

Antibody to streptococcal cell wall peptidoglycan–polysaccharide polymers in seropositive and seronegative rheumatic disease

P. M. JOHNSON,* K. K. PHUA,* H. R. PERKINS,† C. A. HART‡ & R. C. BUCKNALL§ *Departments of *Immunology, †Microbiology, ‡Medical Microbiology and §Medicine, University of Liverpool, Liverpool, UK*

(Accepted for publication 19 August 1983)

SUMMARY

An ELISA has been developed for serum antibodies to streptococcal cell wall peptidoglycan–polysaccharide polymers (PG–GSP). A significantly increased prevalence of serum anti-PG–GSP antibody was found in juvenile chronic arthritis and both seropositive and seronegative rheumatoid arthritis (RA), compared with ankylosing spondylitis, systemic lupus erythematosus, myeloma and healthy controls. Anti-PG–GSP antibody was always of the IgG class and there was no correlation of anti-PG–GSP levels with C reactive protein, rheumatoid factor (RF) or anti-streptolysin O titres. There was no direct cross-reaction of RF with PG–GSP, nor did the presence of IgM–RF significantly interfere with the assay. Examination of paired serum and synovial fluid samples offered no evidence for local production of anti-PG–GSP antibody in synovial tissue. These data are compatible with an increased systemic immunization by bacterial fragments in RA.

Keywords anti-streptococcal peptidoglycan antibody rheumatoid arthritis ELISA rheumatoid factor

INTRODUCTION

An infectious aetiology has often been discussed as most compatible with both the clinical and pathological features of rheumatoid arthritis (RA) (Zvaifler, 1973; Bennett, 1978), although no single micro-organism has been identified as consistently associated with the development of RA (Marmion, 1978). However, the cell wall skeleton of many Gram positive bacteria contains covalently bound polymers of peptidoglycan (PG) and group specific polysaccharide (GSP), and humoral immunity to streptococcal cell wall preparations has been implicated in the pathogenesis of animal models of both chronic erosive synovitis (Greenblatt, Hunter & Schwab, 1980) and rheumatoid factor (RF) like antibody production (Bokisch *et al.*, 1973).

The PG component of bacterial cell walls is itself a polymer composed of chains of repeating di-hexamine subunits of *N*-acetyl muramic acid and *N*-acetyl glucosamine with attached short peptide side chains that are cross-linked through one of a variety of forms of interpeptide bridges (Krause & McCarty, 1961). The side chains of different bacterial species PG are similar pentapeptide or tetrapeptide structures (Schleifer & Kandler, 1972; Schleifer & Seidl, 1977), and that of nearly all staphylococcal and streptococcal PG is L-Ala- γ -D-Gln-L-Lys-D-Ala-(D-Ala). Extensive immunological cross-reactions have been thought to occur between different bacterial species PG as a result of an immunodominant nature of the common terminal -D-Ala-(D-Ala) peptide (Schleifer & Krause, 1971). Most studies on the biological role of streptococcal PG have used purified bacterial cell wall preparations that are now recognized to also contain some residual

Correspondence: Dr P. M. Johnson, Department of Immunology, Duncan Building, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, UK.

methyl pentose polysaccharide covalently complexed with PG (PG-GSP). It is these preparations that are reported to be arthritogenic (Stewart-Tull, 1980; Fox *et al.*, 1982), capable of macrophage activation (Smialowicz & Schwab, 1977a), complement activation via the alternate pathway (Greenblatt, Boakle & Schwab, 1978; Lambris, Allen & Schwab, 1982), and oligoclonal B cell activation (Dziarski, 1982).

The presence of both D-isomers of amino acids and γ -peptide linkage of iso-glutamine renders the peptide component of PG-GSP resistant to mammalian intracellular proteases (Schwab & Ohanian, 1969; Smialowicz & Schwab, 1977b), and its amphipathic property introduces the possibility of acting as a sequestered antigen in the development of a chronic immune response following initial infection and release of bacterial cell wall debris (Dalldorf *et al.*, 1980). We have therefore developed an ELISA for assessment of humoral immunity to PG-GSP preparations derived from group A *Streptococcus pyogenes*, and have also investigated any interrelationship between RF and anti-PG-GSP.

MATERIALS AND METHODS

Preparation of PG-GSP. Cell wall PG-GSP polymer preparations were derived from Gram positive group A *S. pyogenes* by a modification of the method of Petit, Munoz & Ghuysen (1966). Streptococci were killed with 0.05% NaN₃ soon after optimum growth, and harvested by centrifugation at 12,000g. Cells were washed in cold sterile 0.1 M phosphate buffer, pH 7.0, homogenized until Gram staining was negative, and then heated at 60°C for 1 h to inactivate autolysin. The homogenate was then washed twice by centrifugation at 10,000g before being resuspended in sterile 0.1 M phosphate buffer, pH 7.0, containing 10 μ g/ml ribonuclease and 500 μ g/ml trypsin. After overnight incubation at 37°C, the mixture was centrifuged at 40,000g and the pellet washed in sterile distilled water and lyophilized.

