Nonimmune Binding of Equine Immunoglobulin by the Causative Organism of Contagious Equine Metritis, *Taylorella equigenitalis*

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This study identifies nonimmune binding of equine immunoglobulin by the causative organism of contagious equine metritis. Immunoglobulin binding to the bacterium was strongest for immunoglobulin G (IgG) and less for IgM; IgA was not bound. Binding of equine IgG was inhibited by human IgG, but not by IgG of domestic animals. Immunoglobulin binding by the bacterium appeared to be directed towards an epitope in the hinge region of the immunoglobulin molecule.

A previously unknown genital infection of mares was first described in 1977 (3) and became known as contagious equine metritis (CEM). The causative organism was identified as a microaerophilic, gram-negative coccobacillus (17), and its classification has recently been validated as *Taylorella equigenitalis* (11). After experimental or natural infection, CEM is characterized by a mucopurulent uterine discharge, with associated vaginitis. A systemic humoral response has been measured after local infection (4), and specific antibody has been detected in uterine secretions (19, 23). However, the organism may be isolated from recovered mares for extended periods after initial infection (4), and immunization or previous infection may not protect against subsequent challenge (8, 19).

Ultrastructural studies have identified a microcapsule on *T. equigenitalis* (20), and investigations of the phagocytosis of the organism by polymorphonuclear leukocytes has attributed an antiphagocytic role to the bacterial surface components (1, 2).

This study reports on an immunoglobulin-binding property of the bacterial cell surface which may enhance the pathogenicity of this organism.

**MATERIALS AND METHODS**

Bacteria. A streptomycin-resistant strain of *T. equigenitalis* was kindly donated by Mary Mackintosh, Equine Research Station, Newmarket, Suffolk, United Kingdom. Stock cultures were maintained in glycerol at −70°C. An *Escherichia coli* standard strain (NCTC 10418; ATCC 10536) was also used and maintained on blood agar at 4°C.

Bacteria were cultured in modified CEM organism liquid medium (7) at 37°C with gentle agitation for 18 to 24 h. FeSO₄ was omitted from the broth, and fetal calf serum (FCS) (GIBCO Laboratories, Grand Island, N.Y.) was also used as 10% serum supplement in the medium. The stock *T. equigenitalis* culture was grown for four passages in FCS-supplemented medium.

Broth cultures were centrifuged at 1,500 × g for 20 min, washed three times, and suspended in 0.01 M phosphate-buffered saline (pH 7.4) with 0.01% merthiolate. The concentration of the organism in suspension was determined by measuring optical density at 680 nm, standardized by diluting and counting viable bacteria on CEMO chocolate agar plates (Lab M, Salford, United Kingdom). Harvested bacteria for use in enzyme-linked immunosorbent assays (ELISAs) were adjusted to a concentration of 10¹⁰ bacteria per ml and stored in aliquots at −20°C.

Sera and immunoglobulins. Serum for use in bacterial culture were heat inactivated at 56°C for 60 min, sterilized by filtration, and stored at −20°C. The preparation of rabbit antisera specific for the heavy chains of equine immunoglobulin G (IgG), IgA, IgM, and for the whole IgG molecule, and sheep antiserum to rabbit IgG conjugated with alkaline phosphatase, has been described in detail elsewhere (24; F. Widders, C. R. Stokes, J. S. E. David, and F. J. Bourne, Immunology, in press). Briefly, antisera were raised in rabbits by the use of purified solutions of the isotypes and were rendered specific by absorption with insolubilized proteins: antiserum to equine IgG was produced by using the Fc fragment of the immunoglobulin molecule and thus required no absorption; antiserum to IgA and IgM were absorbed with IgG and newborn foal serum; antiserum to whole molecule IgG was used unabsorbed. The specificity of these antisera was confirmed in immunoelectrophoresis with whole serum and by immunodiffusion against the purified immunoglobulin preparations. Antiserum to rabbit IgG was produced in a sheep, using purified rabbit IgG. The resultant antiserum was absorbed with insolubilized equine IgG, and was affinity purified by using rabbit IgG covalently bound to Sepharose 4B. This affinity-purified sheep anti-rabbit IgG was then conjugated with the enzyme alkaline phosphatase (6) (Sigma, Poole, United Kingdom). Rabbit antiserum to equine IgG was similarly affinity purified, using equine IgG bound to Sepharose 4B. All other antisera were used in the ELISA as 40% ammonium sulfate cuts. An antiserum to *T. equigenitalis* was produced by immunizing a rabbit with 10¹⁰ heat-killed organisms in Freund incomplete adjuvant, using three injections, one every three weeks.

