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Identification of a Mycoplasmal Protein Which Binds Immunoglobulins Nonimmunologically

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Immunoblotted protein samples from several strains of *Mycoplasma hominis* and from one strain of *Mycoplasma arginini* each contain a polypeptide of a molecular mass of 95,000 to 105,000 Da which binds immunoglobulin nonimmunologically. Immunoblots from these organisms were probed with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin, conjugated goat immunoglobulin G (IgG) Fab fragments, and conjugated goat IgG Fc fragments. The polypeptide bound the goat anti-rabbit molecules and the Fab fragments but not the Fc fragments. These reactions could be blocked with nonimmune unconjugated goat IgG and unconjugated human IgM. Controls probed with alkaline phosphatase alone did not stain. Binding of the conjugated preparations to whole mycoplasmal cells was dependent on concentrations of both conjugate and cells for the goat anti-rabbit preparation and for Fab. The mycoplasmal polypeptide may be a light-chain-specific reactant.

Nonimmunologic reactions between surface proteins of bacteria and immunoglobulins have been characterized extensively for the classic examples, protein A of *Staphylococcus aureus* (7, 10) and protein G of streptococci (4, 7, 8, 15, 20, 21). It is clear that these proteins bind primarily to Fc domains, although there is weaker binding to Fab domains in both cases. In contrast, the immunoglobulin-binding protein (protein L) of *Peptostreptococcus magnus* is specific for kappa light chains (3, 11, 18).

Mycoplasmas appear to interact nonimmunologically with the immune system in two ways: by binding immunoglobulin or immunoglobulin fragments in a generalized fashion (1, 6, 24) and by influencing the responsiveness of B and T lymphocytes (9, 17, 22). During the course of our previous study of patient response to clinical strains of *Mycoplasma hominis*, one protein reacted in a nonantigenic manner with the antibodies used as probes during immunoblot analysis (1). For instance, when antigens from several strains were separated by polyacrylamide gel electrophoresis (PAGE), electrotransferred to nitrocellulose, and probed with second antibody conjugates alone, one of the proteins (molecular mass, 105,000 Da) was strikingly visible. We show that all the strains of *M. hominis* tested and one *Mycoplasma arginini* strain possess a 95- to 105-kDa protein which reacts with Fab fragments of immunoglobulin G (IgG) from several animal species nonimmunologically.

(An abstract of this paper was presented earlier [1a].)

MATERIALS AND METHODS

Organisms. The organisms used in this study include the following strains from the American Type Culture Collection: *M. hominis* ATCC 14027 and *M. arginini* ATCC 23243. All other strains of *M. hominis* were clinical isolates.

Preparation of cellular antigens and antisera. Immunogens, test antigens, and antisera were prepared as described for past studies (2, 13). For use as test antigens, all strains were cultivated on soy peptone-fresh yeast dialysate broth (12) supplemented with agamma horse serum (obtained from Alpha Gamma Laboratories, Sierra Madre, Calif.) at a final concentration of 17% (wt/vol). Immunogens were cultivated in the same broth supplemented with agamma rabbit serum; the rabbits were immunized with whole-cell preparations which had been harvested by centrifugation and washed three times with *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)-buffered saline (0.1 M TES, pH 7.2). Organisms used as test antigens were harvested after 24 to 48 h of growth, washed three times with 0.25 M NaCl, and resuspended in phosphate-buffered saline (PBS; 0.1 M phosphate, pH 7.2).

Electrophoresis. Analytical polyacrylamide gels were prepared according to the Laemmli procedure (16) with an acrylamide concentration of 7%. Electrotransfer of the separated proteins to nitrocellulose was done with the procedure and buffer described by Towbin et al. (23).

Immunoblot development. Washing and blocking steps for immunoblot development were done as described previously (2, 14). The initial blocking buffer consisted of 0.15 M NaCl, 5 mM Na_4-EDTA, 0.25% gelatin, 10 mM TES, and 0.1% Tween 20. Washes after incubation with primary or secondary antibodies or with fractions of antibodies were done with PBS containing 0.5% Tween 20. Sera used for the probing of the blotted proteins included antimycoplasmal rabbit serum, goat anti-rabbit conjugates with either alkaline phosphatase or hydrogen peroxidase (obtained from Bio-Rad Laboratories, Richmond, Calif., or Kirkegaard & Perry Laboratories, Gaithersburg, Md.), and alkaline phosphatase-conjugated Fab and Fc fragments of IgG from various animal species (obtained from Jackson ImmunoResearch Laboratories, West Grove, Pa.). The conjugated goat anti-rabbit sera had been purified by the manufacturers using affinity chromatography with heavy and light chains of rabbit IgG. Color development was done with 5-bromo-4-chloro-3-indolylphosphate and Nitro Blue Tetrazolium by adapting the method of Blake et al. (5) as described in the protocol supplied with the reagents by Bio-Rad Laboratories. Purified bovine intestinal alkaline phosphatase (EC 3.1.3.1) was obtained from Sigma Chemical Co., St. Louis, Mo. Standards used for determination of relative molecular weights of the separated proteins were biotinylated low-molecular-