Trypsinized cell wall preparations were also derived similarly from three other Gram positive organisms, *Corynebacterium poinsettiae*, *Bacillus megaterium* and *Micrococcus roseus*, all having PG with the same glycan chain as *S. pyogenes*. *M. roseus* peptide chains are very similar to those of *S. pyogenes* whereas those of *B. megaterium* contain di-aminopimelic acid and have no bridge amino acids, while those of *C. poinsettiae* have a very different peptide chain containing glycine, homoserine and D-ornithine (Schliefer & Kandler, 1972). None of these species would have the same attached polysaccharide as *S. pyogenes*.

The protein content of the various cell wall preparations were assessed by the Lowry method; the methyl pentose content was measured by the cysteine-sulphuric acid reaction of Dische & Shettles (1948), which allowed the estimation of rhamnose independent of peptide and hexose contaminants. Final preparations from *S. pyogenes* contained 16–20% rhamnose by weight and, together with amino acid analyses, were compatible with being polymers of PG together with residual group specific polysaccharide and are hence referred to as PG-GSP polymer preparations.

For use, lyophilized PG-GSP was suspended in phosphate-buffered isotonic saline (PBS), pH 7.2, and sonicated at 4°C for a total of 90 min to partially disrupt the polymeric structure and yield a polydisperse suspension of PG-GSP that was subsequently handled as an essentially soluble preparation.

Other preparations. The group specific polysaccharide (GSP) component was prepared from washed whole *S. pyogenes* by the formamide method of Fuller (1938). The final precipitate was dissolved in distilled water to give a 16 mg/ml rhamnose solution. For the preparation of PG, purified *S. pyogenes* PG-GSP was washed in 5% trichloroacetic acid at 37°C overnight, then washed in distilled water and lyophilized. The dried cell walls were then treated with formamide (Fuller, 1938) to remove remaining carbohydrate. The resultant residue was further washed in distilled water and sonicated to an ultrafine suspension as described earlier. Analysis demonstrated less than 1% rhamnose contamination of the formylated PG preparation.

Two tripeptides, L-Lys-D-Ala-D-Ala (LAA) and di-acetyl-L-Lys-D-Ala-D-Ala (Ac₂-LAA), were prepared synthetically (Nieto & Perkins, 1971). Synthetic muramyl dipeptide (MDP; N-acetyl-muramyl-L-Ala- γ -D-Gln) was obtained from Sigma Chemical Co. (Poole, UK).

Periodate cleavage of PG-GSP. PG-GSP (100 µg/ml) was incubated in 1 mM sodium metaperiodate, pH 2.8 for 30 min at room temperature. The reaction was then stopped by dilution in excess 0.05 M carbonate-bicarbonate buffer, pH 9.6, and the final pH adjusted to pH 9.6 with 0.1 M NaOH. As control, PG-GSP was also incubated with buffers without sodium metaperiodate.

Rabbit antiserum to PG-GSP. An adult New Zealand white rabbit was immunized by multiple site intramuscular injection of 1 mg *S. pyogenes* PG-GSP in sterile saline with Freund's incomplete adjuvant. This was repeated after 21 days, and again after another 21 days. Antiserum was obtained 10 days later and anti-PG-GSP antibody assessed by double radial immunodiffusion and ELISA. Pre-immune rabbit serum (NRS) taken prior to the first immunization served as a control serum.

Double radial immunodiffusion (DRID). Antibody to *S. pyogenes* PG-GSP was shown in DRID using 1% agarose gels in 0.05 M tris-barbitone-lactate buffer, pH 8.6. Neat serum samples were diffused against 1-4 mg/ml soluble PG-GSP. Following overnight incubation in a moist chamber, gels were pressed, repeatedly washed and stained with Coomassie brilliant blue.

Enzyme linked immunosorbent assay (ELISA) for anti-PG-GSP antibody. Antibody to *S. pyogenes* was assayed by indirect ELISA using Cooke microtitre plates (M129B MicroElisa; Dynatech, Billingshurst, UK). PG-GSP was adsorbed onto plate wells by overnight incubation of 10 µg/ml soluble PG-GSP (50 µl/well) in 0.05 M carbonate-bicarbonate buffer, pH 9.6. This optimal coating concentration had been determined by prior checkerboard titration against rabbit anti-PG serum. Plates were washed three times with PBS, pH 7.2, containing 0.05% polyoxyethylene sorbitan monolaurate (Tween 20) (PBS/Tween buffer) before incubation at 37°C for 60 min with 100 µl vols/well of dilutions (1:10 to 1:10,000) of test human sera, NRS or rabbit anti-PG-GSP serum in PBS/Tween. Plates were then washed again three times in PBS/Tween before addition of 100 µl/well of the appropriate peroxidase conjugated antiserum diluted 1:1,000 in PBS/Tween. Peroxidase conjugated goat anti-human Ig, IgG, IgA and IgM were obtained from Miles Laboratories (Slough, UK) and peroxidase conjugated swine anti-rabbit Ig from Dakopatts A/S (Copenhagen, Denmark). After 60 min incubation at 37°C, plates were washed as before prior to addition of 100 µl/well of freshly prepared 0.4 mg/ml ortho-phenylenediamine (OPD) in 0.2 M phosphate-citrate buffer, pH 5.5, with 0.012% H₂O₂ and 0.005% NaN₃. After incubation for 6 min at room temperature, the enzyme reaction was terminated by addition of 4 M H₂SO₄ (100 µl/well). The optical absorbance in each well was then determined at 490nm using a Multiskan photometer (Flow Laboratories, Irvine, Scotland).

