Comparison of IgG Fc receptors from clinical isolates of *Streptococcus zooepidemicus*

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Summary. A receptor binding the Fc region of equine immunoglobulin G (IgG) has been isolated from a heat-extracted preparation of a clinical isolate of *Streptococcus zooepidemicus*. This Fc receptor has a M_r of 45×10^3 and was occasionally seen as an apparent trimer of M_r 130×10^3 . Antibodies prepared in horses against the receptor could be adsorbed to and eluted from whole live bacteria, confirming the surface location of this protein. Another 11 isolates of *S. zooepidemicus* from horses with pneumonia, abscesses or endometritis were tested for Fc-receptor activity. Although the M_r of the Fc receptors varied among isolates, their antigenicity was conserved. Thus, the Fc receptor is an attractive candidate for application in the diagnosis of, or protection against, infections with *S. zooepidemicus*.

Introduction

Streptococcus zooepidemicus is the most common bacterial species causing endometritis in mares (Bain, 1966; Shin *et al.*, 1979; Ricketts, 1981; Wingfield Digby and Ricketts, 1982) and is the primary aetiological agent in 15–20% of equine abortions (Roberts, 1971; Welsh, 1984). This level of reproductive wastage in the mare may be minimised through application of specific immunological techniques for protecting against streptococcal infections in the equine reproductive tract. Effective immunological protection requires that the immune response be directed to surface antigens that are conserved among the majority of pathogenic isolates of *S. zooepidemicus*.

S. zooepidemicus possesses a surface Fc receptor which is distinct from those of other streptococci (Myhre and Kronvall, 1982) and from protein A of *Staphylococcus aureus* (Myhre and Kronvall, 1981). The aim of this study was to investigate antigenic conservation among the Fc receptors of clinical isolates of *S. zooepidemicus*. In this paper we describe the isolation of a *S. zooepidemicus* Fc receptor, demonstrate its immunogenicity, and characterise its antigenic homology with receptors from other isolates of *S. zooepidemicus*.

Materials and methods

Bacteria and growth conditions

Twelve isolates of *S. zooepidemicus* were isolated from

horses with clinical conditions that included endometritis (7 cases), pneumonia (2), a cutaneous wound and abscesses (2). Isolates were stored at -70°C in glycerol 60% v/v in 0.1 M phosphate-buffered saline (PBS) pH 7.2. Bacteria were plated on Blood Agar (Difco Laboratories, Detroit, MI, USA) and cultured overnight. The entire growth was resuspended in 2 ml of Todd-Hewitt Broth (Difco) and 0.5 ml was transferred to 50 ml of broth and cultured overnight in a shaking water bath (37°C , 150 rpm). Bacteria, harvested by centrifugation (10 000 g, 10 min) were resuspended in 0.25 M Tris-HCl (pH 6.8). *S. zooepidemicus* isolate no. 1381, isolated from a case of equine endometritis, was used in most experiments.

Heat-extracted preparations

Harvested bacteria were heated at 90°C for 10 min and, after centrifugation at 10 000 g for 10 min, the supernate, which contained a relatively pure preparation of Fc receptor, was dialysed against distilled water.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Bacterial suspensions (c. 5×10^8 bacteria/ml) in 0.25 M Tris-HCl (pH 6.8), or heated extracts, were mixed in a ratio of 2:1 (v:v) with sample buffer (SDS 6% w/v, glycerol 40% v/v, 2-mercaptoethanol 7% v/v in 0.5 M Tris-HCl pH 6.8) and boiled for 3 min. Samples were electrophoresed as described by Laemmli (1970). Separated proteins were electroblotted on to nitrocellulose (Towbin *et al.*, 1979) and allowed to react with equine IgG-Fc fragments, F(ab')_2 fragments or antisera, followed by peroxidase-conjugated anti-equine IgG (Kirkegaard and Perry, Gaithersburg, MD, USA). Blots were developed with chloronaphthol 0.3% w/v in methanol

20 ml, mixed with hydrogen peroxide 0.018% w/v and 0.5 M sodium chloride in 0.02 M Tris-HCl (pH 7.5).

Blots were then stained for proteins with amido black 0.1% w/v in isopropanol:acetic acid:water (25:10:65) and destained with methanol:acetic acid:water (40:10:50).

Isolation of Fc receptor

Heat-extracted preparation of *S. zooepidemicus* no. 1381 was electrophoresed on SDS-polyacrylamide gel with reducing conditions and stained with Coomassie blue. The protein band of $M_r 45 \times 10^3$ was cut from the gel, eluted with a unidirectional electroelutor (model UEA, International Biotechnologies Incorporated New Haven, CT, USA), dialysed against distilled water and concentrated by lyophilisation.

Equine sera

Heat-killed bacteria ($c. 2 \times 10^9$ cells) of *S. zooepidemicus* strain no. 1219 were injected into a horse by the intramuscular (i.m.) route. Serum was collected before immunisation and 2 and 6 weeks after immunisation and stored at -20°C in divided portions.

