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Increased Blood Levels of IgG Reactive with Secreted *Streptococcus pyogenes* Proteins in Chronic Plaque Psoriasis

Rana G. El-Rachkidy[1], Jonathan M. Hales[2], Primrose P.E. Freestone[1], Helen S. Young[3], Christopher E.M. Griffiths[3] and Richard D.R. Camp[1]

A pathogenic role for *Streptococcus* (*S*) *pyogenes* infections in chronic plaque psoriasis is suspected but poorly defined. We separated cellular and supernatant proteins from *S pyogenes* cultures by high-resolution two-dimensional gel electrophoresis, and used immunoblotting to demonstrate the diversity of serum or plasma IgGs that react with elements of the proteome of this bacterium. We have shown that a substantial proportion of IgG-reactive proteins from cultured *S pyogenes* are secreted. The total secreted protein fraction, including diverse IgG-binding elements, was subsequently used in an ELISA to measure blood titers of reactive IgG. This ELISA showed that blood samples from patients with chronic plaque psoriasis contained significantly higher titers of reactive IgG than samples from age- and sex-matched healthy controls ($P=0.0009$). In contrast, neither a standard assay measuring antistreptolysin O titers nor ELISAs measuring titers of IgG reactive with protein fractions from *Staphylococcus aureus* and *Staphylococcus epidermidis*, were able to distinguish between blood samples from the two groups. These findings justify the hypothesis that *S pyogenes* infections are more important in the pathogenesis of chronic plaque psoriasis than has previously been recognized, and indicate the need for further controlled therapeutic trials of antibacterial measures in this common skin disease.

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INTRODUCTION

The pathogenesis of psoriasis depends on genetic susceptibility especially in disease of earlier onset, and mechanisms suggestive of autoimmunity (Griffiths *et al*., 2004). The provocation of autoimmune diseases such as rheumatic fever and acute glomerulonephritis by infection with *Streptococcus* (*S*) *pyogenes* is well recognized and may depend on immunological mimicry between molecules expressed by the bacterium and target tissue (Cunningham, 2000; Bisno *et al*., 2003). There is also a convincing association between streptococcal pharyngitis and exacerbations of acute guttate psoriasis, the less common, small plaque variant (Telfer *et al*., 1994). Some have implicated the expansion of autoreactive T lymphocytes by released bacterial superantigens in the pathogenesis of post-streptococcal guttate psoriasis (Valdimarsson *et al*., 1995) as well as immunological mimicry between microbial surface M proteins (Gudjonsson *et al*., 2004) or the group A-specific carbohydrate *N*-acetyl-*β*-D-glucosamine (Shikhman and Cunningham, 1994) and epidermal keratins. Association between *S pyogenes* infection and the much commoner chronic plaque type of psoriasis is poorly defined but of potential importance, particularly if a subgroup of patients with occult infection can be identified and disease severity improved by appropriate antibacterial measures. This association has been suggested by several uncontrolled reports of improvement following tonsillectomy (Owen *et al*., 2001) and an anecdotal report of improvement following short-term antibiotics in a small number of patients with chronic plaque psoriasis (Rosenberg *et al*., 1986). Two recent reports indicating that streptococcal infections are more frequent in patients with chronic plaque psoriasis than healthy controls remain inconclusive because the two groups were apparently not age matched (Weisenseel *et al*., 2002; Fry *et al*., 2006), an important requirement in such studies as streptococcal infections are known to be commoner in younger people (Cunningham, 2000). Recent epidemiological work also suggests that patients with chronic plaque psoriasis experience more frequent streptococcal sore throats than controls and that these infections may provoke deterioration in disease severity (Gudjonsson *et al*., 2003). Other than these reports, there is little evidence to support an association

[1]*Department of Infection, Immunity and Inflammation, University of Leicester, Leicester, UK;* [2]*Department of Medical and Social Care Education, University of Leicester, Leicester, UK and* [3]*Dermatology Centre, University of Manchester, Manchester, UK*

Correspondence: Dr Richard D.R. Camp, Department of Infection, Immunity and Inflammation, University of Leicester, Medical Sciences Building, University Road, Leicester LE1 9HN, UK.
E-mail: rdrc1@le.ac.uk

Abbreviations: PBS, phosphate-buffered saline; PBS/T, PBS containing 0.05% Tween 20; S, Streptococcus

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between *S pyogenes* infections and chronic plaque psoriasis. However, during a preliminary study aimed at exploring the structure of immunoreactive *S pyogenes* proteins and their homology to skin proteins, we noted increased levels of *S pyogenes*-reactive IgG in serum from chronic plaque psoriasis patients versus healthy controls, and have now extended this finding.

