Characterization of Two *Haemophilus somnus* Fc Receptors

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Haemophilus somnus expresses two types of receptors that bind to the Fc region of bovine IgG, IgA and IgM. In this study, the relationship between these two types of Fc receptors is characterized. The high molecular mass receptors (350, 270 and 120 kDa) were secreted into the culture medium and were also in the insoluble protein fraction of the culture medium. The 41 kDa Fc receptor, which is a major outer-membrane protein, was only present in the insoluble protein fraction. Peptide mapping of the two types of Fc receptors suggests that the 41 kDa receptor is related to the high molecular mass receptor complex. Disulphide linkage is unlikely to be the mechanism of association of the 41 kDa receptor with the high molecular mass receptors since reducing agents had no effect on separating the individual receptors. Although the 41 kDa receptor is a major protein in the outer membrane of *H. somnus*, it does not react with convalescent bovine sera in Western blots. In contrast, convalescent bovine sera reacts intensely with the high molecular mass receptors in Western blots.

INTRODUCTION

Bacterial Fc receptors have been identified on staphylococci (Langone, 1982; Cox *et al.*, 1986) and streptococci (Kronvall, 1973; Myrhe & Kronvall, 1977, 1981; Reis *et al.*, 1984; Yarnall & Boyle, 1986) and more recently on three Gram-negative organisms, *Brucella abortus* (Nielsen *et al.*, 1981), *Tylorella equigenitalis* (Widders *et al.*, 1985) and *Haemophilus somnus* (Widders *et al.*, 1988). The role of Fc receptors in host-parasite interactions is not understood. The staphylococcal Fc receptor, protein A promotes evasion of phagocytosis (Peterson *et al.*, 1977; Dossett *et al.*, 1969), and it has been postulated that other bacterial Fc receptors may be virulence factors (Christensen *et al.*, 1977, 1978, 1981; Schalen, 1982; Ginsburg, 1972). We have recently identified and partially characterized two functionally distinct types of Fc receptors from *H. somnus* based on bovine Ig class reactivity (Yarnall *et al.*, 1988). The receptor with a molecular mass of 41 kDa bound weakly to both bovine IgG subclasses, IgA and IgM, while three high molecular mass receptors (350, 270 and 120 kDa) strongly bound bovine IgG₂, IgA and IgM. All of these Fc receptors were immunologically related (Yarnall *et al.*, 1988).

Although *H. somnus* is a major cause of disease in both dairy and beef cattle, causing thromboembolic meningoencephalitis (TEME) (Bailie *et al.*, 1966; Griner *et al.*, 1956), pneumonia (Corbeil *et al.*, 1986; Corstuet *et al.*, 1973; Saunders *et al.*, 1980), abortion (Corbeil *et al.*, 1987; Chladek, 1975; Humphrey *et al.*, 1982*a, b*) and probably infertility (Keister, 1981; Humphrey & Stephens, 1983), virulence mechanisms and specific host defences are poorly understood. The persistence of *H. somnus* on the respiratory and genital mucosae (Humphrey *et al.*, 1982*a, b*; Slee & Stephens, 1985; Ward *et al.*, 1983), however, suggests that the organism can effectively evade the local defence mechanisms. Characterization of *H. somnus* Fc receptors is a

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prerequisite for determination of the potential roles of these receptors as bacterial virulence factors. Therefore, in this study, *H. somnus* Fc receptors were characterized according to location in the bacterial membrane, peptide mapping profiles and immunogenicity in cattle.

METHODS

Bacterial strain, media and growth conditions. *Haemophilus somnus* strain 2336 was used throughout this study. It was recovered from naturally occurring bovine pneumonia and subsequently used to produce experimental pneumonia (Gogolewski *et al.*, 1987*a, b*). Bacteria were frozen in 60% (v/v) glycerol in 0.01 M-sodium phosphate-buffered isotonic (0.15 M) saline, pH 7.4 (PBS) at -70°C after one subculture of the initial isolate. Bacteria were cultured at 37°C in brain heart infusion broth (BHI, Difco) supplemented with 0.1% Tris and 0.001% thiamin monophosphate (BHI-TT) as described by Inzana & Corbeil (1987), or were cultured on Columbia blood agar (CBA; Difco) plates made with 10% (v/v) bovine blood or fetal bovine serum.

