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Adherence, Fibronectin Binding, and Induction of Cytoskeleton Reorganization in Cultured Human Cells by *Mycoplasma penetrans*

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Mycoplasma penetrans **adhered to cultured human cells, forming clusters that localized to specific areas of the host cell surface. Adherence and cluster formation were inhibited by anti-*M. penetrans* antibodies, suggesting the involvement of specific adhesin-receptor interactions. Ultrastructural studies showed that after 2 h of infection, mycoplasmas attach to and penetrate the host cell surface. *M. penetrans* bound selectively to immobilized fibronectin, an interaction which was not inhibited by a 70-kDa fragment containing a heparin-gelatin-binding domain of fibronectin, other matrix glycoproteins, or an RGD tripeptide, suggesting the recognition of other specific binding sites on the fibronectin molecule. A ca. 65-kDa fibronectin-binding protein of *M. penetrans* was eluted following Sepharose-fibronectin affinity chromatography. Confocal, light, and immunofluorescence microscopy demonstrated that the interaction of *M. penetrans* with target cells triggers a signal that causes recruitment of several cytoskeletal components, including tubulin and α-actinin, and aggregation of phosphorylated proteins. Detergent-soluble mycoplasma proteins with apparent molecular masses of 18, 28, 32, 36, 39, and 41 kDa selectively bound to glutaraldehyde-fixed HEp-2 cells. Our findings offer new insights into understanding the interaction of this human mycoplasma with host target cells.**

The ability to adhere to and penetrate epithelial cells is so characteristic of some bacterial species that it would seem to be an important aspect of their pathogenicity (6, 12, 39). Most bacterial pathogens express surface factors that either mediate direct binding to host cells or indirectly bind host adhesion factors, such as fibronectin, which act as "interlinking" molecules (6, 18, 19, 42). The entry of the pathogen into the host cell is initiated by bacterial binding to the cell surface, which generates an uptake signal that induces internalization into the cytoplasm (11a, 13, 38). Many pathogenic organisms are capable of altering the host cytoskeleton architecture, as manifested by a dramatic rearrangement of microtubule and microfilament proteins (7, 11a, 30, 36, 40, 48).

Pathogenic mycoplasmas are the smallest self-replicating organisms and are capable of inducing acute or chronic debilitating diseases and dramatically altering immune function (21). A wealth of evidence implicates several *Mycoplasma* species in various human diseases such as genitourinary tract infections, pneumonia, and arthritis; lately, they have been suggested to play a putative role in the progression of AIDS (10, 17, 21, 25, 28, 29, 43, 45). *Mycoplasma penetrans* is a new *Mycoplasma* species isolated from the urogenital tracts of AIDS patients (26, 27). The molecular mechanisms whereby *M. penetrans* adheres to and is internalized into host cells are poorly understood. Nevertheless, the interaction between *M. penetrans* and the host target cell is characterized by attachment to the cell surface by a highly oriented process which precedes internalization (26, 27). Intracellular mycoplasmas are later found free in the cytoplasm or localized in membrane-bound vesicles. Extensive invasion and intracytoplasmic multiplication by mycoplasmas within the vesicles are suggested to be responsible for cell disruption and death (26, 27).

For a number of bacterial, parasitic, and viral pathogens, the ability to bind to matrix extracellular glycoproteins such as fibronectin, collagen, laminin, and vitronectin is viewed as an important component of their pathogenic scheme (2, 5, 6, 8, 16, 42, 46). The recognition of these host molecules by mycoplasmas has not yet been fully explored. In this study, we examined the ultrastructural properties and dynamics of *M. penetrans* interaction with cultured epithelial cells. To further our understanding of this process, we examined the binding capabilities of this organism to matrix extracellular glycopro-

TABLE 1. Adherence of *M. penetrans* in the presence of several putative inhibitors