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weight standards from Bio-Rad Laboratories and were developed with their avidin-alkaline phosphatase conjugate. For visual reference only, standards prestained with Coomassie brilliant blue (Bio-Rad Laboratories) were used in most blots.

Binding assay. Determination of binding of the immunoglobulin and fragment conjugates to intact cells was done in 1.5-ml microcentrifuge tubes which had been blocked with the same buffer used for immunoblot blocking for 1 h at 37°C. One hundred microliters of cell suspension and 300 μl of immunoglobulin or fragment conjugate were added to each tube. The concentrations of cells ranged from 0 to 0.5 μg of cell protein per ml in the final volume of reaction mixture; the dilutions of conjugate used were from 1:1,000 to 1:8,000 in the final volume of reaction mixture. Both cells and conjugate were diluted in PBS, pH 7.2. The mixtures were incubated with gentle mixing for 1 h at room temperature. Cells were pelleted by centrifugation at 12,000 × *g* for 5 min. The cells were washed three times with PBS. After the final wash, the cells were resuspended in 1.0 ml of assay buffer (0.05 M Na_2CO_3, 1.0 mM $MgCl_2$, pH 9.8). To this suspension was added 0.1 ml of *p*-nitrophenylphosphate in assay buffer (stock concentration, 10 mg/ml). Incubation was for 30 min at 37°C. At the end of the incubation period, 0.25 ml of 3 N NaOH was added to stop the reaction. The samples were vortexed, and the A_{400} was read. A standard curve for the relationship of A_{400} with respect to concentration of *p*-nitrophenol was prepared with dilutions of *p*-nitrophenol (Sigma Chemical Co.) in assay buffer. The relationship was linear from 0 to 50 nmol of *p*-nitrophenol per ml.

RESULTS

Eight strains of *M. hominis* and one strain of *M. arginini* were compared by using hyperimmune antiserum against one of the *M. hominis* strains, 49L (Fig. 1). This served as a reference for the antigenic cross-reactivity of each strain with respect to strain 49L. A sample of the agamma horse serum used in the growth medium showed no reactions.

Since it was essential to know whether any of the bands seen in the reactions shown in Fig. 1 represented nonspecific reactions, strain 49L was initially probed only with second antibody, i.e., the affinity-purified goat anti-rabbit alkaline phosphatase conjugate (Fig. 2, lane 1). A protein with a relative molecular mass of approximately 100 to 105 kDa reacted prominently with the second antibody probe. Similar control reactions were seen with 14 additional *M. hominis* strains (data not shown).

In order to determine the specificity of the reactions with the second antibody, immunoblots of strain 49L were probed with conjugated fragments of goat IgG. The reaction of the polypeptide at 105 kDa was most specific to the Fab fragment (Fig. 2, lane 2); the Fc conjugate bound primarily to numerous smaller polypeptides, most of which were smaller than 50 kDa (Fig. 2, lane 3).

In order to assess the distribution of the Fab-binding protein in the strains compared earlier, a blot of the proteins from these strains was probed with the Fab alkaline phosphatase conjugate (Fig. 3). The reaction which occurred for each of the strains appeared to be a doublet: a major band with an approximate molecular mass of either 100 or 105 kDa and a faint, minor band approximately 10 kDa smaller. The agamma horse serum control was completely negative.

A 105-kDa polypeptide of strain 49L reacted with conjugated Fab fragments of IgG from several animal species: horses, humans, rabbits, and goats (Fig. 4). The horse Fab