IgGs from human, cat, sheep, cow, dog, pig, mouse, and rabbit serum were prepared in this laboratory, using gel filtration and ion-exchange chromatography (10). Equine IgG, IgM, and 11S IgA were prepared from serum or milk, using gel filtration and ion-exchange chromatography (24). IgG with antibody specificity to dinitrophenylated human serum albumin was purified from hyperimmune horse serum (Widders et al., in press) by affinity chromatography on
WIDDERS developed against rabbit antigens Sepharose 4B molecule (prepared by papain assuming chromatography from undigested The wells Kingdom) coupled with Immunoelectrophoresis macia). Organisms serum Immunoglobulins the developed and volume of mode tions used in this equigenitalis anti-T. 15.3; Fc, mary and affinity-purified as increased with saged four amented with (Fig. ELISA. Bacteria was The Rabbit antiserum Growth of bacteria (Fig. 12.2). However, This ELISA was optimized for coating antigen and primary and conjugate antisera concentrations, using the rabbit anti-T. equigenitalis antiserum. Binding of the reagent antisera to FCS-grown bacteria was negligible at the concentrations used in this assay. Rabbit antiserum to the heavy chain of horse IgG (RAG) bound in the ELISA to bacteria grown in broth supplemented with horse serum. This binding was antigen specific, as affinity-purified RAG bound in ELISA whereas rabbit IgG adjusted to the same concentration did not.

Growth of T. equigenitalis, from the stock culture passed four times in FCS, in a series of broths supplemented by increasing amounts of horse serum diluted in FCS, showed a direct relationship between equine serum concentration in the broth and RAG binding to T. equigenitalis in ELISA (Fig. 2). E. coli grown in parallel in a broth supplemented with 10% horse serum showed some binding of RAG in ELISA (Fig. 2). However, this binding was not IgG specific, and probably represents natural anti-E. coli activity in rabbit serum, as binding of RAG to E. coli or T. equigenitalis grown in horse serum was inhibited by the addition of horse serum to the incubation buffer in the case of T. equigenitalis, but not for E. coli.

RESULTS
The ELISA was optimized for coating antigen and primary and conjugate antisera concentrations, using the rabbit anti-T. equigenitalis antiserum. Binding of the reagent antisera to FCS-grown bacteria was negligible at the concentrations used in this assay.

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Wells coated with T. equigenitalis grown in FCS bound equally polyspecific equine IgG and affinity-purified antibody specific for dinitrophenylated human serum albumin (Fig. 3). IgM was also bound to a lesser extent, but IgA remained unbound (Fig. 3).

This immunoglobulin binding by T. equigenitalis also is species specific. Wells coated with FCS-grown bacteria were incubated with horse IgG (25 μg/ml) mixed with doubling dilutions (from 100 μg/ml initial concentration) of various species IgG, including human, cat, sheep, cow, dog, pig, mouse, and rabbit. Only human IgG inhibited bacterial binding of horse IgG to any extent (Fig. 4). Cross-reaction of RAG with nonequine IgG did not occur in this assay, as only background optical density resulted when 100 μg of IgG per ml from any one of these eight species was incubated with T. equigenitalis in ELISA in the absence of equine IgG. However, the organism was capable of binding immunoglobulin of other species, but apparently to a lesser degree than the binding of horse IgG. Coating ELISA wells with the organism grown with 10% normal cat serum demonstrated specific

FIG. 1. Immunoelectrophoresis of IgG, Fab, and Fc developed against antiserum to IgG (whole molecule).
IMMUNOGLOBULIN BINDING BY T. EQUIGENITALIS

binding of rabbit anti-cat IgG, which was absent with FCS-grown T. equigenitalis.

