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Mycoplasma hyorhinis GDL Surface Protein Antigen p120 Defined by Monoclonal Antibody

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Four antigens of *Mycoplasma hyorhinis* GDL were defined by murine monoclonal antibodies. Components of broth-grown mycoplasmas were separated under reducing conditions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and subsequent protein blots were stained with individual antibodies. Each antibody reacted with a distinct component with relative molecular weights of 120,000, 73,000, 51,000, and 38,000, respectively (termed p120, p73, p51, and p38). Trypsin treatment of protein blots specifically abrogated binding of antibodies, suggesting that the epitopes recognized were associated with proteins. By using indirect immunofluorescence and immunoferritin techniques, mycoplasmas colonizing the surface of chronically infected BW5147 murine T-lymphoblastoid cells were selectively stained with antibody to p120, indicating the localization of the corresponding epitope at the mycoplasma surface. Protein blots of mycoplasmas derived from BW5147 cell cultures were stained with antibody to p120, revealing a component identical to that observed with broth-grown organisms. These results establish the identity of a surface protein antigen of *M. hyorhinis* GDL expressed at the surface of organisms during their colonization of host cells.

The species *Mycoplasma hyorhinis* is comprised of small, wall-less procaryotes possessing a single limiting membrane and is classified largely by serological similarities among its members (reviewed in references 10 and 24). The species includes pathogenic organisms that produce chronic arthritis and serositis in swine (19). This species also represents one of the more common contaminants of tissue culture cell lines (2, 8). These organisms, like many other mycoplasmas, display a marked propensity to colonize the surface of cells, both in tissue culture and in the animal host. This strong association with the host cell surface is probably fundamental in the establishment and persistence of disease, and it may also underlie a number of immunopathological phenomena associated with mycoplasma infections (6, 7, 20, 25–27).

In elucidating the molecular interactions occurring at the apparently dynamic interface between mycoplasma and host cell surfaces, we have been aided by specific immunological tools capable of identifying and monitoring specific host cell components. Disappointingly little is known, however, about the identity and nature of mycoplasma surface constituents contributing to these interactions. We have begun to generate a library of monoclonal antibodies to *M. hyorhinis* GDL to identify and characterize specific surface constituents of these organisms whose role in host cell interactions may be further assessed by using these immunological reagents.

This report describes the use of monoclonal antibodies to define a protein antigen of *M. hyorhinis* GDL expressed at the surface of organisms during their interaction with T-lymphoblastoid cells in vitro.

MATERIALS AND METHODS

Mycoplasma and lymphoblastoid cells. *M. hyorhinis* GDL (5) was purified as described previously (25). Mycoplasmas were grown at 37°C in Spinner flasks (Bellco Glass, Inc., Vineland, N.J.) with modified Hayflick broth medium (12) containing (per liter): 21 g of PPLO broth base without crystal violet (Difco Laboratories, Detroit, Mich.), 5 g of D-glucose, 4 ml of 0.5% phenol red (Flow Laboratories, McLean, Va.), 100 ml of liquid yeast extract (Flow), 5×10^5 U of penicillin G (Parke, Davis & Co., Detroit, Mich.), and 200 ml of heat-inactivated (56°C, 30 min) serum (final pH of medium, 7.8). Horse serum (Flow) was used to prepare mycoplasmas for immunizations, whereas fetal bovine serum (FBS; Flow) was used to prepare organisms for all other purposes. Mycoplasmas were harvested as described previously (21), and were stored at −70°C.

Mycoplasma-free cultures of the BW5147 T-lymphoblastoid cell line and cultures chronically infected after experimental introduction of *M. hyorhinis* GDL have been described previously (26). Logarithmic-phase cultures of BW5147 cells were maintained at

37°C in spinner flasks by using RPMI 1640 medium (Flow) containing 2 mM L-glutamine and 10% heat-inactivated FBS. Mycoplasmas were harvested from the supernatant of infected BW5147 cultures by differential centrifugation and purified by isopycnic sedimentation in a metrizamide gradient as previously described (27).

