Immunodominant antigens of *Streptococcus equisimilis* shared by other β -haemolytic streptococci

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Summary. Three immunodominant antigens of *Streptococcus equisimilis* (Lancefield group C) with approximate mol. wts of 46, 66 and 105 kDa were recognised by human serum IgG and IgA immunoblotting. These antigens were identified consistently by various human sera but immunoblots with IgA (heavy chain) and secretory IgA (J chain) from human respiratory secretions gave more variable results. Antigens with similar migration rates were demonstrated in *S. pyogenes*, large colony human biotype group G streptococci, and streptococci of groups C and G from the “*S. anginosus-milleri* group”. Polyclonal antibody which was eluted from immunoblot substrates that contained the *S. equisimilis* 66-kDa antigen reacted with the 60-kDa antigen of *S. pyogenes*. Both polyclonal and monoclonal anti-vimentin antibodies identified the 46-kDa and 66-kDa antigens of *S. equisimilis*. The homology of these antigens among β -haemolytic streptococci has the potential to complicate both a strategy for the utilisation of immunoblotting for diagnostic purposes and the understanding of how such antigens may be involved in the pathogenesis of post-infectious sequelae.

Introduction

Although a cause-and-effect relationship between group A streptococci (*Streptococcus pyogenes*) and pharyngitis has been recognised for decades, a similar relationship has not been defined for most other β -haemolytic streptococci.¹ In part, the study of the association of β -haemolytic non-group A streptococci with pharyngitis has been complicated by the diversity of species within some Lancefield groups.¹ Furthermore, much of the previous work on a possible role for the β -haemolytic non-group A streptococci has not been able to benefit from the insight provided by relatively recent genetic studies that have demonstrated significant genetic homology between streptococci of different Lancefield groups.^{2,3}

Previously, we published circumstantial evidence that supported a link between large colony streptococci of groups C and G and symptomatic pharyngitis in children.⁴ Later, in a case-control study, we showed that moderate to heavy quantities of *S. equisimilis* (group C) and large colony human biotype group G streptococci were associated with symptomatic pharyngitis.⁵ The latter findings for *S. equisimilis* have been supported recently by the work of Turner *et al.*⁶ A cause-and-effect relationship could be strengthened by various further investigations that could, conceivably,

include infection of human volunteers, assessment of seroconversion and placebo-controlled treatment studies.

The present studies were initiated to improve our knowledge of the human humoral immune response to *S. equisimilis* and to a range of other β -haemolytic streptococci, by immunoblotting.

Materials and methods

Bacterial isolates

The six strains of *S. equisimilis* investigated were isolated from the throats of children with acute pharyngitis.^{4,5} Strain no. 6198 was used for most of the immunoblotting studies as identical SDS-PAGE patterns had been found in *c.* 50% of *S. equisimilis* strains that were obtained from human origin. This strain and the other *S. equisimilis* strains were characterised by Lancefield grouping (group C), carbohydrate fermentation, the Voges-Proskauer test and β -D-glucuronidase activity.⁷ Strains of *S. pyogenes* (group A), large colony human biotype group G streptococci, group C and G streptococci of the “*S. anginosus-milleri* group” and other streptococci were acquired either from our previous studies or from clinical specimens received in the routine diagnostic microbiology laboratory at British Columbia's Children's Hospital. These isolates were also acquired from the throats of symptomatic or asymptomatic children and represent wild-type isolates. Bacteria had been stored at -70°C and were previously passaged only

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Fig. 1. IgG immunoblotting of *S. equisimilis* strain no. 6198 with various samples of human adult donor serum (lanes 1–8). Antigens of 46, 66 and 105 kDa are predominantly recognised. Lane 9 confirms the lack of non-specific Fc binding of the anti-IgG conjugate. The markers on the left indicate mol.-wt standards.

Fig. 2. IgA (heavy chain) immunoblotting of *S. equisimilis* strain no. 6198 with various samples of human adult donor serum (lanes 1–5). Antigens of 46, 66 and 105 kDa are predominantly recognised. Lane 6 confirms the lack of non-specific Fc binding of the anti-IgA conjugate. Mol.-wt markers as for fig. 1.