Substance	Target	Dose	Effect on adherence[a]
D-Fucose		10–100 μg/ml	−
D-Mannose		10–100 μg/ml	−
D-Galactose		10–100 μg/ml	−
D-Glucose		10–100 μg/ml	−
Lactose		10–100 μg/ml	−
N-Acetylglucosamine		10–100 μg/ml	−
N-Acetylgalactosamine		10–100 μg/ml	−
Chloramphenicol	Protein synthesis	10–50 μg/ml	−
Cycloheximide	Protein synthesis	10–50 μg/ml	−
Rifampin	RNA synthesis	10–50 μg/ml	−
Trypsin	Proteins	0.0125–0.25%	±
Neuraminidase	Sialic acid	5–10 U/ml	±
Metaperiodate	Carbohydrate structure	10–10 mM	+
Cytochalasin B	Actin filaments	1–10 μg/ml	−
Cytochalasin D	Actin filaments	1–10 μg/ml	−
Nocodazole	Microtubules	1–10 μg/ml	−
Vinblastine	Microtubules	1–10 μg/ml	−
Colchicine	Microtubules	1–10 μg/ml	−
Tyrphostin	TPKs[b] inhibitor	500 μM	−
Genistein	TPKs inhibitor	50 μM	−
Staurosporine	TPKs inhibitor	1 μM	−

[a] Effect on cluster formation onto HEp-2 cells as determined by Giemsa staining. +, >75% inhibition; ±, 25 to 50% inhibition; −, no inhibitory effect.
[b] TPKs, tyrosine phosphokinase.

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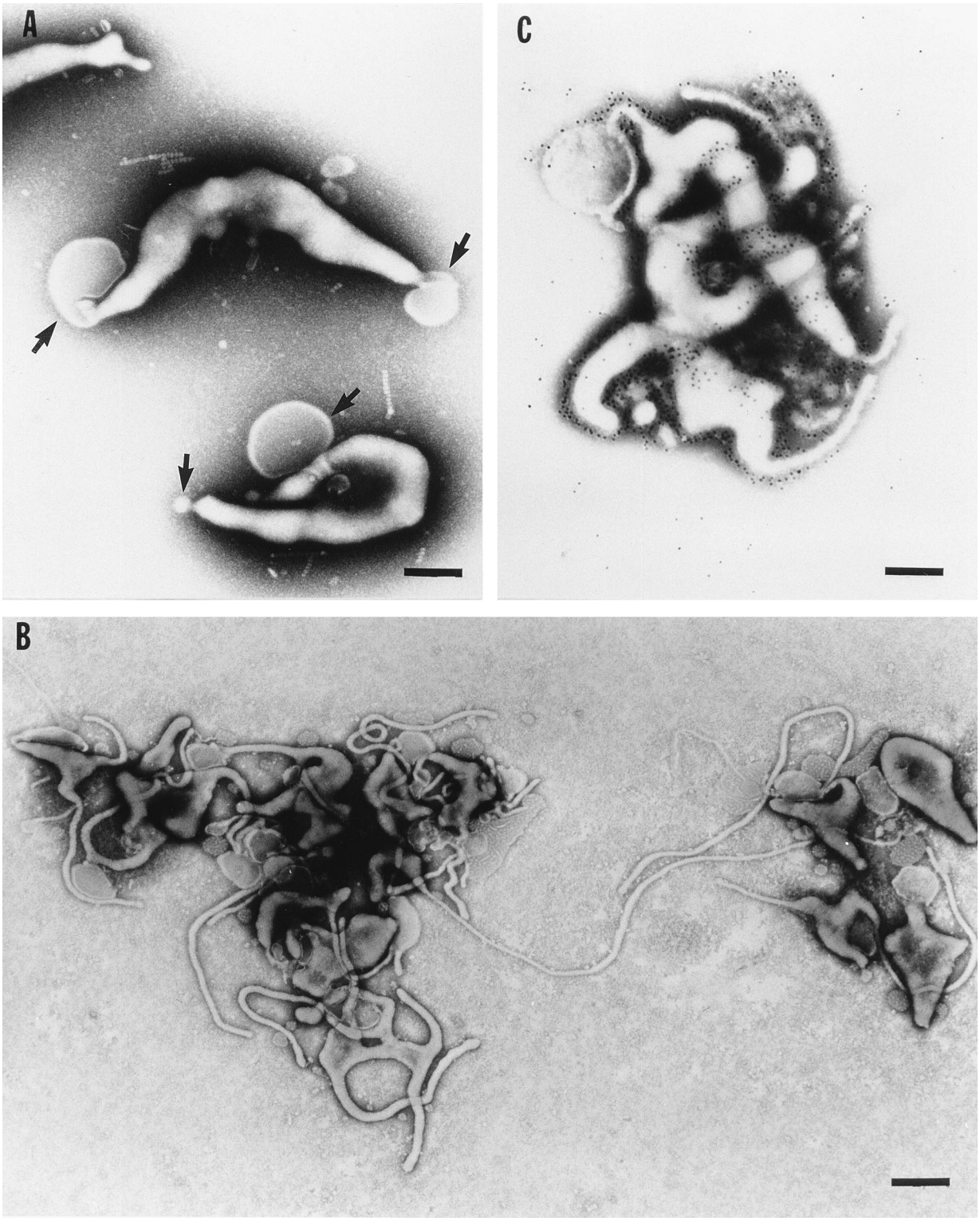