Production and propagation of hybridoma cells. The X63-Ag8.653 mouse myeloma cell line (15) was grown before fusion in myeloma growth medium (RPMI 1640 containing 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol [Fisher Scientific Co., Fairlawn, N.J.], 2.5 μg of amphotericin B [Flow], 100 U of penicillin [Flow], and 100 μg of streptomycin [Flow] per ml, and 20% heat-inactivated FBS [Dutchland Laboratories, Denver, Pa.]).

Three-month-old female BALB/c mice from a National Institutes of Health contract colony (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were immunized at 3-day intervals with 100 μg of mycoplasma protein (17) emulsified in Freund incomplete adjuvant (Difco) by subcutaneous injection (50 μl per site) in rear footpads and four ventral sites. In some experiments, organisms stored at −70°C were used as the source of immunizing material. Other experiments used the same regimen for injection but employed a lysate of mycoplasmas prepared from a mixture (containing 10 mg of mycoplasma protein per ml, 0.5% Nonidet P40 [Particle Data Laboratories, Ltd., Elmhurst, Ill.], and 0.1% sodium dodecyl sulfate [SDS; Bio-Rad Laboratories, Richmond, Calif.]) that was incubated for 30 min at 4°C and clarified by centrifugation at 100,000 × g for 45 min at 4°C. In experiments by either protocol, draining lymph nodes were collected from mice 1 1/2 days after the third inoculation, and a suspension of lymphoid cells was obtained for fusion with myeloma cells.

A mixture of 10^8 lymphoid cells and 3×10^7 myeloma cells was centrifuged, and fusion with polyethylene glycol (molecular weight, 4,000; Sigma Chemical Co., St. Louis, Mo.) was performed by the procedure of Gerhard (11), with minor modifications. After fusion, cells were distributed into 96-well microtiter plates (Costar, Cambridge, Mass.) in myeloma growth medium supplemented with 0.19 μg of aminopterin (Sigma), 13.6 μg of hypoxanthine (Sigma), and 0.38 μg of thymidine (Sigma) per ml. Culture fluid from wells showing colony growth was tested for the presence of antibody to mycoplasmas. Cells from positive wells were cloned by limiting dilution in 96-well microtiter plates by using myeloma growth medium supplemented as above with hypoxanthine, thymidine, and BALB/c thymocyte feeder cells (2×10^5 per well). Cloned hybridoma cells were further propagated in myeloma growth medium.

Detection, concentration, and isotype determination of anti-mycoplasma antibodies from hybridoma cultures. The presence of anti-mycoplasma antibodies in hybridoma cultures was determined in an enzyme-linked immunosorbent assay employing FBS broth-grown mycoplasmas as immobilized antigen. Fifty microliters of a suspension of freeze-thaw-disrupted organisms containing 50 μg of protein per ml in borate-buffered saline (BBS; 50 mM $Na_2B_4O_7$, 150 mM NaCl, pH 8.3) was added to each well of a 96-well polyvinyl chloride microtiter plate (Dynatech Laboratories, Inc., Alexandria, Va.). Plates were incubated for 16 h at 4°C and rinsed with BBS, and 200 μl of BBS containing 1% bovine serum albumin (BSA; Calbiochem-Behring, Corp., La Jolla, Calif.) was added to each well. Plates were incubated for 8 to 24 h at 4°C and then stored frozen at −20°C. To perform an enzyme-linked immunosorbent assay, plates were thawed and rinsed with BBS. Fifty microliters of hybridoma culture fluid was added per well and incubated for 16 h at 4°C. Plates were rinsed with BBS and 50 μl of horseradish peroxidase-conjugated antibody (rabbit) to mouse immunoglobulin G (IgG; Miles Laboratories, Inc., Elkhart, Ind.; diluted 1:2,000 in myeloma growth medium) was then added to each well. Plates were incubated for 6 h at 20°C and rinsed with BBS, and 100 μl of enzyme substrate (0.4 mM 2,2′-azino-di-[3-ethylbenzthiazoline sulfonic acid], 80 mM Na_2HPO_4, 60 mM sodium citrate, and 0.03% H_2O_2, pH 4) was then added to each well. Color development was monitored by measuring the optical density (405 nm) with an enzyme-linked immunosorbent assay plate reader (Bio-Tek Instruments, Inc., Burlington, Vt.).