Fig. 3. IgG immunoblotting of five wild-type *S. equisimilis* strains with a single human serum. These strains are known to differ in their SDS-PAGE profiles. Mol.-wt markers as for fig. 1.

three times or less. We chose to use wild-type strains for our studies because of the uncertainty of passage and the potential for change in virulence factors of highly-passaged type strains.

Sera and respiratory specimens

Serum was collected from 20 healthy volunteer adults and sputum from 20 adolescent paediatric inpatients with cystic fibrosis. To reduce the chance of salivary contamination to a minimum, all sputum samples were examined microscopically for the presence of squamous epithelial cells. Samples containing more than a few epithelial cells by low power microscopy were discarded. Sputum was centrifuged at 1500 *g* and the supernates were stored at -70°C until required for IgA immunoblotting studies. Serum samples were stored under the same conditions.

Gel electrophoresis and Western transfer

Bacteria were inoculated from the frozen state on to sheep blood 5% agar media (Columbia base) and then

incubated overnight at 37°C . The purity of cultures was confirmed and strains were inoculated into Todd-Hewitt broth and incubated aerobically at 35°C for 18–24 h. The biomass was centrifuged, washed three times and then suspended in phosphate-buffered saline (PBS; pH 7.2). These suspensions were standardised by estimating the whole-cell protein equivalent by the Bradford method (BioRad). Initial suspensions were diluted or concentrated to achieve a protein equivalent of *c.* 700 $\mu\text{g}/\text{ml}$. This preparation then served as the stock for subsequent SDS-PAGE. Samples of this stock were boiled in reducing sample buffer (1:4 ratio; 5 $\times$ sample buffer: 0.25 M Tris, glycerol 50%, SDS 10%, β -mercaptoethanol 5%) and then subjected to conventional one-dimensional SDS-PAGE which included 3% stacking and 10% running gels.⁸ After the electrophoresis was completed, the resolved products were transferred to 0.45- μm nitrocellulose in an electrophoresis unit (Hoefer Scientific Instruments, CA, USA) for 30 min with a direct current of 1.5 A. Pre-stained mol.-wt markers (BioRad) were used for the determination of approximate antigen migration rates and corresponding mol. wts.

Fig. 4. IgA (heavy chain) and secretory IgA (J chain) immunoblotting of *S. equisimilis* strain no. 6198 with sputum samples as the antibody source: lanes 1-9 include examples of immunoblot patterns for IgA (heavy chain) reactivity; 10-12 represent secretory IgA patterns; 10, 11 and 12 were with the same sputum samples as 1, 4 and 9, respectively. Note the recognition of a 73-kDa antigen in lanes 1, 4, 10 and 11. Mol.-wt markers as for fig. 1.

Immunoblotting

To assess the presence of antibody to the various bacteria, strips containing the profile of polypeptides for each bacterium were cut from the nitrocellulose. The nitrocellulose strips were blocked with a mixture of skimmed milk 1% and bovine serum albumin 1% in PBS. This stage was crucial because it prevented endogenous streptococcal Fc-binding fragments from interfering with bacterium-specific antibody recognition patterns.⁹ Strips were incubated overnight at 4°C or for 1 h at 35°C. Immunoblotting was performed to assess the presence of IgG, IgA or secretory IgA (sIgA) antibodies from either serum or sputum samples. For serum IgG or IgA assays, the human sera were diluted 1 in 100 in skimmed milk 1% in PBS and then incubated for 1 h at 35°C. The nitrocellulose support was washed three times with PBS and then incubated with a 1 in 500 dilution (in skimmed milk 1% in PBS) of goat anti-human IgG or IgA (heavy chain)-alkaline phosphatase-conjugated antibody (Jackson ImmunoResearch, PA, USA). 1-Nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate substrates were chosen for the alkaline phosphatase. For the detection of antibody in sputum, a 1 in 50 dilution of sputum supernate replaced the serum analogue in the preceding assay. Whereas the anti-IgA (heavy chain) was used as before in the remainder of the assay, the detection of sIgA was accomplished with murine monoclonal antibody