RESULTS

A substantial proportion of IgG-reactive proteins produced in *S pyogenes* cultures are secreted

Total supernatant protein fractions from large cultures of *S pyogenes* were obtained by ammonium sulfate precipitation, and from pelleted bacterial cells by sonication. Both concentrated supernatants and cellular lysates from *S pyogenes* cultures were shown to contain abundant Coomassie-stained proteins following separation by high-resolution two-dimensional gel electrophoresis (Figure 1a and b). Two-dimensional Western blots of gels run simultaneously with those shown in Figure 1a and b were probed with pooled sera from eight chronic plaque psoriasis patients (see next section for details of donors), followed by identification of bound IgGs with alkaline phosphatase-labelled anti-IgG. This analysis consistently showed the presence of numerous IgG-reactive proteins in *S pyogenes* culture supernatant fractions but to a lesser extent in cellular lysate fractions (Figures 1c and d) in spite of the presence of abundant proteins in the cellular lysates (Figure 1b). Probing of two-dimensional Western blots of *S pyogenes* supernatant proteins with pooled sera from eight age- and sex-matched healthy controls showed that the IgGs from this source react with a rather similar profile of proteins to those bound by IgGs in pooled psoriatic sera. However, the staining was less intense when control sera were used, suggesting that titers of *S pyogenes*-reactive IgG are lower in control than psoriatic sera (Figure 1e). These observations were consistent in three separate analyses with cellular and supernatant proteins from different cultures of the same *S pyogenes* and pooled sera from six to eight patients and matched healthy controls.