Monospecific polyclonal antibodies against the Fc receptor of *S. zooepidemicus* strain no. 1381 were prepared by immunising horses with the antigen of $M_r 45 \times 10^3$, electroeluted from SDS-polyacrylamide gels; 10- μg amounts of electroeluted protein in muramyl-dipeptide adjuvant 250 $\mu\text{g}/\text{ml}$ were given by two i.m. injections 4 weeks apart. Serum was collected 2 weeks after the last injection.

Affinity-purified IgG and IgG-fragments

Antiserum specific for the Fc receptor ($M_r 45 \times 10^3$) was affinity-purified by adsorption to and elution from *S. zooepidemicus* strain no. 1381. Antiserum (25 ml) was incubated for 2 h at 20°C with *S. zooepidemicus* (5g wet wt). Bacteria were centrifuged (10 000 g for 10 min) and resuspended in 10 ml of 0.1 M glycine-HCl buffer (pH 2.2). The supernate recovered after centrifugation was neutralised by the addition of Trizma base (Sigma Chemical Co., St Louis, MO, USA) and dialysed against 10 mM Tris-HCl (pH 6.8).

F(ab')_2 fragments of the above affinity-purified antiserum made against the protein of $M_r 45 \times 10^3$ were prepared by pepsin digestion as described before (Yarnall *et al.*, 1988). IgG was incubated in 0.1 M sodium acetate (pH 4.2) with pepsin (Sigma) 5% w/w for 8 h at 37°C . More pepsin was added after 8 h and incubation continued for a further 16 h at 37°C . F(ab')_2 fragments were harvested after absorption of undigested IgG with protein A-sepharose (Pierce Chemical Co., Rockford, IL, USA).

Polyspecific equine IgG was digested with papain and the Fc fragments isolated by ion-exchange chromatography on DEAE-cellulose (Widders *et al.*, 1988). Purity of Ig-fragment preparations was monitored by SDS-PAGE.

Results

Isolation of Fc receptor

Nitrocellulose blots of whole-cell and heat-extracted preparations of *S. zooepidemicus* strain no. 1381 were probed with equine Fc fragments to identify the Fc receptor. A protein of $M_r 45 \times 10^3$ reacted with Fc fragments in both antigen preparations of *S. zooepidemicus* (fig. 1, lanes 1 and 2). In heat-extracted preparations, another protein of $M_r 130 \times 10^3$ was also reactive (fig. 1, lane 2). When the protein of $M_r 45 \times 10^3$ was isolated by electroelution, subjected to SDS-PAGE and probed with Fc fragments, it retained Fc receptor activity (fig. 1, lane 3). When, however, the protein of $M_r 130 \times 10^3$ was electroeluted and subjected to SDS-PAGE, it migrated as a protein of $M_r 45 \times 10^3$ (data not shown).

Fig. 1. Identification of Fc receptor of *S. zooepidemicus* strain no. 1381. Proteins, after SDS-PAGE and electroblotting to nitrocellulose were: stained with amido black, panel A; or probed with equine Fc (40 $\mu\text{g}/\text{ml}$) followed by peroxidase-conjugated goat anti-horse IgG diluted 1 in 1000. Lane 1—whole-cell preparation; lane 2—heat-extracted preparation; lane 3—electroeluted Fc receptor, $M_r 45 \times 10^3$. M_r indicates marker proteins.

Reactivity of the Fc receptor with *S. zooepidemicus* antiserum

Samples of whole-cell and heat-extracted preparations of *S. zooepidemicus* strain no. 1381 were electrophoresed by SDS-PAGE, electroblotted on to nitrocellulose, and probed with pre- and post-immunisation antisera raised against *S. zooepidemicus*. At a dilution of 1 in 20 000, immune serum reacted with >15 proteins in whole-cell preparations of *S. zooepidemicus*, but with <10 proteins in the heat-extracted preparation. Immune serum reacted strongly with a protein of $M_r 45 \times 10^3$ present in both preparations (fig. 2).

To avoid reactivity of the antibody *via* the Fc region of the molecule, $F(ab')_2$ fragments of pre- and post-immunisation antisera prepared against the protein of $M_r 45 \times 10^3$ were used to probe bacterial antigens transferred to nitrocellulose. Both bacterial proteins of $M_r (10^3) 45$ and 130 were

recognised by immune but not pre-immune $F(ab')_2$ fragments ($0.1 \mu\text{g}/\text{ml}$) in blots of whole-cell preparations of strain no. 1381 (data not shown).

Antigenic conservation of Fc receptors from clinical isolates of *S. zooepidemicus*

Heat-extracted preparations from 12 isolates from horses with pneumonia, abscesses or endometritis, were electrophoresed by SDS-PAGE, blotted on to nitrocellulose and probed with equine Fc fragments ($40 \mu\text{g}/\text{ml}$) or with $F(ab')_2$ fragments ($1 \mu\text{g}/\text{ml}$) of affinity-purified antiserum prepared against the protein of $M_r 45 \times 10^3$. All isolates had proteins reacting with the Fc fragments, although the M_r of these Fc receptors varied among isolates (fig. 3, panel A). The $F(ab')_2$ -antibody preparation reacted with the Fc receptors of all isolates (fig. 3, panel B).