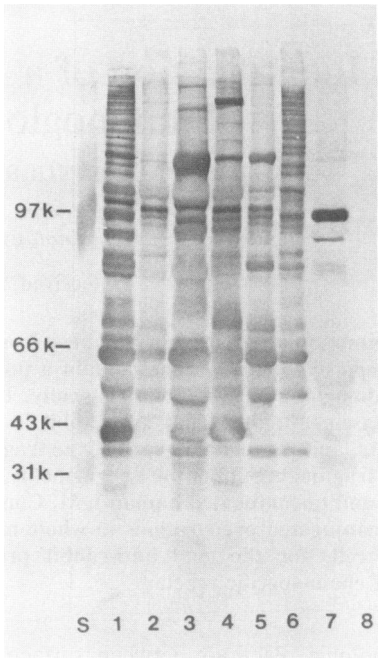


FIG. 1. Immunoblotting of various mycoplasmal strains with rabbit antiserum versus 49L cells (1:10,000). Second antibody was Bio-Rad goat anti-rabbit serum conjugated with alkaline phosphatase (1:3,000). Lanes: 1 through 6, *M. hominis* strains 49L, GX55-1, ATCC 14027, 5, AU41, and AU213; 7, *Mycoplasma arginini* ATCC 23243; 8, agamma horse serum. S, prestained molecular weight standards. The scale refers to the positions of molecular weight standards that were not prestained.

FIG. 2. Reaction of antigens of *M. hominis* 49L with goat IgG and goat IgG fragments each conjugated with alkaline phosphatase. Lanes: 1, Bio-Rad goat anti-rabbit (1:2,000); 2, Fab fragment (1:1,000); 3, Fc fragment (1:1,000); 4, color development reagents only.

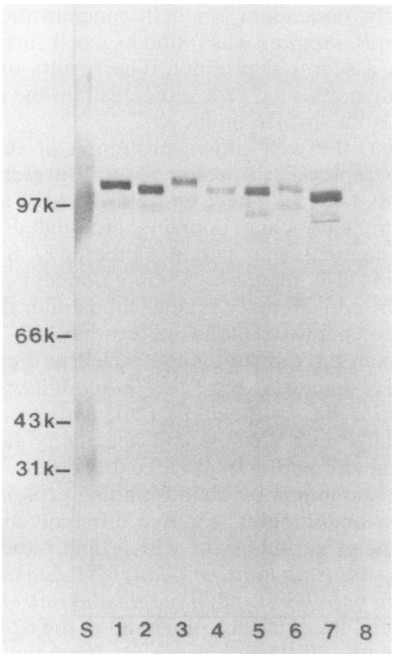


FIG. 3. Immunoblotting of various mycoplasmal strains with goat IgG Fab fragments conjugated with alkaline phosphatase and used at a 1:1,000 dilution. Lanes: 1 through 6, *M. hominis* strains 49L, GX55-1, ATCC 14027, 5, AU41, and AU213; 7, *M. arginini* ATCC 23243; 8, agamma horse serum. S, prestained molecular weight standards. The scale refers to the positions of molecular weight standards that were not prestained.

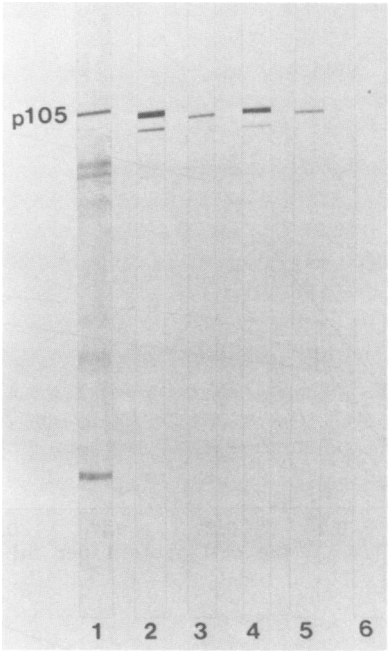


FIG. 4. Reaction of polypeptides of *M. hominis* 49L cells with alkaline phosphatase-conjugated Fab fragments from various animal species. Lanes: 1 through 4, Fab from horse IgG, human IgG, rabbit IgG, and goat IgG, respectively, all at a 1:1,000 dilution; 5, alkaline phosphatase-conjugated goat anti-rabbit (1:3,000); 6, color reagents only.

conjugate also bound to several smaller polypeptides. Each of these latter polypeptides had also been visible on blots probed with goat Fc conjugate (Fig. 2, lane 3). Both unfractionated goat serum and unconjugated goat IgM were tested for their ability to block the reaction with goat anti-rabbit conjugate. After incubation for 1 h with either of these blocking agents, the reaction of strain 49L with goat anti-rabbit conjugate was negative (data not shown).