Competitive inhibition studies. To assess cross-reactivity of antibodies reactive with *S. pyogenes* PG-GSP polymers, anti-PG-GSP positive sera at 1:200 dilution were prior incubated with PG-GSP preparations, LAA, Ac₂-LAA, MDP or formamide extracted PG or GSP. Following incubation at 37°C for 30 min, and then cooling to room temperature, sera were immediately assayed for anti-PG-GSP antibody activity.

Serum pre-treatment to abolish IgM-rheumatoid factor (IgM-RF) activity. The possibility that IgM-RF in RA sera may interfere with the anti-PG-GSP assay was considered. Thus, seropositive RA sera were treated with 5 mM dithiothreitol (DTT) for 30 min at 37°C to abolish IgM-RF activity. After treatment, sera had lost IgM-RF activity as assessed in Rose-Waaler haemagglutination. Sera for anti-PG-GSP antibody estimation were diluted immediately to 1:200 and anti-PG-GSP activity assayed.

Determination of albumin concentration. Albumin concentrations were assessed in sera and synovial fluid samples by single radial immunodiffusion in 1% agarose gels using rabbit anti-human serum albumin (Dakopatts A/S, Denmark). Standardization was by use of serum samples with known albumin levels.

Determination of other antibodies. The Rose-Waaler haemagglutination titre for IgM-RF was determined as described previously (Johnson, 1979). The anti-streptolysin O (ASO) titre (Rantz, Randall & Zuckermann 1956) and Widal test (Cruickshank, 1965) were carried out using reagents obtained commercially (Wellcome Reagents, Beckenham, UK). Anti-herpes simplex virus and anti-cytomegalovirus titres were determined by complement fixation (Grist, Ross & Bell, 1974) with antigens prepared according to the method of Kettering, Schmidt & Lennette (1977).

IgM-RF preparations. IgM-RF was isolated from high titred seropositive RA sera by affinity chromatography using immobilized heat aggregated human IgG as previously described (Johnson,

1979, 1980). All preparations demonstrated strong RF reactivity in Rose-Waaler haemagglutination. As a negative control, an IgM κ paraprotein was isolated from the serum of a patient with Waldenström's macroglobulinaemia (Morgan, Johnson & Dean, 1978): this paraprotein had no RF activity.

Serum samples. Sera were collected from adult patients with seropositive RA ($n=39$), seronegative RA ($n=8$), ankylosing spondylitis (AS) ($n=8$) and systemic lupus erythematosus (SLE) ($n=7$), as well as myeloma sera ($n=19$) containing a pronounced monoclonal Ig paraprotein. Sera from juvenile chronic arthritis (JCA) children with active, systemic disease ($n=10$) were obtained from Mr A. Howard and Dr B. M. Ansell (Clinical Research Centre, Northwick Park). Further sera were collected from patients with pyrexia of unknown origin (PUO) of at least four weeks standing and who subsequently responded to antibiotics: most of these patients developed positive serum Widal or ASO titres despite no pathogenic organism having been detected on culture. Sera were also obtained from patients ($n=19$) with a known viral infection and who all subsequently demonstrated serum antibody against either cytomegalovirus or herpes simplex virus. Normal adult controls ($n=24$) were composed of healthy laboratory staff: C-reactive protein (CRP) estimations on this latter group (Strachan & Johnson, 1982) revealed none with elevated CRP levels. All sera were stored in small aliquots at -20°C .

Statistical analysis. Anti-PG-GSP levels were compared for significance between different