FIG. 1. Ultrastructural analysis of *M. penetrans*. Transmission electron micrographs showing negative staining (A, B, and D) and immunogold labelling with anti-*M. penetrans* antiserum (C and E). Note bleb formation at the poles of mycoplasmas (arrows) in panel A, the formation of long filamentous protrusions in panel B, and the presence of a transverse dividing membrane in panel D. Bars: A, 0.25 μm; B, 0.5 μm; C and D, 0.16 μm; E, 0.25 μm.

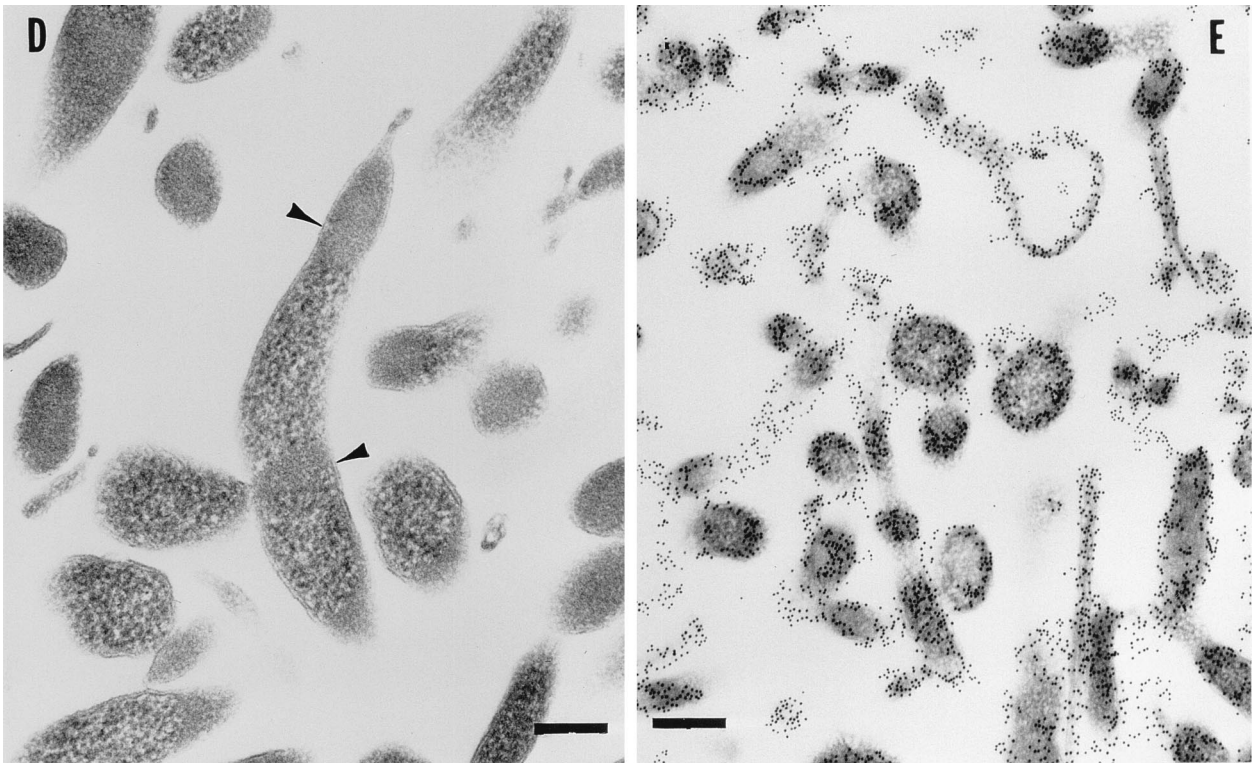

FIG. 1—*Continued.*

teins and its ability to induce cytoskeletal rearrangement in mammalian cells after *M. penetrans* infection. The adherence assays described here may prove useful in studies of the nature of potential receptors and adhesins by using receptor analogs, blocking antibodies, and the isolation of mutants defective in attachment or invasion. The ability of *M. penetrans* to bind immobilized fibronectin and to reorganize the host cell cytoskeleton reveals new approaches to the understanding of the interaction of mycoplasmas with eukaryotic cells.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. penetrans* GTU-54 is a clinical isolate from a human immunodeficiency virus type 1 (HIV-1)-infected patient with AIDS and was kindly provided by Shyh-Ching Lo (26). GTU-54 was routinely grown overnight at 37°C in SP-4 medium (26).

Antisera and cell reagents. Rabbit polyclonal antiserum against *M. penetrans* GTU-54 whole cell was a generous gift from J. Tully, National Institutes of Health, Bethesda, Md. Rabbit preimmunization serum was used as a negative control in the studies described below. Monoclonal antibody (MAb) anti-human α-tubulin (immunoglobulin G1 [IgG1]) and MAb anti-phosphotyrosine (IgG1) were purchased from Zymed, Burlingame, Calif.; MAb anti-human α-actinin (IgG2b; A-5044), polyclonal anti-tropomyosin (T3651), MAb anti-vinculin (IgG1; V-4505), MAb anti-epidermal growth factor receptor (IgG1; E2760), MAb anti-talin (IgG1, T3287), fluorescein isothiocyanate (FITC)-labeled MAb anti-human β_2-microglobulin (IgG1; F1394), and FITC-labeled MAb anti-human CD44 (IgG1; F3647) were purchased from Sigma, St. Louis, Mo. Human AIDS sera were kindly provided by S. Holt, University of Texas Health Science Center at San Antonio.