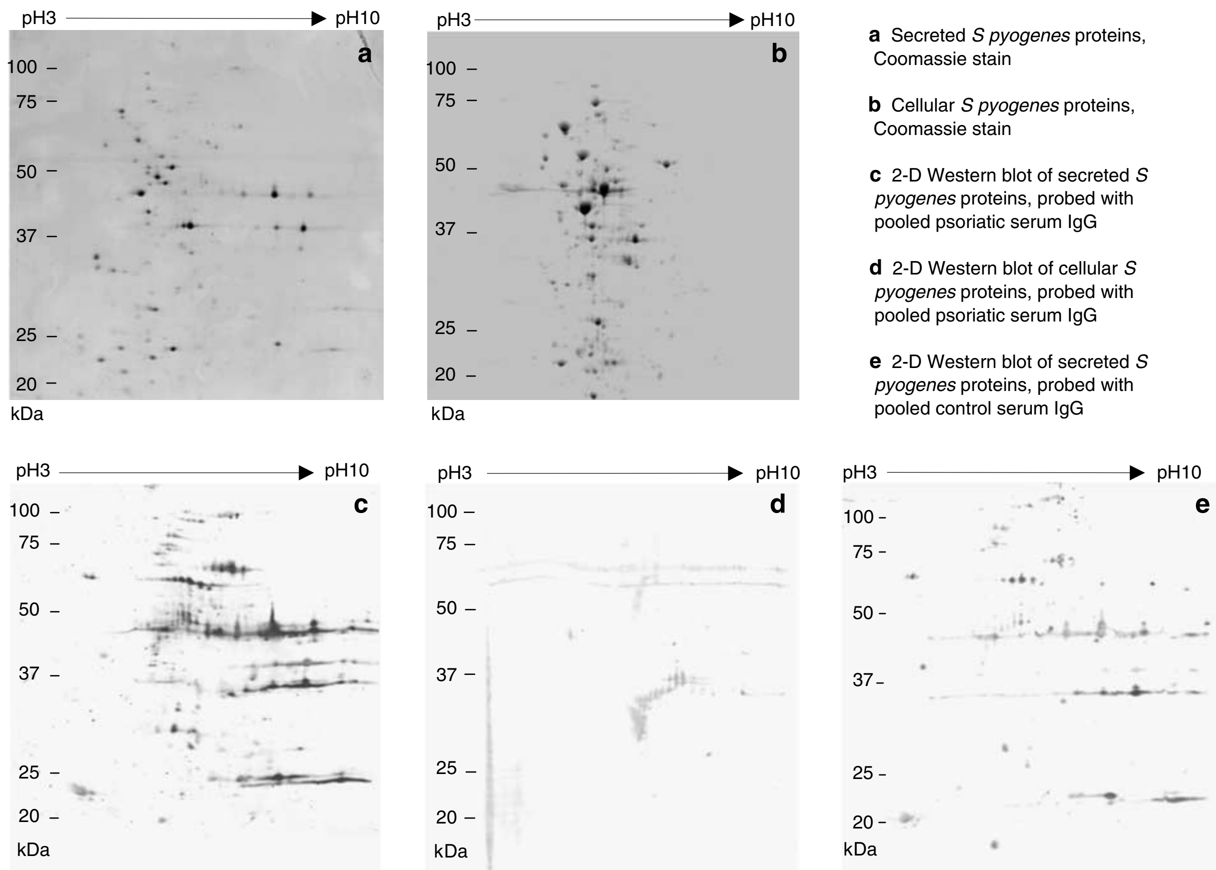

Figure 1. Diversity of IgG-reactive proteins produced by cultured *S pyogenes*. Total secreted or cellular proteins recovered during *S pyogenes* cultures were separated by high-resolution two-dimensional gel electrophoresis. Gels were either treated with Coomassie stain to show all separated proteins ((**a**) secreted *S pyogenes* proteins; (**b**) cellular *S pyogenes* proteins) or blotted onto nitrocellulose and probed with different pooled sera to demonstrate IgG-reactive proteins ((**c**) blot of secreted proteins probed with pooled psoriatic serum IgG; (**d**) blot of cellular proteins probed with pooled psoriatic serum IgG; (**e**) blot of secreted proteins probed with pooled control serum IgG). (**a–e**) Vertical and horizontal axes indicate the molecular weight in kilodaltons and isoelectric points (pH 3–10) of separated proteins, respectively. (**c** and **d**) Pooled sera from eight patients with chronic plaque psoriasis and (**e**) eight age- and sex-matched healthy controls were used in this experiment to probe the Western blots. Data are representative of three separate experiments.

Titers of IgGs that bind to *S pyogenes* total secreted protein fraction are significantly higher in psoriatic than control blood samples

Venous blood was obtained from patients with chronic plaque psoriasis (28 female and 29 male subjects, aged 17–80 years; mean age±SD 43±15 years) who had attended the dermatology clinics of the Leicester Royal Infirmary or Hope Hospital, Manchester. Most of the patients had moderate to severe disease, giving a median psoriasis area and severity index (Fredricksson and Pettersson, 1978) of 13.6±7.0 (SD). None had received systemic anti-psoriatic drugs or UV therapy for at least 2 months before venesection. Control subjects were healthy age- and sex-matched volunteers (20 female and 20 male subjects aged 18–80 years; mean age±SD 42±17 years). We developed a novel, quantitative ELISA that used total proteins secreted by cultured *S pyogenes* as substrate, to show that titers of IgG that reacted with the total secreted protein fraction were significantly higher in serum or plasma samples from the patients with chronic plaque psoriasis (1,000±201, median±SE, $n=57$) than in samples from the age- and sex-matched healthy controls (200±120, median±SE, $n=40$; $P=0.0009$, Mann–Whitney test; Figure 2a). There was no correlation between the titer of IgG reactive with *S pyogenes* total secreted proteins and the psoriasis area and severity index (data not shown). In contrast, analysis of many of the same psoriatic and control samples for antistreptolysin O titers (a standard method used in the clinical diagnosis of recent *S pyogenes* infections) did not distinguish between samples from the two sources (Figure 2b).

We also developed an ELISA that measured blood IgG titers reactive with cellular proteins of *S pyogenes*. This showed a trend towards higher IgG titers in blood samples from psoriasis patients (500±565, median±SE, $n=22$) versus healthy-matched controls (250±201, median±SE, $n=20$), but this did not achieve statistical significance ($P>0.05$, Mann–Whitney test; Figure 2c).