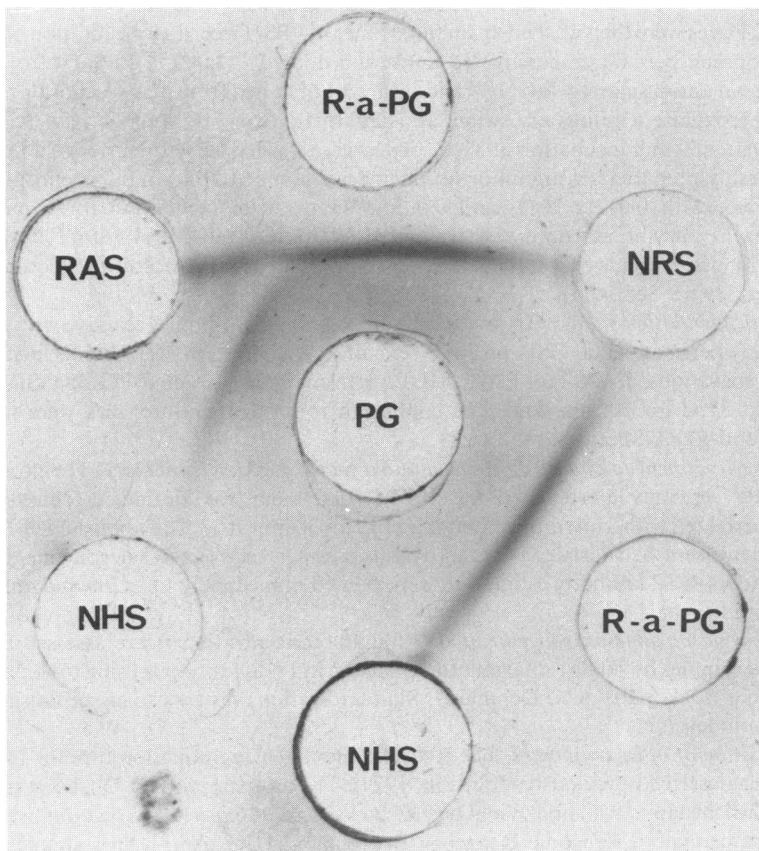


Fig. 1. Immunoprecipitin reaction of rabbit anti-PG-GSP serum (R-a-PG) with solubilized PG-GSP (PG) showing spurring beyond the immunoprecipitin reaction given by an RA serum (RAS). No immunoprecipitin reactivity against PG-GSP was noted for pre-immune rabbit serum (NRS) or two separate normal human sera (NHS).

clinical groupings by the Student's *t*-test for unpaired data, and compared for significance with other serum antibody levels by the Student's *t*-test for paired data.

RESULTS

DRID analyses

The rabbit anti-PG-GSP serum gave strong immunoprecipitin reactions with soluble PG-GSP in DRID, which was not given by pre-immune rabbit serum (NRS) or normal human sera (NHS). Occasional sera from RA patients demonstrated weak immunoprecipitin reactions with soluble PG-GSP. The precipitin reaction of PG-GSP with rabbit anti-PG-GSP serum clearly spurred beyond that with RA sera (Fig. 1), indicating recognition of more antigenic epitopes on PG-GSP by the rabbit antiserum.

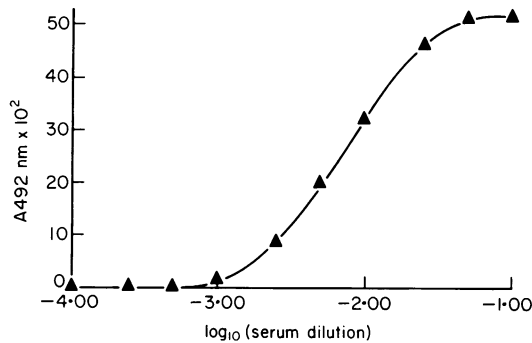


Fig. 2. Reference curve for anti-PG-GSP antibody activity assessed by ELISA and constructed from dilutions of a serum with known precipitating anti-PG-GSP activity.

Establishment of anti-PG-GSP antibody ELISA

Using an RA serum known to give a precipitin reaction with PG-GSP in DRID, a reference curve was established for dilutions (1:10–1:10,000) of this serum in ELISA against soluble PG-GSP adsorbed on MicroElisa plate wells (Fig. 2). One hundred units of anti-PG-GSP antibody activity was arbitrarily assigned to this serum, and all subsequent ELISA estimations of anti-PG-GSP antibody were expressed in these arbitrary units (u) derived from the reference curve (Malvano *et al.*, 1982). The ELISA assay was used for screening test human sera at 1:200 dilution, with peroxidase conjugated anti-human Ig as the developing antibody. Each individual assay plate contained all samples in duplicate, including dilutions of the reference positive serum; negative controls included wells containing no adsorbed PG-GSP or no added test human serum. Also included on each plate were a positive control of rabbit anti-PG-GSP serum (at 1:1,000 dilution) and negative control of NRS (at 1:50 dilution), and for these alone the developing antibody was peroxidase conjugated anti-rabbit Ig. The intra-assay coefficient of variance for the anti-PG-GSP antibody ELISA was 4.2% ($n=8$) and the inter-assay coefficient of variance was 8.3% ($n=7$).

Assessment of anti-PG-GSP antibody in patient sera

A normal range upper limit of 80 u anti-PG-GSP antibody activity was determined as the mean plus two standard deviations for an initial group of sera from healthy controls. Anti-PG-GSP antibody levels for individual sera in different clinical groupings is shown in Fig. 3. There was no significant difference ($P>0.02$) between SLE (mean 56.4 ± 27.5 u), viral infection (mean 44.3 ± 38.9 u), AS (mean 62.8 ± 57.3 u) or myeloma (mean 18.0 ± 24.6 u) patients compared with healthy controls. In contrast, significantly elevated ($P<0.005$) anti-PG-GSP levels were noted in seropositive RA (mean 80.7 ± 64.2 u), seronegative RA (mean 89.9 ± 78.1 u), JCA (mean 135.6 ± 108.3 u) and PUO

