Non-immune immunoglobulin binding by “*Haemophilus somnus*”

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Summary. In-vitro culture of *Haemophilus somnus* in liquid or solid media supplemented with bovine blood or serum resulted in non-immune binding of immunoglobulin (Ig) by the organism. This binding was independent of the antigen-combining site of the Ig molecule, since binding of an IgG preparation specific for the hapten dinitrophenol was unaffected by the presence of the homologous antigen. Quantitative comparison of the binding of Ig fragments Fab and Fc demonstrated that the non-immune binding occurred in the Fc region of bovine IgG. The isotypes of Ig that became bound to *H. somnus* included both bovine IgG subclasses (IgG₁ and IgG₂), which were bound equally, and bovine IgM.

Introduction

Non-immune binding of immunoglobulin (Ig) has been described for staphylococci (Dossett *et al.*, 1969) and streptococci (Kronvall, 1973; Reis *et al.*, 1984; Yarnall and Boyle, 1986), and reported to occur on the surface of other micro-organisms including *Brucella abortus* (Nielsen *et al.*, 1981b), *Tylorella equigenitalis* (Widders *et al.*, 1985), mycoplasmas (Williams *et al.*, 1970), *Schistosoma mansoni* (Torpier *et al.*, 1979), trypanosomes (Ferreira de Miranda-Santos and Campos-Neto, 1981) and Herpes simplex virus (Dowler and Veltri, 1984).

This study describes an Ig binding mechanism on the surface of the gram-negative bacterium “*Haemophilus somnus*”. This organism is a systemic and mucosal pathogen of cattle, causing diseases of the central nervous system, respiratory tract, and reproductive tract (Humphrey and Stephens, 1983). Also, it persists on the respiratory and genital mucosae of apparently normal cattle (Slee and Stephens, 1985). The purpose of this study was to investigate the basis for binding of bovine Ig to the surface of *H. somnus* grown in serum-supplemented media, and to determine the nature of this binding in the context of its possible role in bacterial pathogenicity.

Materials and methods

H. somnus strains

The strains stored at –70°C were as follows: no. 8025, a laboratory strain isolated from a case of thromboembolic meningoencephalitis; no. 649-4, from an aborted bovine fetus used experimentally to reproduce abortion (Widders *et al.*, 1986); and no. 2336, from a case of bovine pneumonia and used experimentally to reproduce pneumonia (Gogolewski *et al.*, 1987). Bacteria were cultured on plates of Columbia blood agar base with bovine blood or fetal bovine serum 10%, or in brain heart infusion broth supplemented with Tris 0.1% and thiamine monophosphate 1 µg/ml.

Bacteria for use as coating antigen in the ELISA were washed three times in isotonic saline containing formalin 1% by centrifugation for 10 min at 10 000 *g*. Plate-grown bacteria were suspended in 0.1M phosphate-buffered saline (PBS), pH 7.2, before being washed.

Immunoglobulins

Bovine IgG₁, IgG₂ and IgM specific for the hapten dinitrophenol (DNP) was a gift from Dr Tom Besser, College of Veterinary Medicine, Washington State University. These antibody preparations were purified from normal bovine serum as previously described (McGuire *et al.*, 1979). Briefly, specific antibody was affinity-purified from normal bovine serum on a column of DNP-lysine-agarose. The Ig preparation was chromatographed on Biogel A 1.5 (Bio-Rad Laboratories, Richmond, CA, USA) to obtain IgM. The IgG subclasses (IgG₁ and IgG₂) were isolated by ion-exchange chromatography on DEAE-cellulose equilibrated with 0.005M

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sodium phosphate buffer, *pH* 8.0. Unbound Ig was IgG₂. IgG₁ fractions eluting with a linear NaCl gradient were pooled, equilibrated with 0.001M sodium phosphate, *pH* 7.5, containing glycine 0.5% and applied to an isoelectric focusing column. Those IgG₁ fractions at the lower end of the *pH* gradient (*pH* 6–8) lacked IgG₂.