Preparation of the insoluble protein fraction of culture medium. *H. somnus* was grown in 300 ml BHI-TT broth for 8 h in a shaking water-bath at 37°C . After centrifugation at 10000 *g* for 10 min, the supernatant was filtered through a 0.45 μm filter and centrifuged at 350000 *g* for 2 h at 4°C in a Beckman T150.2 rotor to sediment insoluble proteins. Pellets recovered from 300 ml of culture were suspended in 2 ml distilled water. The resulting supernatant was concentrated 50 $\times$ by lyophilization and was reconstituted in 6 ml distilled water.

Gel electrophoresis and Western blotting. Insoluble proteins (25 μl) and supernatant (30 μl) were analysed by electrophoresis under denaturing and reducing conditions in polyacrylamide gels containing SDS according to Laemmli (1970). In certain experiments no 2-mercaptoethanol or dithiothreitol was added. The separated proteins were electroblotted onto nitrocellulose (Towbin *et al.*, 1979) and probed either with antisera prepared against the *H. somnus* Fc receptors or with convalescent bovine sera. Rabbit antibody was detected using peroxidase-conjugated goat anti-rabbit IgG (Kirkegaard and Perry Labs) and bovine antibody was detected with peroxidase-conjugated goat anti-bovine IgG (Cooper Biochemicals). The blots were developed with 4-chloro-1-naphthol (0.05%) plus H_2O_2 (0.015%) in 16% (v/v) methanol.

Rabbit and bovine sera. Monospecific polyclonal antibodies to *H. somnus* Fc receptor proteins were prepared by inoculating rabbits or cattle with single antigen bands cut from SDS-PAGE as described previously (Yarnall *et al.*, 1988). In brief, *H. somnus* 2336 insoluble protein pellets were applied to a 3.0 mm SDS-polyacrylamide gel and the gel was stained with 0.2% Coomassie Brilliant Blue after electrophoresis. The 41 kDa band, identified using molecular mass standards from a parallel gel, was cut from the gel and emulsified in approximately equal volumes of complete Freund's adjuvant for intramuscular inoculation into a cow. The bands from two preparative gels were given for each inoculation. Booster doses emulsified in incomplete Freund's adjuvant were given at monthly intervals with serum collected 10 d after each inoculation. The reactivity of the antisera was determined by Western blots of *H. somnus* insoluble protein pellets reacted with peroxidase-conjugated goat anti-bovine IgG (Cooper Biochemicals). Rabbits were immunized with either the 41 kDa band or the 270 kDa band in much the same way, except that the band from one gel was used for each rabbit.

Convalescent bovine sera were obtained from animals challenged experimentally with *H. somnus*. The clinical and isotypic antibody responses to these challenges have been described in earlier reports of experimental abortion (Widders *et al.*, 1986) or pneumonia (Gogolewski *et al.*, 1987*b*). Serum P3 was obtained from a cow in an experimental abortion study 6 weeks after intrabronchial inoculation of 10^{10} colony forming units (c.f.u.) of *H. somnus* isolate 649-4. Serum E5 was collected from a calf 5 weeks after intrabronchial inoculation of 10^7 c.f.u. *H. somnus* isolate 2336.

Limited proteolysis. Peptide maps were constructed by limited proteolysis using the procedure of Josefsson & Randall (1983). Briefly, the 270 kDa and 41 kDa proteins were cut from an SDS-polyacrylamide gel, placed in the wells of another gel with 0.1 μg *Staphylococcus aureus* V8 protease (Miles Scientific) and electrophoresed into the stacking gel. Electrophoresis was then stopped for 45 min to allow digestion of the proteins which had been eluted from the gel piece. Electrophoresis was restarted and the peptides which were generated during the digestion were separated in the gel, electroblotted onto nitrocellulose, and probed with rabbit anti-41 kDa sera. The blot was developed as described above.