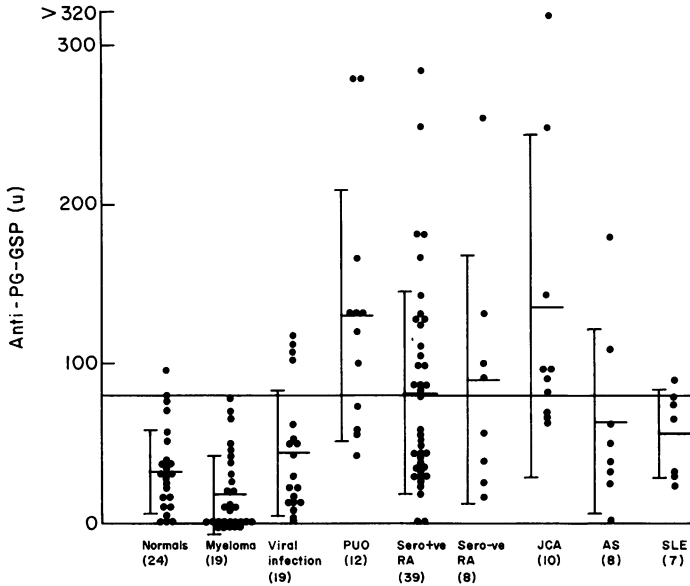


Fig. 3. Distribution of anti-PG-GSP antibody in arbitrary units for individual serum samples from different clinical groupings. The mean \pm standard deviation are marked for each grouping, and the horizontal line denotes the upper limit of the normal range for anti-PG-GSP activity (i.e. 80 u).

(mean 130.6 ± 78.7 u) patients compared with healthy controls. Forty-six per cent of seropositive RA, 50% of seronegative RA, 70% of JCA and 67% of PUO patients had serum anti-PG-GSP levels above the upper limit of the normal range.

There was no correlation ($P > 0.05$) of serum anti-PG-GSP antibody in RA sera with age, sex, clinical assessment or laboratory measurement (ESR or CRP level) of joint inflammation. There

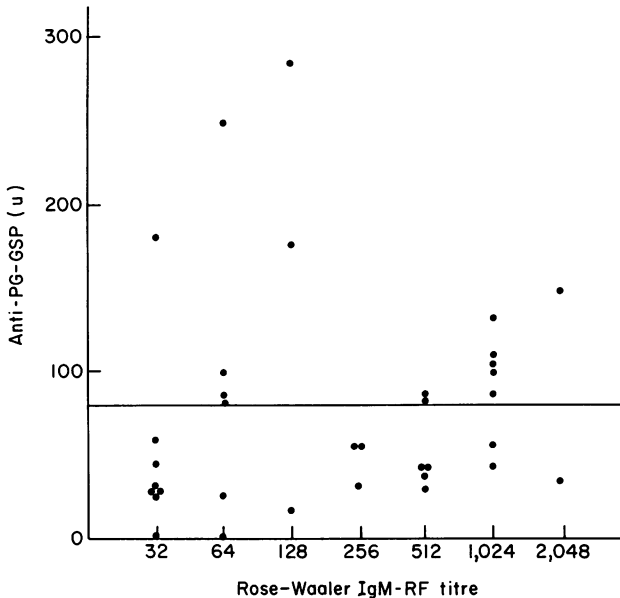


Fig. 4. Correlation of anti-PG-GSP antibody activity with Rose-Waaler IgM-RF titre for individual sera. The horizontal line denotes the upper limit of the normal range for anti-PG-GSP activity (80 u).

was also no significant correlation of serum anti-PG-GSP antibody with ASO titre (Spearman's rank correlation coefficient $R_s=0.09$, $P>0.05$; $n=18$). In 11 paired serum and synovial fluid samples, the anti-PG-GSP antibody level in serum always exceeded that of the corresponding synovial fluid sample. When anti-PG-GSP levels in paired serum and synovial fluids were expressed as a ratio to the albumin level, in no case was there any indication of local production of anti-PG-GSP in synovial tissues. Serum and synovial fluid anti-PG-GSP antibody was always of the IgG class: no reactivity could be determined when peroxidase conjugated anti-human IgM or IgA were used as developing antibodies in the ELISA system.

Table 1. Inhibition of anti-PG-GSP antibody activity in RA sera

Inhibitor	Number of anti-PG-GSP positive sera tested	% Inhibition \pm s.d.
5mM DTT	10	1.4 \pm 8.4
2mM LAA	10	5.6 \pm 4.2
2mM Ac ₂ -LAA	10	0.2 \pm 6.5
10 μ g/ml MDP	4	-1.1 \pm 0.8
100 μ g/ml <i>M. roseus</i> PG-GSP	10	2.8 \pm 2.8
100 μ g/ml <i>B. megaterium</i> PG-GSP	10	18.9 \pm 10.7
100 μ g/ml <i>C. poinsettiae</i> PG-GSP	10	1.3 \pm 7.8
10 μ g/ml <i>S. pyogenes</i> PG-GSP	10	56.0 \pm 11.0
20 μ g/ml <i>S. pyogenes</i> PG-GSP	6	70.5 \pm 8.8
100 μ g/ml <i>S. pyogenes</i> PG-GSP	4	83.5 \pm 9.5
20 μ g/ml formamide extracted <i>S. pyogenes</i> PG	6	63.3 \pm 13.3
20 μ g/ml formamide extracted <i>S. pyogenes</i> GSP	6	38.4 \pm 11.5