Electron microscopy, immunogold labeling, and scanning microscopy. For ultrastructural studies of *M. penetrans*, 10 μl of a mycoplasma suspension (10^6 CFU/ml) was placed onto a carbon-Formvar coated 300-mesh copper grid (Electron Sciences, Fort Washington, Pa.), negatively stained with 1% phosphotungstic acid (pH 7.4), and examined in a JEOL JEM 1200 EX II transmission electron microscope at 80 kV (14). For immunogold labeling studies, mycoplasmas were reacted with anti-*M. penetrans* antiserum and goat anti-rabbit IgG conjugated with 10-nm-diameter gold particles (Amersham, Arlington Heights, Ill.), and stained as described previously (15). Cell monolayers infected with GTU-54 were fixed with 2% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) and processed for thin sectioning and immunogold labeling with anti-*M. penetrans* antibodies or for scanning electron microscopy (15, 32).

Adherence assays and kinetics. The kinetics of GTU-54 adherence to epithelial cells was studied with cultures of HEp-2 (human larynx carcinoma cells; ATCC CCL 23), normal human colon mucosal (NCM/SM; kindly provided by M. Moyers, University of Texas Health Science Center at San Antonio), and WI-38VA13 (human lung fibroblasts, ATCC CCL 75.1) cell lines. Before the assay, mycoplasmas were washed in PBS and suspended to a concentration of 10^8 CFU/ml. An inoculum of 10^6 CFU/ml was incubated with a monolayer of cultured human cells for 0, 2, 4, 6, 8, 10, 12, 14, 16, and 24 h at 37°C under 5% CO_2 in Dulbecco's minimal essential medium (Gibco, Bethesda, Md.) containing 10% fetal bovine serum (Hyclone, Logan, Utah). Infected cells were washed three times with PBS and fixed with methanol, and cell-associated mycoplasmas were visualized by Giemsa staining and light microscopy under oil immersion with a Zeiss Axioscope microscope (11, 14, 41).

Similar kinetics experiments were performed with mycoplasmas metabolically labeled with [^{35}S]methionine and mammalian cells grown on Thermanox coverslips. After nonadherent mycoplasmas were washed off, individual coverslips were counted in a scintillation counter (Beckman, Palo Alto, Calif.).