Control ELISAs were also carried out with protein fractions from *Staphylococcus aureus* and *Staphylococcus epidermidis* cultures. Preliminary work showed that abundant Coomassie-stained proteins are demonstrable on two-dimensional gels of concentrated supernatant and sonicates of pelleted bacteria from overnight cultures of *Staphylococcus aureus*. In contrast, only lysate of pelleted bacteria from overnight culture of the

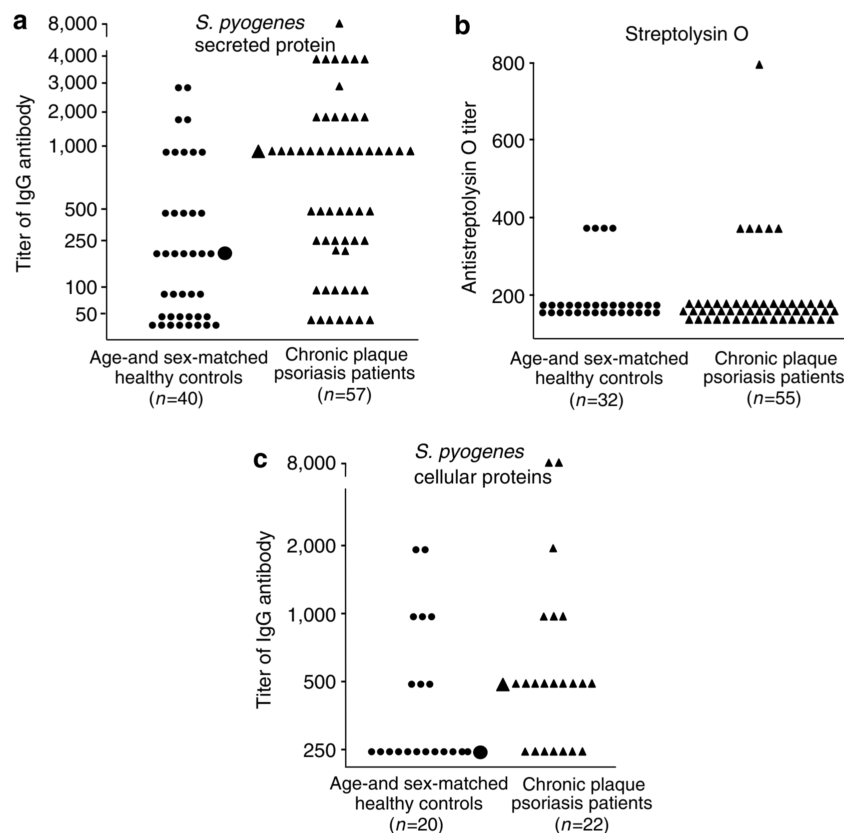

Figure 2. Titers of IgG reactive with *S pyogenes* proteins. (**a**) Titers of IgG reactive with total secreted protein fraction of *S pyogenes*, (**b**) antistreptolysin O titers, and (**c**) titers of IgG reactive with total cellular protein fraction of *S pyogenes*, in blood samples from individual patients with chronic plaque psoriasis (closed triangles) or healthy controls (closed circles). The protein source used in each set of assays is indicated above each graph. Titers of IgG in (**a** and **c**) were measured by the novel ELISAs described in the Materials and Methods section. Titers of IgG reactive with secreted (**a**) *S pyogenes* proteins were significantly higher in the psoriatic blood samples than in controls ($P=0.0009$, Mann–Whitney test). Antistreptolysin O titers in (**b**) were measured by a standard polystyrene particle agglutination assay and were no different in psoriasis versus control blood samples. (**c**) Titers of IgG reactive with cellular *S* pyogenes proteins were not significantly different in samples from the two donor groups. (**a** and **c**) The large symbols show median values.

commensal *Staphylococcus epidermidis* contained abundant proteins on two-dimensional gels, concentrated supernatant being essentially protein-free (data not shown), a finding consistent with evidence that pathogenicity depends on protein secretion in many bacteria (Rosch and Caparon, 2004). In contrast to the findings with *S pyogenes* proteins, ELISAs with protein fractions from these alternative bacteria showed little or no difference between levels of reactive IgG in pooled sera from patients with chronic plaque psoriasis versus controls (Figure 3).