Association with IgM-RF

No direct binding of isolated IgM-RF preparations to *S. pyogenes* PG-GSP was shown in ELISA by comparison with a non-RF IgM κ paraprotein over the concentration range of 1-100 μ g/ml. There was no significant correlation of serum anti-PG-GSP antibody in RA sera with Rose-Waaler haemagglutination RF titre ($R_s=0.29$, $P>0.05$; $n=35$) (Fig. 4). No interference by IgM-RF in the anti-PG-GSP ELISA was indicated following pre-treatment of test seropositive RA sera with DTT to abolish IgM-RF activity. DTT treatment did not significantly affect the assay of serum anti-PG-GSP antibody (Table 1).

Inhibition studies

The specificity of anti-PG-GSP antibody found in different RA sera for antigenic determinants on PG-GSP was examined by competitive inhibition studies (Table 1). Pre-treatment of test sera with LAA, Ac₂-LAA, MDP, *M. roseus*, *B. megaterium* or *C. poinsettiae* cell wall PG-GSP did not substantially inhibit the antibody activity of RA sera reactive with *S. pyogenes* PG-GSP. In contrast, *S. pyogenes* PG-GSP at 10 μ g/ml (i.e. same concentration as that used in overnight PG-GSP adsorptions to MicroElisa wells) gave significant inhibition of serum anti-PG-GSP antibody activity, and *S. pyogenes* PG-GSP at 100 μ g/ml gave virtually complete inhibition (Table 1). Partial inhibition was shown for formamide extracted *S. pyogenes* GSP preparations, whereas the inhibition was more pronounced for formamide extracted PG preparations, suggesting that most antibody may be reactive against the peptide component although at least some expresses reactivity against the polysaccharide component. This was supported by the observation that pre-treatment of *S. pyogenes* PG-GSP with 1 mM periodate (which attacks the polysaccharide component, but does not modify the PG component) decreased the mean anti-PG-GSP binding activity of 10 RA sera by 29.7 \pm 11.2%.

DISCUSSION

An increased prevalence and level of IgG antibody activity reactive in an ELISA with PG-GSP polymers derived from the *S. pyogenes* cell wall skeleton has been detected in RA and JCA sera compared with AS, SLE and healthy control sera. The lack of antibody reactive with *S. pyogenes* PG-GSP preparations in AS sera has also been confirmed in a recent larger series studied in collaboration with A. Ebringer (unpublished data). Detection of anti-PG-GSP antibody in this assay was independent of serum immunoglobulin concentration as, for example, manifest by the low anti-PG-GSP levels in myeloma sera.

Studies based on experimental animals immunized with bacterial cell wall preparations have indicated immunological cross-reactivity between PG preparations from different bacterial species (Schleifer & Krause, 1971). It was surprising therefore that three different Gram positive bacterial cell wall PG-GSP preparations, and also MDP and the two tripeptide preparations, were unable to inhibit significantly the antibody activity in RA sera reactive with *S. pyogenes* PG-GSP polymers. It is clear from these results that anti-PG-GSP antibody activity present in RA and JCA sera is not directed against the repeating disaccharide backbone of the PG structure. The periodate sensitivity, however, suggests partial antibody reactivity with the rhamnose component of PG-GSP polymers as well as the antibody reactivity with the peptide component of *S. pyogenes* PG-GSP preparations.

A previous study has reported increased levels of anti-PG antibody in the sera of JCA patients using a radioimmunoassay for antibody binding to the synthetic hapten L-Ala- γ -D-Glu-L-Lys-D-Ala-D-Ala (Heymer *et al.*, 1976). A brief report has also noted an increased incidence of antibody in RA and JCA sera compared with normal controls that was reactive with the synthetic hapten L-Ala- γ -D-Glu-L-Leu-D-Ala-D-Ala, which is the analogue of the pentapeptide of group A variant streptococcal PG (Pope *et al.*, 1979). Nevertheless, bacteria other than streptococci may be involved in the immunization process since no significant correlation of anti-PG-GSP antibody with ASO titre has been noted in the present study. Other reports of similar antibodies in rheumatic disorders have also failed to demonstrate any correlation with ASO titre (Heymer *et al.*, 1976; Pope *et al.*, 1979).