Concentrates of culture fluids were prepared by precipitation at 4°C with 50% ammonium sulfate followed by extensive dialysis against phosphate-buffered saline (PBS).

Antibody isotype was determined by two-dimensional double diffusion (18) in 2% agar. Concentrates of hybridoma culture fluids were allowed to react with rabbit antisera against mouse immunoglobulin heavy chain class IgG1, IgG2a, IgG2b, IgG3, IgM, or IgA (Litton Bionetics, Inc., Kensington, Md.) or goat antisera against mouse λ or κ light chains (kindly provided by J. Kearney).

Electrophoresis and protein blot assays. Components of *M. hyorhinis* were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (16) under reducing conditions (21), and nitrocellulose filter blots were prepared by the procedure of Towbin et al. (22), with slight modifications (21). Filter blots were treated for 1 h at 37°C with Tris-saline (10 mM Tris, 150 mM NaCl, pH 7.4) containing 3% BSA. Strips of the filter blots were then incubated as previously described (21) with culture fluid from individual hybridoma cultures or with mouse antiserum diluted in myeloma growth medium. Blots were then rinsed, treated with peroxidase-conjugated antibody (rabbit) against mouse IgG (21), and incubated with enzyme substrate solution containing O-dianisidine and hydrogen peroxide to allow color development at the sites of antibody binding (22).

The apparent molecular weight of immunologically stained components was determined by comparison with molecular weight standards electrophoresed and transferred to the same blots containing mycoplasma samples. Filter strips containing protein markers were stained with naphthol blue black (Sigma) immediately after transfer (22). Molecular weight standards included β-galactosidase (130,000), phosphorylase (95,000), BSA (68,000), ovalbumin (45,000), carbonic anhydrase (30,000), and cytochrome c (12,500).

Indirect immunofluorescence. All procedures were carried out at 0 to 4°C. BW5147 cells from logarithmic-phase cultures were sedimented at 500 × g for 5 min, suspended in myeloma growth medium, and centrifuged again. Washed pellets were dispersed in the same medium, and 50 μl of the suspension (2×10^7

TABLE 1. Characteristics of monoclonal antibodies to *M. hyorhinis* GDL

Hybridoma clone	Antibody isotype		Immunizing preparation[a]	Antigen recognized[b]
	Heavy chain	Light chain		
F146C11B	IgG1	κ	Whole organisms	p120
F22C32D	IgG1	κ	Detergent lysate	p73
F81C39R	IgG1	κ	Detergent lysate	p51
F24C4Fg	IgG1	κ	Detergent lysate	p38

[a] Mycoplasma preparations used to immunize mice are described in detail in the text.
[b] Nomenclature is based on size of component immunologically stained on protein blots of mycoplasma as described in the text and in Fig. 1.

cells per ml) was incubated with 50 μl of culture fluid from hybridoma (or control myeloma) cells for 30 min. Cells were then washed and suspended in 50 μl of fluorescein-conjugated antibody (rabbit) against mouse immunoglobulin (Cappel Laboratories, Cochranville, Pa.) diluted 1:200 in myeloma growth medium. Cells were incubated for an additional 30 min, washed again as described, and immediately observed under cover slips, using a Leitz Laborlux 12 fluorescence microscope equipped with epi-illumination and an I2 filter block (Ernst Leitz Wetzlar GmbH, Wetzler, West Germany).