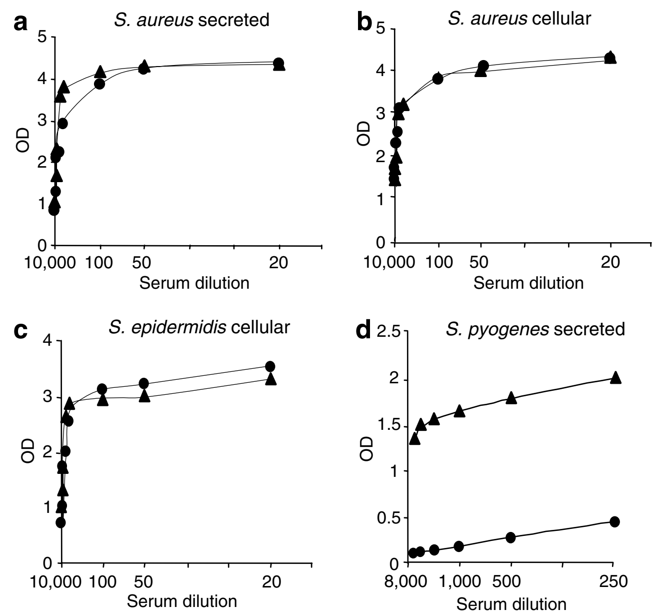

Figure 3. ELISAs showing binding of IgG in pooled sera to protein fractions from *Staphylococcus aureus* and *Staphylococcus epidermidis*. Binding of IgG in pooled sera to protein fractions from (**a** and **b**) *Staphylococcus aureus* and (**c**) *Staphylococcus epidermidis* is shown. ELISAs were performed with different bacterial protein fractions as substrate, as indicated above each graph. These protein fractions were probed with different dilutions of pooled sera from eight patients with chronic plaque psoriasis (closed triangles) or eight age- and sex-matched healthy controls (closed circles). For comparison, (**d**) shows the result of ELISAs using high-titer serum from a patient with psoriasis (closed triangles), low-titer serum from a healthy control (closed circles) and the total secreted protein fraction from *S pyogenes*. Bound IgG was determined by addition of alkaline phosphatase-labelled anti-IgG and optical density measurement. Vertical axes indicate levels of bound IgG and horizontal axes the dilutions of sera. Unlike the results shown in (**d**), levels of IgG reactive with the alternative bacterial protein fractions were similar in (**a–c**) psoriatic and control sera.

DISCUSSION

The finding that IgG responses of psoriasis patients focus significantly on proteins in supernatants of *S pyogenes* cultures is compatible with the known protein-secreting capacity of this bacterium (Cunningham, 2000) and thus the availability of these proteins to the immune system. Serum IgG responses of psoriasis patients to secreted *S pyogenes* proteins were also found to be higher than IgG responses to cellular proteins in this work, as shown initially by immunoblotting (Figure 1). Furthermore, 6/22 psoriatic blood samples (27%) gave IgG ELISA titers of >500 in response to the cellular protein fraction, whereas 29/57 psoriatic samples (51%) gave titers of >500 in response to the secreted protein fraction (Figure 2). These differences could be due to the described antiphagocytic properties of *S pyogenes*, which appear at least in part to be associated with hyaluronate encapsulation and with the ability of streptococcal surface M protein to bind serum factor H and fibrinogen, which inhibit complement activation (Cunningham, 2000; Bisno *et al.*, 2003). Thus, it is possible that failure of opsonization and decreased phagocytosis of intact streptococci by antigen-presenting cells are associated with impaired T-cell activation by streptococcal cellular proteins and thus reduced T-cell-dependent synthesis of antibodies (DeFranco, 1987) that recognize these cellular proteins.

The *S pyogenes* cultures used in in the present work were derived from the same parent culture, which expressed M protein (serotype M5), a fibrous surface protein that has well-recognized immunogenic properties (Cunningham, 2000; Gudjonsson *et al.*, 2004). However, the levels of M protein in the different total protein fractions we have used to develop our assays have not been established, and the contributions of this protein to the IgG ELISA reactivities described are not known. Nevertheless, it is likely that a substantial component of the *S pyogenes* cellular fraction used in the ELISAs illustrated in Figure 2c (which included insoluble cellular fragments) contained M protein. This highlights the potential importance of our finding of higher levels of IgG reactivity amongst secreted *S pyogenes* proteins than cellular proteins, a finding that appears not to have been described previously and may have relevance in vaccine design.

Our work shows that a significantly greater number of patients with chronic plaque psoriasis than healthy controls have raised blood titers of IgG reactive with total proteins secreted by *S pyogenes*. The significance of these findings is enhanced by the fact that age-matched controls were used, as *S pyogenes* infections are known to be more common in younger subjects (Cunningham, 2000), and also by the fact that blood from the two sets of donors contained similar titers of IgG reactive with protein fractions from the other bacteria tested.