There would appear to be no direct correlation between anti-PG-GSP antibody and RF. Unusual cross-reactions of RF have been described (Johnson, 1979, 1981; Hobbs *et al.*, 1983), and a cross-reaction of RF like antibodies with PG has also been reported for experimental rabbits immunized with streptococcal cell walls (Bokisch *et al.*, 1973). However, it has now been shown in the present study that RF isolated from seropositive RA sera does not bind *S. pyogenes* PG-GSP. The results of Bokisch *et al.* (1973) could instead be explained by interactions at other levels such as through anti-idiotypic specificities (Phua & Johnson, in preparation), especially since RF and anti-PG-GSP antibody may be clonally restricted antibody specificities. Nevertheless, anti-PG-GSP antibody appears to occur in a comparable incidence in seropositive RA as in seronegative RA or JCA. Furthermore, the anti-PG-GSP assay developed in this study was not influenced by the concomitant presence of IgM-RF activity in test sera since the determination of IgG anti-PG-GSP levels was not significantly affected by prior DTT treatment of sera to destroy IgM-RF activity.

The raised anti-PG-GSP antibody level in RA and JCA sera reflect immunization by bacterial agents. This could indicate an increased susceptibility due to impaired host clearance of bacterial infection resulting from immunological alterations that may be part of rheumatoid disease process. However, our preliminary studies have indicated that RA patients within the first 3 months of clinical presentation also often have raised serum anti-PG-GSP antibody levels (Jones *et al.*, 1983) and that these levels may remain relatively stable throughout the course of the disease. Hence, it is suggested that anti-PG-GSP antibody may have a more fundamental role in the pathogenesis of chronic synovitis, as has been indicated from experimental animal models (Greenblatt *et al.*, 1980). By analogy with such animal models (Dalldorf *et al.*, 1980), it is possible that cell wall PG-GSP derived from bacterial infection and ineffective host clearance of cell wall debris (Fox *et al.*, 1982) could become a source of sequestered antigen in joint tissues providing a stimulus for chronic immune stimulation. Against this, however, no evidence was obtained in the present study for local synthesis of anti-PG-GSP antibody in rheumatoid synovia, although synovial fluid antibody levels could have been lower than expected due to trapping by antigen in the local milieu.

Thus, in conclusion, the present study offers evidence for increased systemic immunization (but not necessarily infection) by bacterial components in RA and JCA; the microbiological source of bacterial PG-GSP involved in this process remains to be clarified.

We would like to thank Mr A. Howard, Drs B.M. Ansell, L.D.C. Halliday and R.M.R. Barnes for providing some of the serum samples and clinical evaluation of patients included in this study, and Professor J.M. Ghuysen for the sample of *M. roseus* walls. This work was supported by a project grant from the Arthritis and Rheumatism Council.