Indirect immunoferritin staining. All procedures were performed at 0 to 4°C. BW5147 cells were washed (as described for immunofluorescence staining) with PBS containing 0.5% BSA (PBS-BSA). One hundred microliters of a suspension containing 10^7 cells per ml in PBS-BSA was incubated for 30 min with 50 μl of hybridoma (or control myeloma) culture fluid concentrate diluted 1:2 in this same buffer. Cells were washed once with PBS-BSA and suspended in 50 μl of ferritin-conjugated antibody (goat) to mouse immunoglobulin (Cappel) diluted 1:2 in PBS-BSA. After incubation for 30 min, the suspension was diluted with PBS-BSA to a volume of 1.5 ml and centrifuged. The pellet was fixed in glutaraldehyde and OsO_4 and processed for examination by electron microscopy (27).

Preparation of anti-mycoplasma antiserum. BALB/c mice were inoculated at weekly intervals by intraperitoneal injection of 0.5 ml of PBS containing 100 μg of protein from a mycoplasma detergent lysate (described above). Hyperimmune serum was obtained by retro-orbital bleeding after five injections.

RESULTS

Hybridoma clones secreting monoclonal antibodies to *M. hyorhinis* GDL. From a current library of approximately 40 stable hybridoma cell lines, four monoclonal antibodies were utilized: F146C11B, F22C32D, F81C39R, and F24C4Fg. Properties of the antibodies are presented in Table 1.

Characterization of mycoplasma components recognized by monoclonal antibodies. Identification of mycoplasma constituents recognized by these monoclonal antibodies was achieved by immunologically staining filter blots of broth-grown organisms subjected to SDS-PAGE (Fig. 1). Reference hyperimmune mouse serum to *M. hyorhinis* stained numerous mycoplasma components separated by this procedure, whereas, preimmune serum showed no staining (Fig. 1, lanes 1 and 2). In contrast to polyclonal hyperimmune serum, monoclonal antibodies from each of the four hybridoma clones stained individual mycoplasma components of different sizes (Fig. 1, lanes 4 through 7). Culture fluid from myeloma cells served as a negative control for the specific reactions of monoclonal antibodies (Fig. 1, lane 3). These results demonstrated that each monoclonal antibody reacted with a distinct epitope and that the epitopes recognized were borne on discrete macromolecular species. Since mycoplasma samples used for SDS-PAGE were heated (100°C, 5 min) in 2% SDS solution containing mercaptan (21), the results shown in this experiment also indicated that (i) the epitopes recognized by the monoclonal antibodies were stable to heat and detergent (i.e., not irreversibly destroyed by this treatment), and (ii) secondary structure involving disulfide bonds was not required for the integrity of the epitope structure. A provisional nomenclature for the antigens recognized by monoclonal antibodies was established based on the apparent molecular weight ($\times 10^3$) of the component stained in protein blots (see Table 1).

Localization of p120 antigen at the surface of mycoplasmas. It was anticipated that only a portion of the monoclonal antibodies of the library would react with intact mycoplasmas since many would recognize inaccessible internal components of the organisms. As an initial means of identifying monoclonal reagents reacting with mycoplasma surface antigens, indirect immunofluorescence was utilized to stain BW5147 cells chronically infected with *M. hyorhinis* GDL. Figure 2 illustrates the results of these experiments. Indirect immunofluorescent staining of intact mycoplasma-infected BW5147 cells with monoclonal antibody from clone F146C11B (recognizing the p120 antigen) resulted in a bright fluorescent pattern distributed in a patchy array at the surface of lymphoblastoid cells (Fig. 2, panel 1), characteristic of the mycoplasma staining pattern previously identified in this infected cell line (25, 27). BW5147 cells free from mycoplasma infection showed no