Demonstration of the large diversity of IgG-reactive proteins secreted by *S pyogenes* (Figure 1c) justified the use of the total secreted protein fraction in our novel ELISA and is the likely explanation for its greater sensitivity in detecting humoral immunoreactivity to the bacterium, than that of a standard assay in clinical use that measures immune responses to only a single protein reactant, streptolysin O. Our results do not determine whether the ELISA-positive chronic plaque psoriasis patients have suffered from recent *S pyogenes* infections, are exposed to long-lived streptococcal antigens for example as a result of low level bacterial carriage, or whether the raised IgG titers might represent past infection and ongoing immunity. Nevertheless, the findings implicate these infections in the pathogenesis of psoriasis, possibly through immunological mimicry.

We have recently identified a number of serum IgG-binding antigens in the proteome of psoriatic lesional epidermis,

including arginase 1, enolase 1, keratin 10, and 60 kDa heat-shock protein (unpublished data). NCBI BLAST searches show that human arginase 1, enolase 1 and 60 kDa heat-shock protein all share substantial homology with similar proteins of *S pyogenes*. A credible molecular basis for immunological mimicry between proteins expressed by *S pyogenes* and psoriatic epidermis therefore exists, in addition to the previously proposed mimicry between surface M proteins of *S pyogenes* or the group A-specific carbohydrate *N*-acetyl-β-D-glucosamine and epidermal keratins (Rosenberg *et al.*, 1986; Shikhman and Cunningham, 1994; Gudjonsson *et al.*, 2004).

Our findings suggest that better recognition, long-term prevention and treatment of *S pyogenes* infections may be important in the management of chronic plaque psoriasis. There is a need for randomized, controlled trials of appropriate long-term antibiotics or even tonsillectomy, as suggested by systematic review (Owen *et al.*, 2001), especially targeted at those patients with raised titers of *S pyogenes*-reactive IgG. The need for such trials is highlighted by the recent report of remarkable improvement of chronic plaque psoriasis in an uncontrolled study of 30 Indian patients treated with repeated intramuscular injections of benzathine penicillin for at least 48 weeks (Saxena and Dogra, 2005). Such treatment may provide a cost-effective alternative to potentially toxic systemic agents including the emerging high-cost biologic therapies.

MATERIALS AND METHODS

Blood samples

This study was approved by the local research ethics committee and was conducted in accordance with the Declaration of Helsinki principles. Written, informed consent was obtained from each donor. Following venesection, blood samples were allowed to clot in glass test tubes; serum was removed and centrifuged to deplete cells. In some cases, venous blood was mixed with heparin and plasma obtained following centrifugation. Serum and plasma samples were stored at −80°C in aliquots.

Production and separation of bacterial proteins

A single, prevalent M5 serotype of *S pyogenes* (expressing type 5 surface M-protein), was obtained from the Public Health Laboratory Service, Colindale, UK, and cultured overnight in up to 6 l of freshly prepared, chemically defined, protein-free medium (van de Rijn and Kessler, 1980). Culture supernatant containing released proteins was recovered after centrifugation at 2,000 *g* and concentrated by ammonium sulfate precipitation. Precipitate was redissolved in pH 7.7 buffer containing 8 mM sodium phosphate, 1.5 mM potassium phosphate, 2.5 mM potassium chloride, 0.5 mM phenylmethylsulphonyl fluoride and 1 mM EDTA. This was dialyzed in 3.5 kDa cutoff Slide-A-Lyzer™ cassettes (Perbio, Cramlington, UK) with up to six 2 l volumes of the same buffer (without phenylmethylsulphonyl fluoride or EDTA), depending on the concentration of ammonium sulfate in the dialysis fluid, which was monitored with Nessler's reagent (Sigma, Poole, UK). Dialyzed proteins were freeze-dried, the residue redissolved in the same dialysis buffer and desalted on Pierce spin minicolumns (product code 89866, Perbio), eluting with buffer containing 7 M urea, 2 M thiourea and 4% 3-[(3-cholamido propyl)-dimethylammonio]-2-hydroxy-1-propanesulfonic acid in accordance with the supplier's instructions. The eluate was mixed with rehydration buffer containing the above concentrations of urea, thiourea, and 3-[(3-cholamido propyl)-dimethylammonio]-2-hydroxy-1-propanesulfonic acid, 2% ampholytes (Bio-Rad, Hemel Hempstead, UK) and 100 mM DeStreak reagent (Amersham, Little Chalfont, UK) to a final protein concentration of 1 μg/μl. Batches of proteins (300 μg) were separated on 17 cm pH 3-10 ReadyStrip IPG strips in a Protean IEF cell (Bio-Rad). Strips were equilibrated according to the Bio-Rad instruction manual then laid on up to six 20 cm × 20 cm × 1 mm 12% acrylamide gels, which were run in parallel in an Ettan™ DALT*six* electrophoresis unit (Amersham) under denaturing conditions. Pelleted bacteria from the same *S pyogenes* cultures were resuspended in PBS containing 0.5 mM phenylmethylsulphonyl fluoride and 1 mM EDTA and repeatedly sonicated on ice (up to 10 times) for 15 seconds at 8 μm amplitude and 45 seconds intervals. Sonicates were desalted and subjected to two-dimensional electrophoresis as above.