(MAb) directed against the J chain of IgA (MAb GA-1; ICN Biomedicals, CA, USA). For the latter, a 1 in 250 dilution (in skimmed milk 1% in PBS) of the MAb was used after incubation of the sputum supernate with the nitrocellulose strip. Goat anti-mouse IgG-peroxidase conjugate (dilution 1 in 500; BioRad) was used as the final antibody and 4-chloronaphthol served as the peroxidase substrate. For anti-vimentin immunoblotting, both murine MAb (clone V9; Dako-patts, Denmark) and rabbit polyclonal antibody (ICN Biomedicals) were used. The MAb was diluted 1 in 300 and the procedure followed essentially the same protocol as used for the J chain MAb. Rabbit polyclonal anti-vimentin antibody was diluted 1 in 600 and then detected with goat anti-rabbit IgG-horseradish peroxidase (1 in 1000 dilution; BioRad).

Affinity purification and re-blotting

To determine whether the 66-kDa antigen of *S. equisimilis* shared immunoreactive epitopes with the 60-kDa antigen of *S. pyogenes*, the following experiment was performed. The immunoreactive polypeptides of *S. equisimilis* were localised by IgG immunoblotting. Unused portions of contiguous nitrocellulose that contained the 66-kDa antigen were excised, blocked with skimmed milk 1% in PBS, and then incubated in 1 in 100 dilutions of human serum for 2 h at 35°C. After washing five times in PBS, the polyclonal human immunoglobulin was eluted from

Fig. 5. IgG immunoblotting of various streptococci: lanes A–D, *S. pyogenes*, group B streptococcus, *S. equisimilis* strain no. 6198 and *Enterococcus faecalis*. Note the variation of *S. equisimilis* (66 kDa) and *S. pyogenes* (60 kDa) antigens. Mol.-wt markers as for fig. 1.

the nitrocellulose by exposure to 0.2 M glycine-HCl (pH 2.8) for 15 min at 4°C. After the elution, the antibody in solution was dialysed overnight against PBS (pH 7.2). The dialysed eluate (undiluted) was then used as antibody in immunoblotting with the *S. pyogenes* substrate.

Results

Human serum IgG and IgA identified three main antigens of *S. equisimilis* strain no. 6198 with migration rates equivalent to mol. wts of c. 46, 66 and 105 kDa (figs. 1 and 2). There were minor variations of immunoblot patterns with different human sera and antigens < 46 kDa were rarely recognised. Although c. 50% of *S. equisimilis* strains of human origin (30 wild-type, epidemiologically unlinked isolates) that we have studied yielded SDS-PAGE profiles identical to strain no. 6198, at least seven other SDS-PAGE patterns could be defined by major or minor vari-

ations. Sera were used to probe immunodominant antigens among other *S. equisimilis* strains that had SDS-PAGE patterns different from that of strain no. 6198. Similar antigens were immunodominant (fig. 3). These strains represent c. 80% of SDS-PAGE patterns among our wild-type *S. equisimilis* isolates. Control immunoblots, with bacterial growth media as the substrate for antibody recognition, were negative.

Half of the sputum samples (10 of 20) had specific IgA to *S. equisimilis* that could be demonstrated with anti-heavy chain conjugates. Examples with stronger resolution are illustrated in fig. 4. The main antigens recognised were similar to those detected with serum samples. Among the 10 sputum samples that contained anti-*S. equisimilis* IgA (heavy chain), only four had evidence of anti-*S. equisimilis* sIgA. In addition to variable recognition of the three dominant antigens, an antigen of 73 kDa was occasionally recognised by IgA (heavy chain) or sIgA from sputum. This antigen was not prominently highlighted in any of the serum immunoblotting.

Fig. 6. IgG immunoblotting of various streptococci: lanes 1–3, three different strains of large colony human biotype group G streptococci that have been demonstrated previously to possess different SDS-PAGE profiles; 4–6, three different strains of group C “*S. anginosus-milleri* group”. Mol.-wt markers as for fig. 1.

Further studies with serum IgG immunoblotting identified what appeared to be the same three immunodominant antigens in *S. pyogenes*, large colony group G streptococci, and group C and G streptococci of the “*S. anginosus-milleri* group” (figs. 5 and 6). The patterns obtained with group B streptococci and enterococci showed that these did not possess the 46-, 66- or 105-kDa antigens.