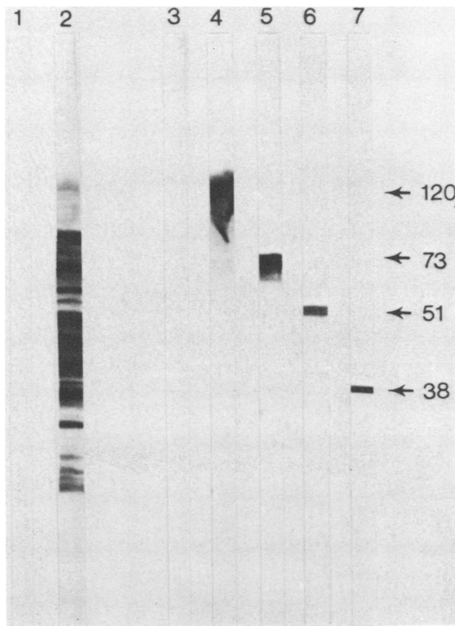

FIG. 1. Immunological staining of protein blots of mycoplasma components separated by SDS-PAGE. *M. hyorhinis* GDL grown in FBS-supplemented broth was isolated and components were separated by SDS-PAGE and transferred to nitrocellulose filters as described in the text. After treatment with protein to saturate binding sites, adjacent replicate filter strips were treated with mouse serum diluted 1:200 in myeloma growth medium or with culture fluid from individual hybridoma clones. Strips were then processed for indirect staining of bound antibody as described in the text. In one experiment (lanes 1 and 2), strips were treated with preimmune mouse serum (lane 1) or hyperimmune serum from the same animal after injection of *M. hyorhinis* GDL grown in HS-supplemented broth (lane 2). In a separate experiment (lanes 3 through 7), strips were treated with supernatant fluid from X63-Ag 8.653 myeloma cell cultures (lane 3) or from cultures of hybridoma clone F146C11B (lane 4), F22C32D (lane 5), F81C39R (lane 6), or F24C4Fg (lane 7). The approximate molecular weight ($\times 10^3$) is indicated from components stained by hybridoma culture supernatants based on comparison with molecular weight standards as described in the text.

fluorescent staining with this antibody (data not shown). Mycoplasma-infected BW5147 cells treated with supernatant from myeloma cells (Fig. 2, panel 3) showed no fluorescent staining, nor did cells treated with the other monoclonal antibodies listed in Table 1, or with any of an additional 25 separate monoclonal antibodies from our hybridoma library (data not shown). The selective staining obtained with monoclonal antibody to p120 suggested that this antigen may reside at the surface of mycoplasmas since the specific epitope recognized was available for antibody binding. The expression of this epitope on mycoplasmas colonizing host cells in vitro was therefore established by these experiments.

Localization of the p120 antigen was further investigated by electron microscopy by using an indirect immunoferritin technique (Fig. 3). Staining of infected BW5147 cells with antibody from clone F146C11B resulted in a marked deposition of ferritin at the perimeter of mycoplasmas colonizing lymphoblastoid cells (Fig. 3, panel 1). In contrast, cells treated identically with a supernatant preparation from myeloma cells showed only very sparse background staining with ferritin (Fig. 3, panel 2). The specific staining with antibody to p120 was densely and uniformly distributed around the entire periphery of virtually all organisms observed and was restricted to a layer external to the mycoplasma membrane. No ferritin deposition above background levels was observed on the host cell membrane except where organisms were in close juxtaposition to the cell surface. These results confirmed that the epitope recognized by antibody from clone F146C11B was expressed at the mycoplasma surface.

Comparison of p120 antigen on mycoplasmas grown in broth and in BW5147 cell cultures. To firmly establish the properties of the molecule bearing this epitope on organisms infecting lymphoblastoid cell cultures, we prepared protein blots of mycoplasmas derived from infected BW5147 cells and stained them with monoclonal antibody from clone F146C11B (Fig. 4). Blots of organisms from cell cultures revealed an antigenic component of molecular weight 120,000 indistinguishable from the p120 antigen from broth-grown organisms. Simultaneous staining of these two preparations with monoclonal antibody to p73 was included as a control and further demonstrated the similarity of this distinct antigenic component derived from each of the mycoplasma sources. The results of these experiments established that the epitope expressed at the surface of mycoplasmas during infection of host cells was associated with the p120 molecule.