Since the reaction observed with immunoblot could be an artifact, it was essential to determine whether the immunoglobulin conjugates bind to intact cells. The binding of conjugated goat anti-rabbit antibodies and conjugated goat Fab and Fc fragments to cells of strain 49L was measured as nanomoles of *p*-nitrophenol formed: the assumption was that the formation of this product was directly proportional to the amount of immunoglobulin conjugate bound to the cells (Fig. 5). When cells were mixed with either goat anti-rabbit conjugate (goat IgG) or goat Fab conjugate, the amounts of conjugate bound increased with increases in the concentrations of both cells and conjugate. In contrast, the reaction of cells with goat Fc conjugate appeared to be less dependent on cell concentration. However, binding of the Fc conjugate increased with increasing conjugate concentration.

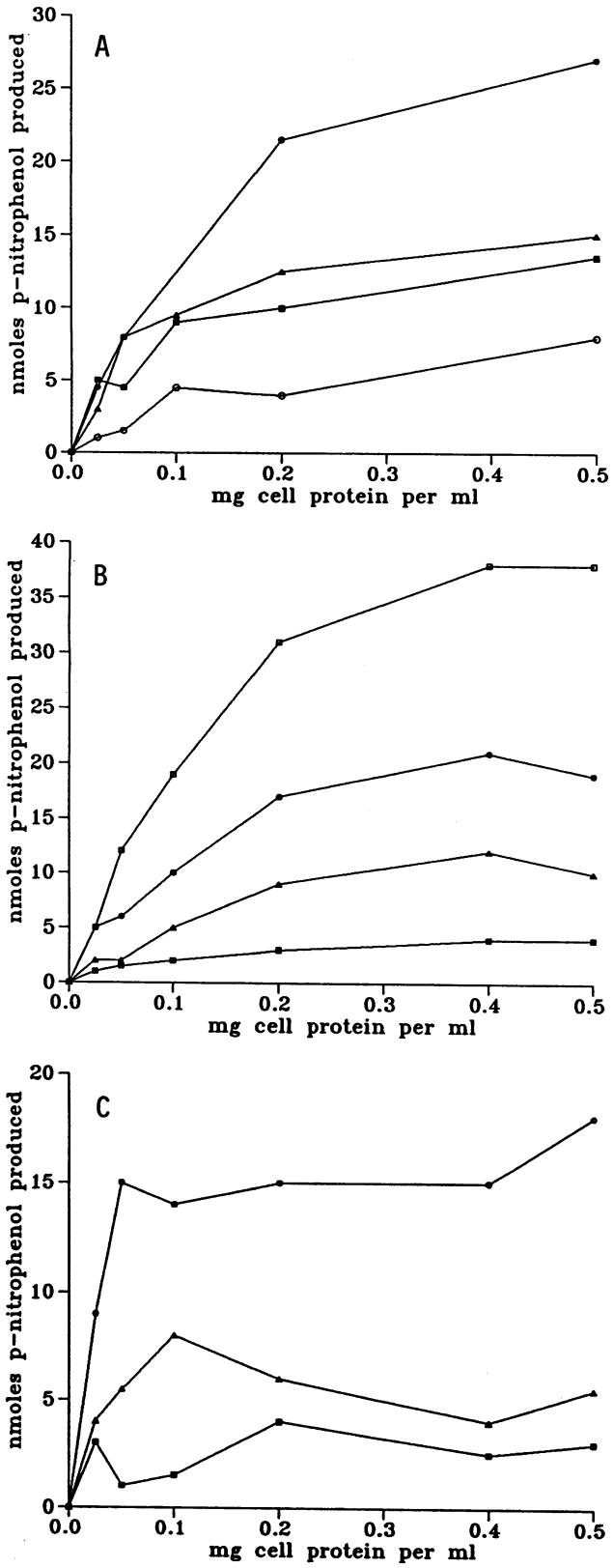
In all the immunoprobes on nitrocellulose, treatment of the blots with color reagents alone resulted in no color development. In addition, immunoblotted agamma horse serum at a concentration of 60 μg per well in the original polyacrylamide gel showed no positive bands when probed with both first and second antibodies or with second antibody alone. Finally, reaction of electroblotted mycoplasmal proteins with purified bovine intestinal alkaline phosphatase (EC 3.1.3.1) followed by the color reagents was completely negative (data not shown).

DISCUSSION

The results of this study indicate that the strains of *M. hominis* and *M. arginini* possess a protein of an approximate molecular mass of 100 to 105 kDa, p105, which binds immunoglobulin Fab fragments nonimmunologically. Since the commercial goat anti-rabbit conjugate had been affinity purified with heavy and light chains of rabbit IgG, it is unlikely that the mycoplasmal p105 is reacting antigenically. The finding that unconjugated goat IgM blocks the binding of conjugated goat IgG (as goat anti-rabbit conjugate) leads to the hypothesis that binding is directed at light chains, perhaps with a degree of specificity for the C_L region.