Finally, the binding of fragments of the IgG molecule by T. equigenitalis in the ELISA was investigated. Wells were coated with FCS-grown bacteria, and the binding of doubling dilutions of the immunoglobulin preparations IgG, Fab, and Fc, from 70 µg/ml, was measured by using RAG (whole molecule specific). For each preparation, an endpoint to binding was calculated from the binding curves, as the protein concentrations, in micrograms per milliliter, at 1× the background optical density. Since the RAG antiserum did not recognize the three immunoglobulin preparations equally, correction for this bias in the assay was necessary. This was effected by directly coating microtiter wells with doubling dilutions of the three immunoglobulin fractions from 10 µg/ml, and again, for each preparation, calculating an endpoint for RAG binding as described above. With the IgG endpoints set at 1, the relationship between the three preparations, IgG-Fab-Fc, was 1:9.40:2.21 for binding to T. equigenitalis and 1:10.76:1.55 for binding directly to the microtiter wells. Thus, correcting for differences in recognition of the three fractions by RAG, Fab bound to T. equigenitalis at 114% of whole IgG binding, with Fc binding at 70% of whole IgG.

DISCUSSION

These results identify a nonimmune immunoglobulin binding mechanism on the surface of T. equigenitalis, characterized by maximal binding of equine IgG compared with other equine immunoglobulin classes and other IgG of other species, and by binding of both the Fab and Fc fragments of equine IgG, although not equally. Such a mechanism may enhance the pathogenicity of this organism in equine tissues. The longevity of T. equigenitalis in the genital tract after natural or challenge infection (4), in the face of a detectable local immune response (15; Widders et al., in press), suggests some bacterial mechanism for the evasion of the immune system. Earlier studies have identified a microcapsule on the bacterial cell surface (20) which may possess antiphagocytic activity (1, 2). The results of the present study suggest that this antiphagocytic activity may be related to the nonimmune binding of immunoglobulin to the cell surface, thereby minimizing the effectiveness of specific antibody, and possibly complement, in phagocytosis.

The reactivity of T. equigenitalis for immunoglobulin is greatest for IgG, although IgM is also bound to some extent. That both Fab and Fc fractions were bound to the bacterial cell surface, albeit to differing degrees, suggests that the binding specificity may be directed towards an epitope in the hinge region of the immunoglobulin molecule, although this is by no means proven. Alternative explanations include multiple binding sites on the immunoglobulin molecule, or a single repeating sequence represented on both the Fab and Fc fragments. The increased binding of Fab compared with whole IgG in this study probably reflects steric hindrance of whole molecule binding.

The specificity of this immunoglobulin binding by the bacterial cell surface is manifest not only in the class of immunoglobulins bound, but also in the species of origin of IgG. Of a range of animal species, only human IgG inhibited the binding of equine IgG to T. equigenitalis. However, even at concentrations of human IgG ×4 that of equine IgG, this inhibition was incomplete. Whether single or multiple immunoglobulin epitopes are involved in binding, this result suggests the sharing of imperfectly matched determinants by human and equine IgG which are absent from the IgGs of the other species studied.

This species specificity of immunoglobulin binding by T. equigenitalis may be significant in light of the specific pathogenicity of this organism. Transmission studies in the genital tracts of sheep, cows, sows, rabbits, mice, and guinea pigs (21, 22) succeeded in establishing infection only in mice, although the bacteria could also be isolated for a limited period after intrauterine inoculation in rabbits and guinea pigs. In no case, however, did inflammation accompany the infection. Thus, the pathogenicity of this organism in the equine genital tract may be directly related to this immunoglobulin binding mechanism. Actively coating the bacterial cell surface with host protein may limit the antigenicity of the organism, and so minimize a specific immune response. Clearance of the organism by phagocytic cells in the tract may also be reduced. Similar mechanisms have evolved in other microbial species, although their precise role in pathogenesis remains largely underexplored. Protein A of Staphylococcus aureus binds to the Fc region of immunoglobulin molecules from a range of mammalian species (9). Soluble protein A inhibits phagocytosis in vitro (5), although in normal serum IgG aggregation by protein A may activate complement (13). Similar nonimmune reactivity for IgG, IgA, and human serum albumin has been demonstrated in the surface components of streptococci (12, 14), and Trichomonas vaginalis also has the capacity to bind human serum proteins (16). This is the first report, however, of immunoglobulin binding by a gram-negative bacterial pathogen. Such mechanisms represent a specific adaptation for microbial survival in a potentially hostile environment and, in the case of T. equigenitalis, this evolution may be a significant factor in its pathogenicity in the equine genital tract.
FIG. 4. Inhibition of T. equigenitalis binding of equine IgG by other species IgG. Plates were coated with 2 × 10^7 FCS-grown CEM organisms per well and incubated with dilutions of these species IgG (from 100 μg/ml) in a solution of horse IgG (25 μg/ml). Only human IgG inhibited the binding of horse IgG by T. equigenitalis.

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LITERATURE CITED