Sensitivity of p120 antigen to trypsin treatment. To investigate the chemical nature of the p120 antigen, we incubated filter blots of mycoplasma components with a variety of enzymes immediately after electrophoretic transfer and then treated them with monoclonal antibody. Figure 5 illustrates the effect of trypsin on this antigen, and shows that treatment with this enzyme completely abrogated subsequent binding of monoclonal antibody (Fig. 5, lane 2). The presence of large protein excess (3% BSA or 20% FBS) was maintained during immunological staining to prevent degradation of immunoglobulin by residual enzyme. This possibility was further ruled out by showing that the monoclo-

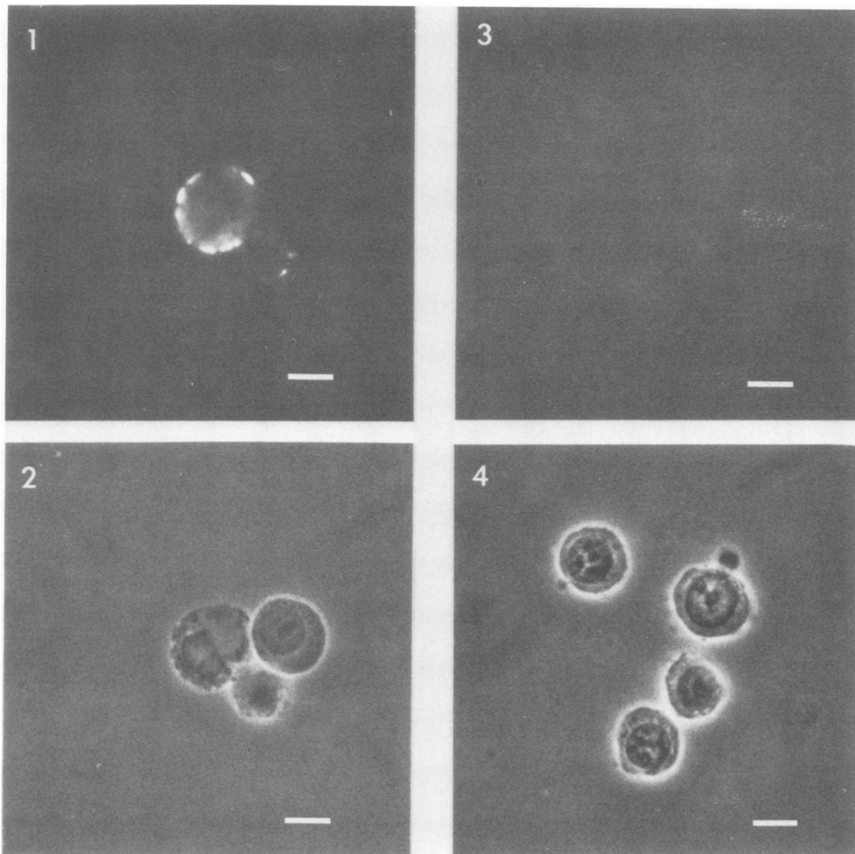

FIG. 2. Indirect immunofluorescent staining of *M. hyorhinis*-infected lymphoblastoid cells with monoclonal antibody. BW5147 cells chronically infected with *M. hyorhinis* GDL were treated with hybridoma (or myeloma) cell culture fluid and further incubated with fluorescein-conjugated antibody to mouse immunoglobulin as described in the text. Fluorescent images (panels 1 and 3) of cells treated with supernatant from hybridoma clone F146C11B (panel 1) or X63-Ag 8.653 myeloma cells (panel 3) are shown in the top row. Corresponding phase contrast images of the respective fields are shown directly below (panels 2 and 4). Bar = 10 μm.