Polyspecific IgG₂ and IgG (IgG₁ + IgG₂) were similarly purified from normal bovine serum by ion-exchange chromatography on DEAE-cellulose.

The purity of the immunoglobulin preparations was monitored by immunoelectrophoresis developed with rabbit anti-bovine whole serum (fig. 1) and by enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies specific for bovine IgG₁, IgG₂, IgA (supplied by Dr A. Guidry, USDA, Beltsville, MD, USA) and IgM (supplied by Dr W. Davis, Washington State University) as described below. Contaminating serum proteins and other Ig isotypes were undetectable in the IgG preparations by immunoelectrophoresis and ELISA respectively. The IgM antibody specific for DNP produced a single precipitin line on immunoelectrophoresis (data not shown) but a minor IgG₁ contaminant was detectable by ELISA (data not shown).

Polyspecific IgG (IgG₁ and IgG₂) was digested with papain (Porter, 1950) for 6 h. The Fab and Fc fragments were isolated by ion-exchange chromatography on DEAE-cellulose equilibrated with 0.005M sodium phosphate, *pH* 8.0. Fab was not bound to the column in starting buffer. Undigested IgG and some Fc was eluted with 0.05M sodium phosphate, *pH* 8.0. Fc was eluted with 0.1M sodium phosphate, *pH* 8.0, with 1.0M NaCl. Purity of the fractions was monitored by immunoelectrophoresis (fig. 1).

ELISA

To test for Ig purity and Ig binding to bacteria, the Ig preparations (10 µg/ml) or formalinised bacteria (22%

transmission at 610 nm) were used to coat the wells of a polystyrene microelisa plate (Costar) by incubation in PBS with 0.02M MgCl₂ overnight at 4°C. Antigen concentrations that gave optimal coating were determined by titrating antigen in coating buffer. Omission of coating antigen resulted in only background readings in the ELISA.

Monoclonal antibodies used to monitor Ig purity were diluted in PBS with Tween 20 0.05%. Ig preparations used to measure binding to bacteria were diluted in PBS with Tween 20 0.05% containing polyvinyl pyrrolidone 2% and ovalbumin 0.2%. These solutions were incubated with antigen-coated plates for 1 h at 37°C.

Binding of bovine IgG to bacteria was measured by means of peroxidase-conjugated rabbit-antibovine IgG (heavy and light chain specific; Cappell Worthington). Binding of IgM was measured with IgM monoclonal antibody. Monoclonal binding in ELISA was measured with peroxidase-conjugated goat-antimurine IgG (Kirkgaard and Perry Laboratories). Reactions were developed with H₂O₂ in 5 aminosalicic acid and plates were read in the dual wavelength mode at 490 and 630 nm in an ELISA reader. End-points for ELISA titrations were determined by calculating the protein concentration that resulted in an optical density (OD) of 0.20 (3 × background OD).

Results

In developing an ELISA to measure bovine antibody specific for *H. somnus*, high background ODs were encountered when the coating antigen consisted of bacteria grown in bovine serum-supplemented media. This high background was seen with both plate- and broth-grown bacteria (fig. 2), and was eliminated by growing the organism in media deficient in bovine Ig (Columbia blood agar

Fig. 1. Immunoelectrophoresis of bovine IgG, Fab and Fc fragments developed with antiserum to bovine whole serum (WS).

Fig. 2. IgG bound by *H. somnus* grown in liquid or solid media with or without a 10% supplement of heat-inactivated normal bovine serum.

with fetal bovine serum instead of bovine blood, or liquid medium with no serum supplement; fig. 2). Clearly, growth of *H. somnus* in culture media containing bovine Ig resulted in Ig binding to the bacterial cell surface.