RESULTS

Release of Fc receptors into the culture supernatant. Our previous studies showed that Fc receptors are found in the supernatant of exponential phase *H. somnus* cultures (Yarnall *et al.*, 1988). The initial effort of this study was to determine if both types of Fc receptors were secreted as soluble proteins directly into the medium or were sedimented with the insoluble protein fraction of the culture supernatant. Reacting these bacterial antigens with antisera specific for

Fig. 1

Fig. 2

Fig. 1. *H. somnus* Fc receptors in culture supernatant or insoluble pellet fraction. Culture supernatant concentrated $50\times$ (lane 1) or insoluble pellet fraction (lane 2) were electrophoresed on an SDS-polyacrylamide gel containing 2-mercaptoethanol, electroblotted onto nitrocellulose, and probed with a mixture of rabbit anti-270 kDa (1/2000 dilution) plus rabbit anti-41 kDa sera (1/2000 dilution). Rabbit antibodies were detected with peroxidase conjugated goat anti-rabbit IgG (1/2000 dilution).

Fig. 2. Peptide maps of *H. somnus* 270 kDa and 41 kDa Fc receptors. Peptides generated by *Staphylococcus aureus* V8 protease were detected by probing the nitrocellulose blot with rabbit anti-41 kDa sera (1/1000 dilution) followed by peroxidase-conjugated goat anti-rabbit IgG (1/2000 dilution). Lane 1, 270 kDa peptide profile. Lane 2, 41 kDa peptide profile. Arrows indicate identically migrating peptides from both Fc receptors.

the Fc receptors revealed that the 41 kDa Fc receptor was found only in the insoluble protein pellet. However, the high molecular mass Fc receptors were detected both in the pellet and in the supernatant (Fig. 1).

Treatment with reducing agents. Since preliminary results suggested that the 41 kDa Fc receptor may be a subunit or degradation product of the high molecular mass receptors (Yarnall *et al.*, 1988), we determined the effect of reducing and non-reducing conditions on the Fc receptors: neither dithiothreitol nor 2-mercaptoethanol had any effect (results not shown).

Peptide mapping of Fc receptors. Peptide maps of the two types of Fc receptors probed with anti-41 kDa serum are shown in Fig. 2. Some antigenic peptides from the 270 kDa Fc receptor (Fig. 2, lane 1) migrated identically to antigenic peptides from the 41 kDa Fc receptor (Fig. 2, lane 2); however, not all of the antigenic peptides detected in the 41 kDa peptide profile were detected in the 270 kDa peptide profile. No additional peptides in the 41 kDa peptide profile

Fig. 3

Fig. 4

Fig. 3. Detection of *H. somnus* antigens using convalescent sera. *H. somnus* insoluble pellet fraction was electrophoresed on an SDS-polyacrylamide gel, electroblotted onto nitrocellulose, and probed with convalescent sera from a case of experimental abortion (lane 1) or from a case of experimental pneumonia (lanes 2 and 3). Lane 3 was counterstained with amido black for detection of proteins.

Fig. 4. Bovine anti-41 kDa serum activity. Antiserum prepared against the 41 kDa Fc receptor was tested for reactivity with *H. somnus* insoluble pellet proteins by Western blot analysis. Blots were developed using peroxidase-conjugated goat anti-bovine IgG. Lane 1, preimmune serum (1/8000 dilution); lane 2, anti-41 kDa serum (1/8000 dilution).

were detected when the nitrocellulose was stained with amido black. Some high molecular mass peptides (> 41 kDa) in the 270 kDa peptide profile, however, were not detected with the anti-41 kDa sera (data not shown).

Reactivity with convalescent sera. Detection of *H. somnus* antigens with convalescent bovine sera is shown in Fig. 3. Both abortion (lane 1) and pneumonia (lane 2) convalescent sera recognized the high molecular mass Fc receptors, but neither serum recognized the 41 kDa Fc receptor even though it is a major protein in the insoluble protein pellet (lane 3).