nal antibody preparation incubated with the trypsin-treated blot was subsequently able to stain p120 on an untreated blot (Fig. 5, lane 3). As an additional control for these experiments, trypsin treatment of identical blots had no effect on the binding of some other monoclonal antibodies within our library (K. Wise, unpublished data). These results suggested that the epitope recognized by monoclonal antibody to p120 was comprised of or was covalently linked to a trypsin-sensitive polypeptide. Treatment of blots (37°C, 30 min) with PBS containing 1 U of *Clostridium perfringens* neuraminidase (Sigma) per ml had no effect on antibody binding (data not shown). In a series of similar experiments, trypsin treatment of blots subsequently stained with the other monoclonal antibodies listed in Table 1 indicated that antigens p73, p51, and p38 were also associated with protein (data not shown).

DISCUSSION

We identified four distinct protein antigens of *M. hyorhinis* GDL by using monoclonal antibodies from our current library. The p120 antigen characterized in this initial analysis is of particular interest due to its prominent expression at the surface of mycoplasmas and its close proximity to the host cell plasma membrane during in vitro colonization of BW5147 lymphoblastoid cells by these surface parasites. In previous studies, we have shown that host cell surface glycoproteins associate with the mycoplasma surface during this interaction (25–27). The possibility is raised by these observations that host components may be associated with specific mycoplasma surface structures. The identification of the p120 mycoplasma surface antigen and the simultaneous development of a specific immunological tool recognizing this structure

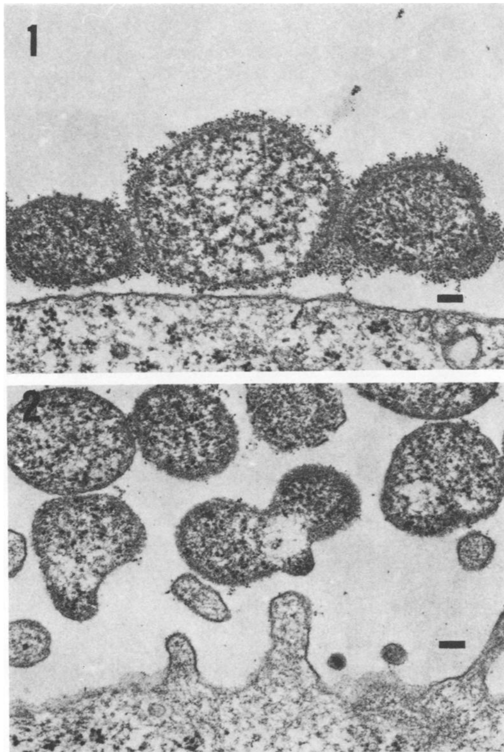

FIG. 3. Transmission electron micrograph of mycoplasma-infected lymphoblastoid cells stained with monoclonal antibody by indirect immunoferritin technique. BW5147 cells infected with *M. hyorhinis* GDL were treated with concentrates of hybridoma (or myeloma) cell culture fluid and further incubated with ferritin-conjugated antibody to mouse immunoglobulin as described in the text. Cells were treated with concentrates from cultures of F146C11B cells (panel 1) or from X63-Ag 8.653 myeloma cells (panel 2). Mycoplasmas are shown residing at the lymphoblastoid cell surface. Bar = 100 nm.

should facilitate analysis of its involvement in these processes. In addition, the role of p120 in attachment of *M. hyorhinis* to host cells may be readily assessed by blocking the process with the corresponding antibody, as has been demonstrated with antibodies to surface structures of other mycoplasmas (3, 9, 14).