In an attempt to clarify the role of antibody specificity in the binding of Ig to *H. somnus*, the binding of monospecific (anti-DNP) and polyspecific Ig preparations was titrated by the ELISA (table) with antigen consisting of bacteria grown in serum-free medium. There was no difference in

binding of IgG₁ and IgG₂ anti-DNP antibody to *H. somnus*, binding being detectable in solutions of 38 µg/ml (table). IgM also was bound in this assay (table), with an end-point at 19 µg/ml. IgG binding to *H. somnus* was measured in a direct ELISA with peroxidase-conjugated antiserum to bovine IgG (heavy and light chain specific). IgM binding was measured in an indirect ELISA with monoclonal anti-bovine IgM followed by peroxidase-conjugated antimurine IgG. Since different amplification steps and different antisera were used to measure IgG binding and IgM binding, quantitative comparison of IgG and IgM binding is not possible.

Binding of polyspecific IgG₂ was recorded with an end-point to binding at 2.3 µg/ml as compared with an end-point of 38 µg/ml for anti-DNP IgG. To define the role of the antigen binding sites of Ig in mediating binding to *H. somnus*, the effect of DNP on binding of antibody specific for DNP was investigated in ELISA. With coating antigens consisting of DNP-BSA (bovine serum albumin) or *H. somnus*, binding of IgG₂ anti-DNP was titrated in the presence of BSA, or DNP-BSA (fig. 3). When DNP-BSA was used as the coating antigen, Ig binding was inhibited by soluble DNP-BSA, but not by BSA (fig. 3). In contrast, with *H. somnus* as

Fig. 3. Participation of the antigen binding site of anti-DNP IgG in binding to *H. somnus*. Anti-DNP IgG was titrated in the presence of DNP-BSA or BSA in the wells of a microtitration plate coated with DNP-BSA or *H. somnus*. Binding of anti-DNP IgG to DNP-BSA was inhibited by co-incubation with DNP-BSA but not BSA, while binding of anti-DNP IgG to *H. somnus* was not inhibited by DNP-BSA or BSA.

Table. Ig and Ig-fragment binding to *H. somnus*

Immunoglobulin	End-point*
Anti DNP IgG ₁	38
Anti DNP IgG ₂	38
Polyspecific IgG ₂	2
Anti DNP IgM	19
Fab	6†
Fc	100

* The end-point for binding of whole Ig preparations is expressed as the minimal Ig concentration (µg/ml) at which binding could be detected.

† Binding of the Ig fragments was corrected for differential recognition of the Fab and Fc fragment by anti-bovine IgG (see text). Accordingly, Fc binding is set at 100, with Fab binding at 6% the level of Fc binding.

coating antigen, Ig binding was comparable in solutions of BSA or DNP-BSA (fig. 3).

Relative binding of the Fab and Fc fragments of bovine IgG was estimated by titrating binding to *H. somnus* in ELISA. The peroxidase-conjugated rabbit-antibovine Ig used to measure binding did not recognise the Ig fragments equally, and a correction for this bias was therefore necessary. This was effected by directly coating the microtitration wells with decreasing concentrations of the Ig fragments, and calculating an endpoint for binding of the conjugated antibovine Ig. With Fc endpoints set at 1, the relative binding of the Ig fragments, Fc:Fab, was 1:0.6 for binding to *H. somnus*, and 1:10 when coated directly on to the microtitration wells. Therefore, after correcting for differences in recognition of the Ig fragments by the conjugated antiserum, Fab fragment was bound to *H. somnus* at only 6% of the level of Fc binding.

To monitor growth-phase variation in Ig-binding activity, a bacterial culture in serum-free liquid medium was sampled hourly from 0 to 9 h and at 24 h. The washed bacteria were used as coating antigen in an ELISA to measure binding of anti-DNP IgG₂. There was minimal variation in the end-point of Ig binding, from the lag phase, through log phase, to the stationary phase of growth.