Immunofluorescence and confocal microscopy. Adherence of *M. penetrans* to tissue culture cells was further characterized by indirect immunofluorescence with specific anti-*M. penetrans* antibodies. Briefly, after the adherence assay described above, infected cells were fixed with 3% formalin (30 min at room temperature) and incubated for 1 h with a 1:1,000 dilution of the primary antibodies in PBS containing 1% bovine serum albumin (BSA; Sigma). The cells were washed with PBS and incubated for an additional 1 h with goat anti-rabbit IgG conjugated to FITC (Sigma). Immunofluorescence was examined under oil immersion with a Zeiss Axioscope microscope (13, 34, 41).

To study cytoskeletal changes that occur upon *M. penetrans* infection, infected-cell monolayers were fixed as above, washed in PBS, and permeabilized in 0.1% Triton X-100 for 5 min. After the washing step, primary anti-microtubule or anti-receptor antibodies were added for 1 h, unbound antibodies were removed by washing in PBS, and then secondary antibodies (FITC or rhodamine labeled) were added for 1 h each. Indirect immunofluorescence was examined under oil immersion with a Zeiss Axioscope microscope and with a Carl Zeiss LSM10 confocal microscope equipped with helium/neon laser using line 543 along with an LG 590 cutoff filter and rhodamine optical filter set (9, 11a, 40, 48).

Inhibition of adherence. For adherence inhibition experiments, 10 μl of a mycoplasma suspension (10^6 CFU/ml) was incubated for 20 min at room temperature with 1:10, 1:25, 1:50, and 1:100 dilutions of anti-*M. penetrans* antiserum or normal rabbit serum. This mix was then added to HEp-2 cell monolayers containing the same dilution of antibodies. After 6 h of infection at 37°C, the