Discussion

In this study, we have isolated a surface protein from *S. zooepidemicus* that reacts with the Fc portion of equine IgG. Although many proteins were demonstrated in the protein profile of *S. zooepidemicus* strain no. 1381, only a few were strongly reactive with antiserum from a horse immunised with a different isolate (strain no. 1219) of *S. zooepidemicus*. This limited reactivity may reflect either poor immunogenicity of many bacterial antigens, or limited conservation of antigens among different isolates. One antigen apparently shared is the Fc receptor of $M_r 45 \times 10^3$. As reported before (Myhre and Kronvall, 1982; Reis *et al.*, 1988), the Fc receptor is present on the surface of the bacterium. That observation was confirmed in the present study because antibody specific for the Fc receptor ($M_r 45 \times 10^3$) could be affinity purified by adsorption to and elution from live *S. zooepidemicus*.

Antigenic conservation among the Fc receptors of clinical isolates of *S. zooepidemicus* was indicated by the reactivity of the antiserum to the protein of $M_r 45 \times 10^3$. Despite the considerable heterogeneity among isolates in the M_r of proteins with Fc-receptor activity, binding of equine Fc fragment was paralleled by binding of the $F(ab')_2$ antibody preparation. Binding of this $F(ab')_2$ preparation was antigen specific, because the $F(ab')_2$ preparation of preimmunisation serum did not react with the protein of $M_r 45 \times 10^3$ at a comparable dilution (data not shown). $F(ab')_2$ fragments were used to probe blots at a concentration one-fortieth of that of Fc fragments, indicating that any contaminating

Fig. 2. Proteins in whole-cell preparations (panel A) or heat-extracted preparations (panel B) of *S. zooepidemicus* strain no. 1381 probed with equine antiserum against *S. zooepidemicus* strain no. 1219 and detected with peroxidase-conjugated goat anti-horse IgG diluted 1 in 1000. Sera were tested at a dilution of 1 in 20 000; lane 1—pre-immunisation; lane 2—post-immunisation.

Fig. 3. Heat-extracted preparations of 12 clinical isolate of *S. zooepidemicus*, after SDS-PAGE and electroblotting to nitrocellulose, were probed with: equine Fc fragments, 40 µg/ml, panel A; or F(ab')₂ fragments, 1 µg/ml, against the protein of M_r 45 × 10³, panel B. Blots were developed as described in fig. 1. Isolates were: lane 1, no. 10837 from cutaneous wound; lane 2, no. 10217 from abscess; lane 3, no. 8527 from abscess; lane 4, no. 7243 from pneumonia; lane 5, no. 2049 from endometritis; lane 6, no. 1854 from endometritis; lane 7, no. 1731 from endometritis; lane 8, no. 1219 from endometritis; lane 9, no. 1849 from endometritis; lane 10, no. 1826 from endometritis; lane 11, no. 6747 from pneumonia; lane 12, no. 1381 from endometritis.

whole IgG molecules present in the F(ab')₂ preparation, undetectable by SDS-PAGE, could not account for the binding observed with this preparation.

Conservation of antigenic epitopes among streptococcal Fc receptors is predicted by the immunoglobulin-binding characteristics of these proteins. Streptococcal Fc receptors recognise a limited domain in the Fc region of IgG (Christensen *et al.*, 1976; Schroder *et al.*, 1987), suggesting that the structure and, therefore, antigenicity of the binding site of the bacterial Fc receptors is conserved. Although the present results confirm that antigenicity is conserved, it is not known if the antibody against the protein of M_r 45 × 10³ reacts with the specific binding site of the Fc receptor.

The role of the streptococcal Fc receptor in bacterial virulence is uncertain, but comparable receptor molecules in other gram-positive pathogens have been associated with enhanced virulence. In group-A streptococci, the M protein binds fibrinogen (Whitnack *et al.*, 1984) and thereby restricts complement deposition on the bacterial cell surface (Whitnack and Beachey, 1982), probably by masking surface antigens. In the absence of specific antibody, this mechanism limits phagocytosis *in vitro* (Whitnack and Beachey, 1982) and may enhance bacterial survival *in vivo* by limiting effective opsonisation. Of particular significance is the antiphagocytic effect of the staphylococcal Fc receptor, protein A (Dossett *et al.*, 1969; Peterson *et al.*, 1977). This receptor activity limits phagocytosis either by reducing effective opsonisation (Stahlenheim *et al.*, 1973; Peterson *et al.*, 1977; Schalen *et al.*, 1985) or by competing with the phagocyte Fc receptor for binding to the Fc region of specific antibody (Dossett *et al.*, 1969; Stahlenheim *et al.*, 1973). Staphylococci are killed after internalisation by phagocytic cells (White and Walker, 1981). Evasion of phagocytosis, therefore, will enhance bacterial survival *in vivo*. Antibody specific for these receptor molecules may modify their receptor function and, therefore, may enhance clearance of these organisms by host defences. On the basis of the surface location of the protein of M_r 45 × 10³, its immunogenicity, and its conservation among clinical isolates, the Fc receptor of *S. zooepidemicus* is an attractive candidate in both protection against, and rapid diagnosis of, infections by *S. zooepidemicus*.

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