Western blotting

Acrylamide gels were either stained directly with Coomassie blue G250 (Candiano *et al.*, 2004) or electroblotted onto 20 × 20 cm Hybond-ECL™ nitrocellulose membranes (Amersham). Membranes were blocked with 5% skimmed milk powder in PBS at 4°C overnight, washed four times in PBS containing 0.05% Tween 20 (PBS/T), then probed with pooled sera diluted 1:2,000 with PBS containing 1% skimmed milk powder for 2 hours at room temperature. Following four washes in PBS/T, membranes were incubated with alkaline phosphatase-labelled goat anti-human IgG (product code A 3188, Sigma) diluted 1:4,000 with PBS containing 1% skimmed milk powder for 90 minutes at room temperature, followed by four further washes and addition of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium chloride substrate solution (Sigma) in accordance with the supplier's instructions.

ELISAs and agglutination assay

Batches of secreted *S pyogenes* proteins were concentrated from overnight culture supernatants, dialyzed and freeze-dried as described above. Residues were redissolved in coating buffer (50 mM sodium bicarbonate buffer pH 9.6), 500 ng aliquots were added to the wells of Maxisorb microtiter plates (Nunc, Roskilde, Denmark) and left at 4°C overnight. Pelleted *S pyogenes* bacteria from the same overnight cultures were sonicated as described above, in PBS containing EDTA and phenylmethylsulphonyl fluoride. The sonicate was then diluted in a great excess of coating buffer ($>$2,000:1) to a final protein concentration of 500 ng in 100 μl, and 100 μl aliquots used to coat microtiter plate wells. Wells were then washed five times with PBS/T, blocked with 5% skimmed milk powder in PBS for 2 hours at 37°C, then again washed five times with PBS/T. Four duplicate dilutions (250-, 500-, 1,000-, and 2,000-fold) of each serum or plasma sample in PBS containing 1% skimmed milk powder were added to coated wells for 2 hours at 37°C, wells were again washed five times with PBS/T, and alkaline phosphatase-labelled goat anti-human IgG (Sigma) diluted 1:4,000 with 1% skimmed milk powder in PBS added for 90 minutes at 37°C. Following a further five washes in PBS/T, substrate *p*-nitrophenyl phosphate (Sigma) was added according to the supplier's instructions and the optical density read at 405 nm. Curves of dilution against

optical density were plotted for each sample. The titer of reactive IgG was taken as the dilution giving half-maximal optical density, rounded to the nearest actual dilution made. Maximal optical densities were established by including the same high-titer standard serum in each assay. Samples giving titers of more than 2,000 were re-assayed in duplicate at dilutions of 1,000, 2,000, 4,000, and 8,000 and titers expressed to the nearest 1,000. When analyzing IgG responses to secreted *S pyogenes* proteins, samples giving titers of less than 250 were re-assayed at dilutions of 50, 100, 200, and 400 and titers rounded to the nearest dilution made. Very similar titers were repeatedly obtained when plasma or serum from the same blood samples were used (data not shown). *Staphylococcus aureus* and *Staphylococcus epidermidis* (local clinical isolates) were cultured overnight in serum-free Rosewell Park Memorial Institute-1640 medium supplemented with 3 mM glutamine (Qbiogene, Cambridge, UK). Supernatant and cellular proteins from these cultures were prepared as described for *S pyogenes* and ELISAs were performed with these protein fractions (500 ng per well). Serum or plasma samples were assayed for antistreptolysin O IgG titers in the Clinical Microbiology Department, Leicester Royal Infirmary, by use of a polystyrene particle agglutination assay (RapiTex ASL, Dade Behring Limited, Milton Keynes, UK).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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