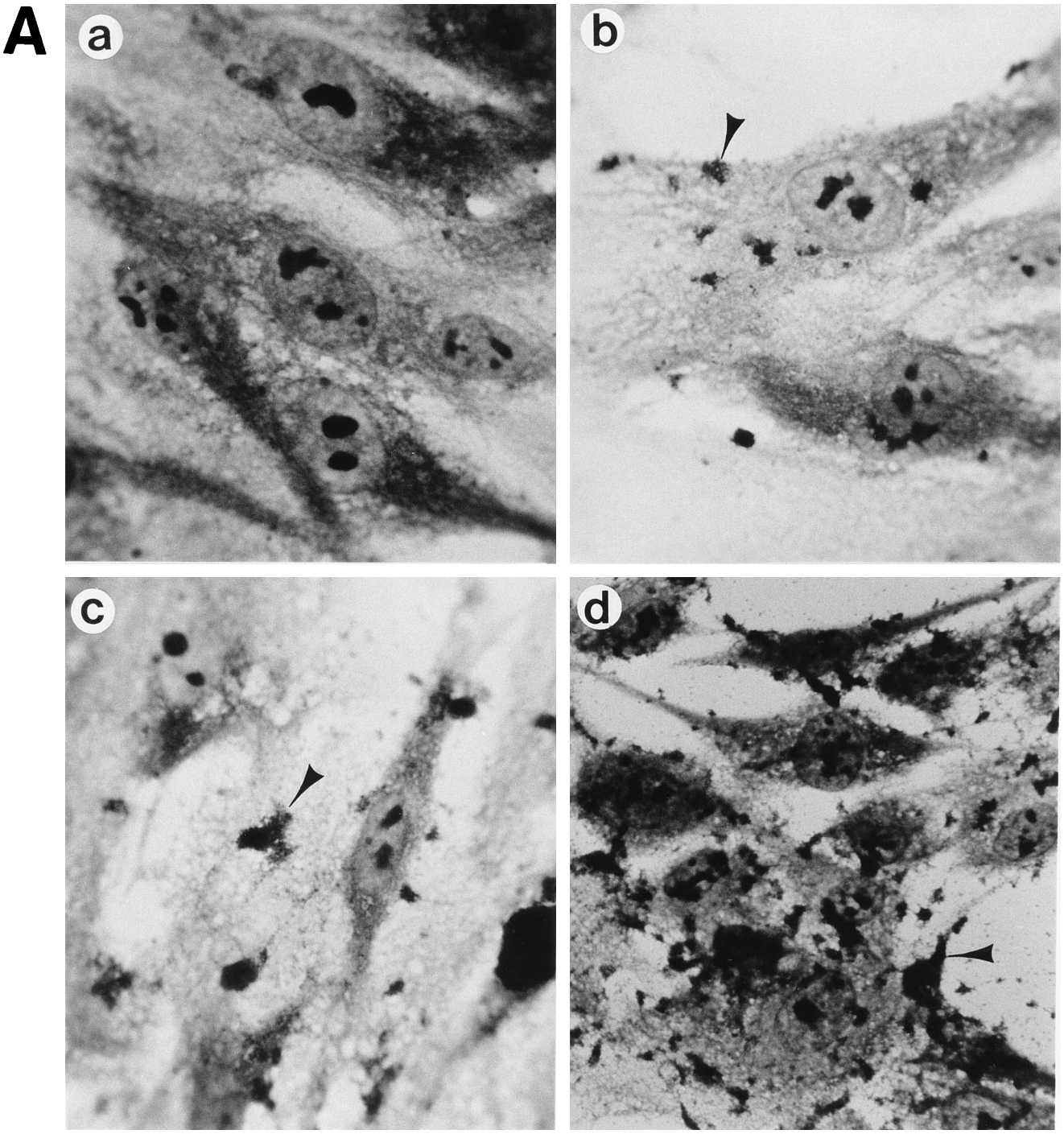

FIG. 2. Morphological characterization of *M. penetrans* adherence to HEp-2 cells. Giemsa staining, (A), fluorescence staining (B), and scanning electron microscopy (C) of HEp-2 cells infected with *M. penetrans* for 0 h (a), 2 h (b), 4 h (c), and 6 h (d) at 37°C. (B) Mycoplasmas were labeled with anti-*M. penetrans* antiserum and goat anti-rabbit FITC conjugate. Magnification, ×100. (C) Typical *M. penetrans* organisms (Fig. 1) are seen clustering onto target cells, and in some instances, mycoplasmas appear to penetrate the cell surface (a to c, curved arrows). Some mycoplasmas are seen rounding up into donut shapes (b to d, arrowheads). Bars, 1 μm. In all panels, note the formation of localized clusters or microcolonies which grow in size and number with the time of infection.

cells were washed, fixed, and visualized as indicated above (11, 14, 49). In addition, the dose effect of putative inhibitors or drugs (listed in Table 1) was similarly studied. The degree of adherence in the presence of the inhibitor(s) was compared with that in the control wells containing only mycoplasmas and epithelial cells (49).

Binding of biotinylated *M. penetrans* proteins to glutaraldehyde-fixed mammalian cells. Intact mycoplasmas were surface labeled with sulfosuccinimidyl 6-(biotinamido)hexanoate (NHS-LC-biotin) as recommended by the manufacturer (Pierce, Rockford, Ill.). The biotinylated organisms were suspended in solubilization buffer (35 mM Tris [pH 8.2], 0.25 M NaCl, 16 mg of sodium deoxycholate per ml, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride) and incubated for 30 min at 37°C with gentle mixing (20). The suspension was centrifuged at 100,000 × *g* for 1 h at 4°C to obtain a supernatant fraction of biotinylated-detergent-soluble proteins (BDSP). Glutaraldehyde-