Discussion

This study identified a non-immune Ig binding mechanism on the surface of *H. somnus*. Ig binding was detected in the presence of a 50-fold excess of ovalbumin and a 500-fold excess of polyvinyl pyrrolidone, suggesting that binding was specific for Ig. Subsequent studies have identified bacterial surface proteins with Fc binding activity and have characterised the species-specificity of Ig binding (Yarnall *et al.*, in press).

Antibody specific for the hapten DNP became bound to *H. somnus* in the ELISA, and this binding was unaffected by the presence of the homologous antigen, DNP-BSA. Thus, the antigen combining sites of the Ig molecule play no part in this interaction. Binding of polyspecific IgG was greater than that for monospecific (anti-DNP) IgG. This difference in binding is likely to have been due to natural antibody in the polyspecific Ig preparation purified from normal bovine serum. Quantitative comparison of Fab and Fc binding by *H. somnus* suggests that the specificity of the non-immune binding mechanism is directed towards a site in the Fc region of bovine IgG, since Fab was bound to *H. somnus* at only 6% of the binding of Fc (table).

This level of Fab binding probably reflects the presence of cross-reactive antibody specific for *H. somnus* in normal bovine serum.

The Fc-directed binding by *H. somnus* parallels the reactivity of similar binding mechanisms described for *Staphylococcus aureus*, *Streptococcus* spp., *Schistosoma mansoni*, trypanosomes, and Herpes simplex virus. In the present study, both bovine IgG and IgM were bound by a non-immune mechanism, since both IgG and IgM antibody specific for DNP were bound to the surface of *H. somnus*. It is unclear, from the present study, whether IgG and IgM binding to *H. somnus* is mediated by the same or distinct bacterial surface components. Fc-specific, non-immune binding of bovine IgM has also been reported for *B. abortus* (Nielsen *et al.*, 1981b), but the binding mechanism in this instance was specific for a subpopulation of bovine IgM molecules. Similarly, a proportion of bovine sera displayed ethylenediaminetetraacetic acid (EDTA)-labile binding of IgG₁ to *B. abortus* (Nielsen *et al.*, 1981a). The role of the Fc and Fab fragments in binding was not investigated for this isotype.

In the present study, the assays used to quantitate IgG and IgM binding employed different antisera and different amplification steps, preventing a quantitative comparison of IgG and IgM binding. However, there was no difference in reactivity between the bovine IgG subclasses (table), suggesting that the Ig-binding mechanism of *H. somnus* recognises a class-specific rather than a subclass-specific determinant on bovine Ig. In comparison, type III streptococcal-Fc receptors bind both bovine IgG₁ and IgG₂ (Reis *et al.*, 1983), and protein A of *S. aureus* binds well to bovine IgG₂ but only weakly to IgG₁ (Goudswaard *et al.*, 1978).

Non-immune Ig binding by microbial pathogens probably represents a specific adaptation for evasion of host effector functions. Non-immune Ig binding has been associated with virulence in schistosomes (Torpier *et al.*, 1979) and trypanosomes (Ferreira de Miranda-Santos and Campos-Neto, 1981). Ig binding by staphylococcal protein A has been associated with a reduction in phagocytosis (Dossett *et al.*, 1969) by an effect on Fc-mediated interaction with phagocytes (Peterson *et al.*, 1977). Similarly, binding of fibrinogen by the M protein of group A streptococci resulted in limited deposition of complement on the bacterial cell surface (Whitnack *et al.*, 1984). Non-immune binding of Ig might function similarly to mask bacterial surface structures targeted by specific antibody or complement, thereby reducing opsonisation and susceptibility to killing and phagocytosis.

sis. The significance of non-immune Ig binding as a virulence determinant of *H. somnus* warrants further investigation in view of the organism's (1) resistance to complement-mediated killing, (2) persistence at mucosal surfaces, (3) capacity to

evade host effector functions *in vivo*, and (4) ability to cause a range of bovine infections.

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