Other laboratories have recently generated monoclonal antibodies reacting with *M. hyorhinis*, either inadvertently during immunization with contaminated cell culture lines (13, 23) or as a means of producing specific reagents for the diagnosis of cell culture contamination by a variety of mycoplasma species (4). These diagnostic antibody reagents showed selective reactivity within the species *M. hyorhinis*, but the characteristics and precise location of the antigens recognized were not determined. The monoclonal antibodies described in the present report have been partially analyzed for specificity among species of related mycoplasmas (1), and recent experiments comparing protein blots of *M. hyorhinis* GDL, *Mycoplasma pulmonis*, and *Mycoplasma fermentans* have shown that none of the four antigens defined in Table 1 are expressed in the latter two species (K. Wise, unpublished data). Detailed analysis of species specificity (and possible strain specificity within the species *M. hyorhinis*) is currently under investigation.

A potentially novel use of the monoclonal antibodies in our library may be the precise definition of gene products encoded in DNA sequences of *M. hyorhinis* as a means of identifying specific genomic markers in this organism. We have recently constructed a library of cloned genomic DNA fragments from *M. hyorhinis* in bacteriophage expressing mycoplasma antigens in an *Escherichia coli* host (21). Immunological definition of antigenic gene products by monoclonal antibodies would provide a unique and extremely specific set of markers within this

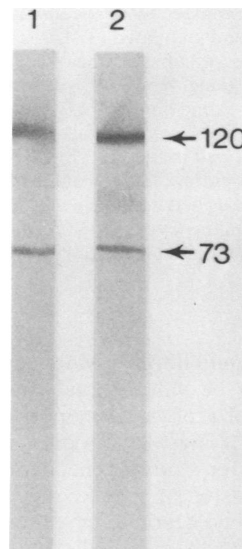

FIG. 4. Comparison of antigen p120 derived from mycoplasmas grown in broth culture and lymphoblastoid cell culture. *M. hyorhinis* GDL was prepared from broth culture or from the supernatant of BW5147 cells infected with this organism as described in the text. Protein blots of each preparation were performed as described in the legend to Fig. 1, and blots were treated with the combined culture fluids from F146C11B and F22C32D hybridoma cells. Stained strips representing mycoplasmas derived from broth culture (lane 1) and from BW5147 cell cultures (lane 2) are shown. The amount of mycoplasma protein loaded is approximately eightfold less than that shown in Fig. 1. The positions of antigens p120 and p73 are indicated by arrows.

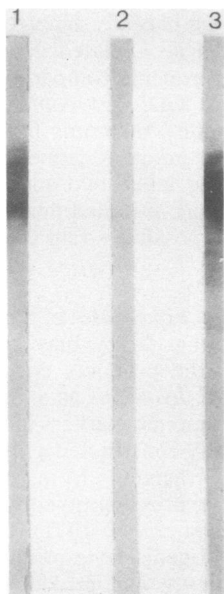

FIG. 5. Effect of trypsin treatment on p120 antigen. Protein blots of *M. hyorhinis* GDL were prepared as described in the legend to Fig. 1. Immediately after electrophoretic transfer to nitrocellulose filter paper, strips were cut and incubated (37°C, 30 min) with PBS (lane 1) or PBS containing 0.5 mg of acetylated trypsin (Sigma) per ml (lane 2). Blots were then separately rinsed with Tris-saline containing 3% BSA and further incubated with this solution before immunological staining as described in the text. Strips were separately stained with culture fluid from F146C11B hybridoma cells as described in the legend to Fig. 1. Culture fluid used to stain the trypsin-treated strip (lane 2) was reused to stain a strip not treated with enzyme (lane 3).

recombinant gene library. Monoclonal reagents may therefore be valuable aids in the ongoing construction of a physical map of the *M. hyorhinis* genome. These antibodies may also be ideally suited for studying mechanisms controlling the expression of specific mycoplasma proteins from cloned genomic DNA sequences, and should facilitate the detailed structural analysis of mycoplasma (surface) antigens by identifying the corresponding genes encoding these proteins.

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