Immunogenicity of the 41 kDa Fc receptor in cattle. Although we have prepared antisera against the 41 kDa Fc receptor in rabbits (Yarnall *et al.*, 1988), no antibody was detected in Western blots with serum from cattle previously infected or immunized with *H. somnus*. Therefore we immunized a cow to determine whether the protein itself is non-immunogenic in cattle. Fig. 4 shows the results of the immunization with the 41 kDa Fc receptor. Antibody against the 41 kDa Fc receptor was produced and this antiserum also reacted strongly with the high molecular mass receptors.

DISCUSSION

Recently we demonstrated that the binding of bovine IgG subclasses IgA and IgM to *H. somnus* was a function of two distinct types of Fc receptors (Yarnall *et al.*, 1988). One type consisted of a family of high molecular mass proteins (a 350 kDa protein, a 270 kDa protein, and a series of proteins in the 120 to 270 kDa range) which bound strongly to bovine IgG₂, IgA and IgM. The other type of Fc receptor (a 41 kDa protein) bound weakly to bovine IgG₁, IgG₂, IgA and IgM. In this report, we have further characterized these two types of Fc receptors. The high molecular mass Fc receptors were present in the culture medium in both soluble and insoluble forms (Fig. 1), whereas the 41 kDa Fc receptor was found only in the insoluble portion of the supernatant. Although we hypothesized that the 41 kDa protein may be a subunit of the high molecular mass proteins, treatment with reducing agents had no effect on any of the Fc receptors. The peptide profiles of the two types of Fc receptors, however, suggested that the 41 kDa Fc receptor is related to the high molecular mass receptor since digestion of the 270 kDa Fc receptor produced peptides which migrated identically and were antigenically related to peptides from the digestion of the 41 kDa Fc receptor (Fig. 2). Digestion of the 270 kDa protein was always less efficient than digestion of the 41 kDa protein even if the concentration of protease was increased. Therefore, the apparently weak reactivity of the peptides from the 270 kDa Fc protein (Fig. 2) probably reflects the low concentration of peptides which were generated, rather than a lower affinity *per se*.

Although the 41 kDa Fc receptor appears to be a major protein in the insoluble protein pellet of *H. somnus* (Fig. 3), and is immunogenic in rabbits and cattle as a purified protein from an SDS polyacrylamide gel (Fig 4), it fails to react with sera from animals convalescing from *H. somnus* disease (Fig. 3). This result is in keeping with those of our previous studies, when we found no reactivity with the 41 antigen among five out of five sera from cattle with experimental pneumonia (Gogolewski *et al.*, 1987b), and eighteen out of eighteen cattle with abortion (Corbeil *et al.*, 1987), or one cow hyperimmunized with whole formulated *H. somnus* (Corbeil *et al.*, 1987). The reason for the recognition of the high molecular mass Fc receptors, but not the 41 kDa Fc receptor by convalescent bovine sera (Fig. 3) is not known. The 41 kDa protein may not be immunogenic in cattle infected or immunized with *H. somnus* because the protein is not exposed in the outer membrane. This would be consistent with the lack of shedding of soluble 41 kDa protein into the supernatant. However, it may also be that the 41 kDa protein was denatured by boiling in SDS prior to SDS-PAGE and excision of the band from the gel in our immunization studies with gel purified protein. The denatured protein may be immunogenic whereas the native protein may not be immunogenic. Whatever the reason for lack of an immune response to the 41 kDa protein by infected animals, the lack of response may be significant in bacterial evasion of the host response. Since antibody to *H. somnus* enhances phagocytosis (Czuprinski & Hamilton, 1985) and protects against experimental pneumonia (Gogolewski *et al.*, 1987), failure of this major membrane protein to stimulate a humoral response during the course of an infection may enhance bacterial survival *in vivo*. Further studies are in progress to characterize the biological activities of these Fc receptors, to determine their distribution among *H. somnus* strains, and to establish if the presence of these receptors plays any role in the pathogenesis of *H. somnus*.

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