The striking difference in the reactivity of this protein with the Fab conjugates compared with its reactivity with the Fc conjugates rules out the possibility that the binding is occurring between the 105-kDa protein and alkaline phosphatase itself. In addition, the same reaction pattern was seen in our earlier study in which sera from both hyperimmune rabbits and infected patients were used as primary antibody reagents and goat anti-rabbit immunoglobulin conjugated with horseradish peroxidase was used as the second antibody (2). Thirdly, the direct control with alkaline phosphatase alone was completely negative.

This mycoplasmal protein resembles the protein L identified and purified by Myhre and Erntell (18), Bjorck (3), and Kastern et al. (11) from *Peptostreptococcus magnus*. The peptostreptococcal protein is similar in size (95 to 100 kDa), and it binds light chains of human IgG, particularly kappa



chains. Binding of protein L to radiolabelled kappa chains was similarly dependent on cell concentration (11). The protein from *P. magnus* was found as a cell surface molecule and also as a secreted product. The results of the binding assay with mycoplasmal cells indicates that the mycoplasmal protein is also a surface molecule.

In contrast, the well-known protein A of staphylococcal origin and streptococcal protein G both preferentially bind Fc fragments (4, 15). These proteins clearly interact most strongly with heavy-chain domains, although domains found on $F(ab')_2$ fragments may enhance binding (7, 8, 10). Both of these proteins also appear by sodium dodecyl sulfate-PAGE analysis to be considerably smaller than either the mycoplasmal protein or peptostreptococcal protein L (4, 20, 21). The proteins which have been characterized as Fc receptors in gram-negative bacteria, e.g., in *Haemophilus somnus* (25, 28, 29), *Taylorella equigenitalis* (26), and *Brucella abortus* (19), also differ in both size and specificity from both the mycoplasmal and peptostreptococcal proteins.

Nonantigenic immunoglobulin-binding proteins have been observed on immunoblots for five other mycoplasmal species. Proteins of various sizes which bind radiolabelled IgA occur in *Mycoplasma gallisepticum*, *Mycoplasma mycoides*, *Mycoplasma pulmonis*, and *Mycoplasma putrefaciens* (20a). Vu et al. noted the presence of a band at the 62-kDa position on immunoblots of *Mycoplasma pneumoniae* probed with goat anti-rabbit peroxidase conjugate alone (24). The specificity of that latter protein has not been determined. Finally, assessment of the occurrence in immune complexes of antigens of *M. hominis*, *M. pneumoniae*, *Mycoplasma arthriditis*, *Mycoplasma orale*, *Mycoplasma fermentans*, and *Mycoplasma salivarius* was complicated by nonspecific Fc binding to the mycoplasmal antigens (6).

The biological role of the mycoplasmal protein is uncertain. Since its reaction appears to be similar in spatial orientation to normal reactions between antigens and their specific antibodies, it is unlikely that this protein would shield the mycoplasmal cells from effects of complement or from antibody-enhanced phagocytosis or cytolytic attack. If the molecule is found to be a secreted product, it could be effective as a soluble competitive inhibitor against immunoglobulins specific for other, more prominent mycoplasmal surface components. It is also plausible that the binding of this protein to immunoglobulin is a secondary reaction; its primary target may be some other host molecule in the immunoglobulin superfamily, e.g., those with V region homology (27). Finally, although several mycoplasmal species, including *M. arginini*, have substantial effects on the functions of T and B lymphocytes (8, 17, 22), the proteins from *M. arginini* which seem to inhibit the differentiation of B lymphocytes are in a range of sizes from 61 to 84 kDa (22) and appear not to be functionally related to the protein we describe here.

Finally, the mycoplasmal protein may confound the results of serological assays in which mixtures of the proteins from entire cells are used as the test antigens without

FIG. 5. Binding of alkaline phosphatase-conjugated IgG and fragments to intact cells of *M. hominis* 49L. (A) Goat anti-rabbit serum. (B) Goat Fab. (C) Goat Fc. Concentrations of conjugated immunoglobulin or fragment: ○, 0.05 μg/ml; ■, 0.1 μg/ml; ▲, 0.2 μg/ml; ●, 0.4 μg/ml; □, 0.8 μg/ml. Nanomoles of *p*-nitrophenol produced by bound conjugate are plotted against the concentration of cell protein present in 400 μl of reaction mixture.

resolution of the individual proteins, e.g., enzyme-linked immunosorbent assays with whole-cell antigens.

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