvaril, F., Steinberg, A.G., van Loghem, E. & Terry, W.O. (1972) Correlations between the concentrations of the four sub-classes of IgG and Gm allotypes in normal human sera. *J. Immunol.* **108**, 195.

Muller-Eberhard, H.J. (1968) Chemistry and reaction mechanisms of complement. *Adv. Immunol.* **8**, 1.

Natvig, J.B. & Kunkel, H.G. (1973) Human immunoglobulins: classes, sub-classes, genetic variants and idiotypes. *Adv. Immunol.* **16**, 1.

Nelson, J., Ayoub, E.M. & Wannamaker, L.W. (1968) Streptococcal antideoxyribonuclease B: microtechnique determination. *J. Lab. clin. Med.* **71**, 867.

Ouchterlony, O. (1958). Diffusion in gel methods for immunological analysis. *Progr. Allergy*, **5**, 1.

Scheidegger, J. (1965) Une microméthode de l'immunoélectrophorèse. *Int. Arch. Allergy appl. Immunol.* **7**, 103.

Schmitz, H., Shimizu, H., Kampa, D. & Doerr, H.W. (1975) Rapid method to detect Rubella immunoglobulin M and immunoglobulin A antibodies. *J. clin. Microbiol.* **1**, 132.

Schulman, S.T., Ayoub, E.M., Victoria, B.E., Gessner, I.M., Tamer, O.F. & Hernandez, F.A. (1974) Differences in antibody response to streptococcal antigens in children with rheumatic and non-rheumatic mitral valve disease. *Circulation*, **50**, 12, 44.

Sonozaki, H., Takizawa, S. & Torisu, M. (1970) Immunoglobulin analysis of anti-streptolysin-O antibody. *Clin. exp. Immunol.* **7**, 519.

Spiegelberg, H.L. (1974) Biological activities of immunoglobulins of different classes and sub-classes. *Adv. Immunol.* **19**, 259.

Stollerman, G.H. (1975) *Rheumatic fever and streptococcal infection*, p. 35. Grune and Stratton, New York, San Francisco and London.

VAN DE RIJN, I. & ZABRISKIE, J.B. (1977) Purification of heart reactive antibodies from acute rheumatic fever patient's sera. *Annual Meeting of the American Society of Microbiology, May* 1977, *New Orleans.* (Abstract No. E98).

VOSTI, K.L., JOHNSON, R.H. & DILLON, M.F. (1971) Further characterisation of purified fractions of M protein from a strain of group A, type 12 streptococcus. *J. Immunol.* **107**, 104.

WIDDOWSON, J.P., MAXTED, W.R. & PINNEY, A.M. (1971) An M-associated protein antigen (MAP) of group A streptococci. *J. Hyg. Camb.* **69**, 553.

WIDDOWSON, J.P., MAXTED, W.R., NEWRICK, C.W. & PARKIN, D. (1974a) An outbreak of streptococcal sore throat and rheumatic fever in a Royal Air Force training camp; significance of serum antibody to M-associated protein. *J. Hyg. Camb.* **72**, 1.

WIDDOWSON, J.P., MAXTED, W.R., NOTLEY, C.M. & PINNEY, A.M. (1974b) The antibody responses in man to infection with different serotypes of group-A streptococci. *J. med. Microbiol.* **7**, 483.

YOUNT, W.J., KUNKEL, H.G. & LITWIN, S.D. (1967). Studies of the Vi (γ2c) sub-group of γ-globulin. *J. exp. Med.* **125**, 177.

ZABRISKIE, J.B., HSU, K.C. & SEEGAL, B.C. (1970) Heart-reactive antibody associated with rheumatic fever: characterisation and diagnostic significance. *Clin. exp. Immunol.* **7**, 147.