REFERENCES

- BENNETT, J.C. (1978) The infectious etiology of rheumatoid arthritis. *Arthrit. Rheum.* **21**, 531.
- BOKISCH, V.A., CHIAO, J.W., BERNSTEIN, D. & KRAUSE, R.M. (1973) Homogeneous rabbit 7S anti-IgG with antibody specificity for peptidoglycan. *J. exp. Med.* **138**, 1184.
- CRUICKSHANK, R. (1965) *Medical Microbiology* (11th edn.) p. 907. Churchill Livingstone, Edinburgh.
- DALLDORF, F.G., CROMARTIE, W.J., ANDERLE, S.K., CLARK, R.L. & SCHWAB, J.H. (1980) Relation of experimental arthritis to the distribution of streptococcal cell wall fragments. *Am. J. Pathol.* **100**, 383.
- DISCHE, Z. & SHETTLES, L.B. (1948) A specific colour reaction of methyl-pentoses and a spectrophotometric micromethod for their determination. *J. biol. Chem.* **175**, 595.
- DZIARSKI, R. (1982) Preferential induction of auto-antibody secretion in polyclonal activation by peptidoglycan and lipopolysaccharide. *J. Immunol.* **128**, 1018.
- FOX, A., BROWN, R.R., ANDERLE, S.K., CHETTY, C., CROMARTIE, W.J., GOODER, H. & SCHWAB, J.H. (1982) Arthritopathic properties related to the molecular weight of peptidoglycan-polysaccharide polymers of streptococcal cell walls. *Infect. Immun.* **35**, 1003.
- FULLER, A.T. (1938) The formamide method for the extraction of polysaccharides from haemolytic streptococci. *Br. J. exp. Path.* **19**, 230.
- GREENBLATT, J.J., BOACKLE, R.J. & SCHWAB, J.H. (1978) Activation of the alternate complement pathway by peptidoglycan from streptococcal cell wall. *Infect. Immun.* **19**, 296.
- GREENBLATT, J.J., HUNTER, N. & SCHWAB, J.H. (1980) Antibody response to streptococcal cell wall antigens associated with experimental arthritis in rats. *Clin. exp. Immunol.* **42**, 450.
- GRIST, N.R., ROSS, C.A. & BELL, E.J. (1974) Diagnostic Methods. In *Clinical Microbiology* (2nd edn.) p. 80. Blackwell Scientific Publications, Oxford.
- HEYMER, B., SCHLEIFER, K.-H., READ, S., ZABRISKIE, J.B. & KRAUSE, R.M. (1976) Detection of antibodies to bacterial cell wall peptidoglycan in human sera. *J. Immunol.* **117**, 23.
- HOBBS, R.N., LEA, D.J., PHUA, K.K. & JOHNSON, P.M. (1983) Binding of isolated rheumatoid factors to histone proteins and basic polycations. *Ann. rheum. Dis.* **42**, 435.
- JOHNSON, P.M. (1979) IgM-rheumatoid factors cross-reactive with IgG and a cell nuclear antigen: apparent 'masking' in original serum. *Scand. J. Immunol.* **9**, 461.
- JOHNSON, P.M. (1980) IgM-rheumatoid factors cross-reactive with IgG and a cell nuclear antigen: immunopathological implications? *Ann. rheum. Dis.* **39**, 586.
- JOHNSON, P.M. (1981) Molecular nature and cross-reactions of rheumatoid factor. *Clin. Immunol. Allergy*, **1**, 103.
- JONES, V.E., JACOBY, R.K., JOHNSON, P.M., PHUA, K.K. & WELSH, K.I. (1983) Association of HLA-DR4 with definite rheumatoid arthritis but not with susceptibility to arthritis. *Ann. rheum. Dis.* **42**, 223 (abstr.).
- KETTERING, J.D., SCHMIDT, N.H. & LENNETTE, E.H. (1977) Improved glycine-extracted complement fixing antigen for human cytomegalovirus. *J. clin. Microbiol.* **6**, 647.
- KRAUSE, R.M. & MCCARTY, M. (1961) Studies on the chemical structure of the streptococcal cell wall. *J. exp. Med.* **114**, 127.
- LAMBRIS, J.H., ALLEN, J.B. & SCHWAB, J.H. (1982) *In vivo* changes in complement induced with peptidoglycan-polysaccharide polymers from streptococcal cell walls. *Infect. Immun.* **35**, 377.
- MALVANO, R., BONIOLLO, A., DOVIS, M. & ZANNINO, M. (1982) ELISA for antibody measurement: aspects related to data expression. *J. Immunol. Meth.* **48**, 51.
- MARMION, B.P. (1978) Infection, autoimmunity and rheumatoid arthritis. *Clin. rheum. Dis.* **4**, 565.
- MORGAN, M.R.A., JOHNSON, P.M. & DEAN, P.D.G. (1978) Electrophoretic desorption of immunoglobulins from immobilised protein A and other ligands. *J. Immunol. Meth.* **23**, 381.
- NIETO, M. & PERKINS, H.R. (1971) Modifications of the acyl-D-alanyl-D-alanine terminus affecting complex formation with vancomycin. *Biochem. J.* **123**, 789.
- PETTIT, J.R., MUNOZ, E. & GHUYSEN, J.M. (1966) Peptide cross-links in bacterial cell wall peptidoglycans studied with specific endopeptidases from *Streptomyces albus* G. *Biochemistry*, **5**, 2764.
- POPE, R.M., RUTSTEIN, J.E., STRAUSS, D.C. & CHANG, D. (1979) Antibodies to the immunodominant portion of streptococcal mucopeptide (pentapeptide) in patients with rheumatic disorders. *Arthr. Rheum.* **22**, 648 (Abstr.).
- RANTZ, L.A., RANDALL, E. & ZUCKERMANN, A. (1956) Haemolysis and haemagglutination by normal and immune sera treated with non-specific bacterial substances. *J. Infect. Dis.* **98**, 211.
- SCHLEIFER, K.-H. & KANDLER, O. (1972) Peptidoglycan type of bacterial walls and their taxonomic implications. *Bacteriol. Rev.* **36**, 407.
- SCHLEIFER, K.-H. & KRAUSE, R.M. (1971) The im-

- muchochemistry of peptidoglycan. I. The immunodominant site of the peptide subunit and the contribution of each of the aminoacids to the binding properties of the peptides. *J. biol. Chem.* **246**, 986.
- SCHLEIFER, K.-H. & SEIDL, H.P. (1977) Structure and immunological aspects of peptidoglycans. In *Microbiology-1977* (ed. by D. Schlessinger) p. 339. American Society of Microbiology, Washington.
- SCHWAB, J.H. & OHANIAN, S.H. (1969) Degradation of streptococcal cell wall antigens *in vivo*. *J. Bacteriol.* **94**, 1346.
- SMIALOWICZ, R.H. & SCHWAB, J.H. (1977a) Cytotoxicity of rat macrophages activated by persistent or biodegradable bacterial cell walls. *Infect. Immun.* **17**, 599.
- SMIALOWICZ, R.H. & SCHWAB, J.H. (1977b) Processing of streptococcal cell walls by rat macrophages and human monocytes *in vitro*. *Infect. Immun.* **17**, 591.
- STEWART-TULL, D.E.S. (1980) The immunological activities of bacterial peptidoglycans. *Ann. Rev. Microbiol.* **34**, 311.
- STRACHAN, A.F. & JOHNSON, P.M. (1982) Protein SAP (serum amyloid P component) in Waldenström's macroglobulinaemia, multiple myeloma and rheumatic diseases. *J. clin. lab. Immunol.* **8**, 153.
- ZVAIFLER, N.J. (1973) The immunopathology of joint inflammation in rheumatoid arthritis. *Adv. Immunol.* **16**, 265.