Although the presence of the triplet fingerprint of immunodominant antigens among the different streptococci was most striking, it was apparent that antigens putatively homologous between species did not have identical migration rates, e.g., the 66-kDa antigen of *S. equisimilis* appeared to have a homologue of 60 kDa in *S. pyogenes* (fig. 5). Minor variation of the same antigen was also noted among *S. equisimilis* strains (fig. 3). When polyclonal antibody was eluted from the 66-kDa antigen of *S. equisimilis*, it was found to bind to the 60-kDa antigen of *S. pyogenes*, thereby confirming the suspicion that these antigens shared epitopes.

Both monoclonal and polyclonal anti-vimentin antibodies recognised the 46- and 66-kDa antigens of *S. equisimilis* (not shown). Controls for growth medium components were uniformly negative.

Discussion

The three immunodominant antigens described were consistently present among different *S. equisimilis*

strains and were recognised by human serum of different origins. There was some variation in the demonstration of these antigens when IgA or sIgA from sputum was tested, but further study seems warranted to assess their potential role in pathogenesis and in the development of immunodiagnostic assays. However, the frequency and degree of their recognition by the humoral immune responses implies that qualitative immunoblotting assays alone will be insufficient. Nevertheless, it is conceivable that quantitative assays that examine humoral responses to these antigens may be of value. In addition to the triplet of commonly recognised antigens, studies of IgA in sputum demonstrated a 73-kDa antigen. It is remarkable that serum IgG and IgA did not highlight this same antigen. Immunoblotting has been employed to investigate the association of various streptococci and guttate psoriasis,¹⁰ but the immunodominant antigens described here were not reported. Rather, the latter paper proposed that differences in the immunoblotting response among patient groups could be defined on the basis of both the number and intensity of staining of the antigens recognised.

The presence of the triplet of immunodominant antigens among strains of *S. equisimilis* and large colony group G streptococci is not surprising, as they are probably homogeneous.^{2,3,7,9} The fact that the antigens were also found in *S. pyogenes* could be related to all three species being primary pharyngeal pathogens with the ability to cause infections of

sufficient severity to stimulate systemic and mucosal antibody responses to these antigens. Previous studies have proposed homology between streptococci of Lancefield groups A, C and G by the demonstration of common M-like proteins.^{7,11,12} The association of the "*S. anginosus-milleri* group" with symptomatic pharyngitis is in doubt⁵ and it is known that mucosal colonisation is common and increases with age.⁴ Therefore, the presence of the triplet of immunodominant antigens in this group of streptococci detracts somewhat from the hypothesis that these antigens are related to pathogenesis.

Several studies have demonstrated evidence of immunological cross-reactivity between *S. pyogenes* M proteins, vimentin, cardiac antigens and the *S. pyogenes* 60-kDa antigen.¹³⁻¹⁷ Some of these studies have proposed that these immunological similarities have significant implications for the understanding of both rheumatic fever and enteroviral myocarditis. Whereas it is believed that the M proteins of *S. pyogenes* are prime candidates for stimulating an auto-antibody response that may be the fundamental pathology in acute rheumatic fever, the 60-kDa antigen also cross-reacts with heart tissue. Our results seem to suggest

that the *S. pyogenes* 60-kDa antigen and the *S. equisimilis* 66-kDa antigen share epitopes, and that this antigen in addition to the 46-kDa antigen shares epitopes with vimentin. The presence of these antigens in less pathogenic but commonly found streptococci indicates that the role of such antigens in the pathogenesis of rheumatic heart disease may be less than previously believed. Ayakawa *et al.*¹⁴ have also used anti-*S. pyogenes* MAbs to identify a 67-kDa antigen in *S. mutans*; the latter organism rivals bacteria of the "*S. anginosus-milleri* group" in frequency of human oropharyngeal colonisation. A function remains to be defined for the 60-kDa antigen of *S. pyogenes*, although proteins of similar mol. wt have included protein kinases, actin-like filaments and streptolysin O.¹⁸⁻²⁰

The value of the immunodominant antigens described here for serodiagnostic purposes remains to be fully investigated. Furthermore, the complex antigenic relationships of the β -haemolytic streptococci that have been demonstrated warrant further study in the context of auto-antibody formation and the pathogenesis of post-infectious sequelae of streptococcal infections.

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