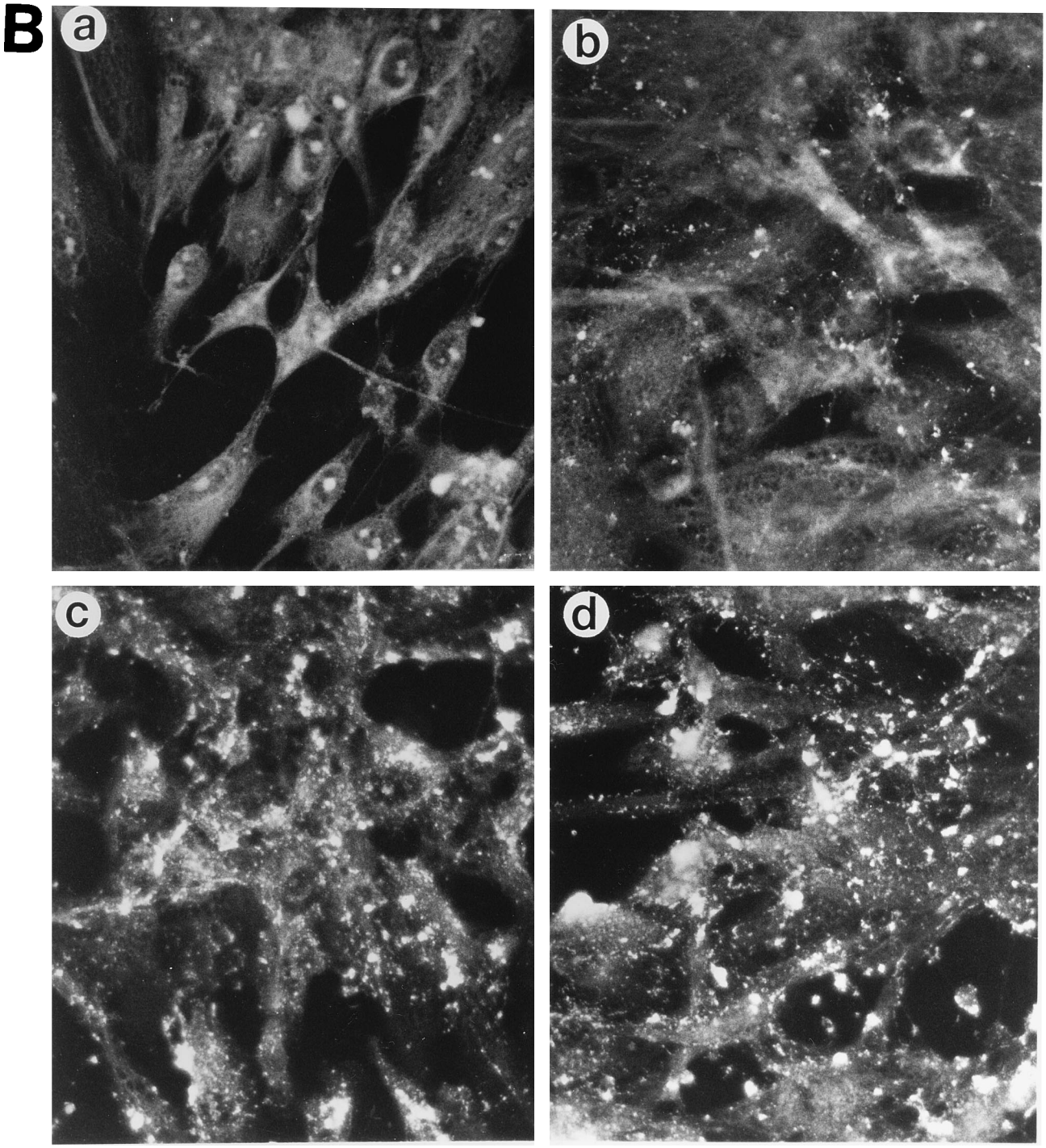

FIG. 2—*Continued*

fixed cells were incubated in microcentrifuge tubes with different concentrations of BDSP for 1 h at 37°C with gentle rocking (20, 24) and washed three times with PBS containing 0.5% Tween 20. The BDSP, which selectively bound to glutaraldehyde-fixed cells, were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (22) in 14% polyacrylamide gels. After the proteins were blotted onto nitrocellulose membranes, streptavidin conjugated to alkaline phosphatase was added and the reaction was visualized with a mixture of nitroblue tetrazolium and 5-bromo-chloro-indolyl phosphate (Sigma).

The reactivity of BDSP with rabbit anti-*M. penetrans* antiserum or antisera from humans with AIDS was studied by immunoblotting. The membranes were reacted with goat anti-rabbit or anti-human IgG conjugated to alkaline phosphatase (Sigma). After being washed, the blots were developed as described above.

Binding of *M. penetrans* to matrix glycoproteins. Binding of *M. penetrans* to basement membrane matrix glycoproteins was studied by a standard enzyme-linked immunosorbent assay (ELISA) method or with ^{35}S-labeled mycoplasmas. Briefly, 96-well Immunolon II (Dynatech, Detroit, Mich.) ELISA plates were coated with different concentrations of matrix glycoproteins (fibronectin; collagen types III, VI and X; laminin; and vitronectin) (Sigma) in carbonate buffer (pH 9.6), blocked with PBS containing 1% BSA, and then incubated for 1 h with mycoplasmas (10^5 CFU per well). The reaction was monitored by incubation with anti-*M. penetrans* antibodies and goat anti-rabbit Ig alkaline phosphatase

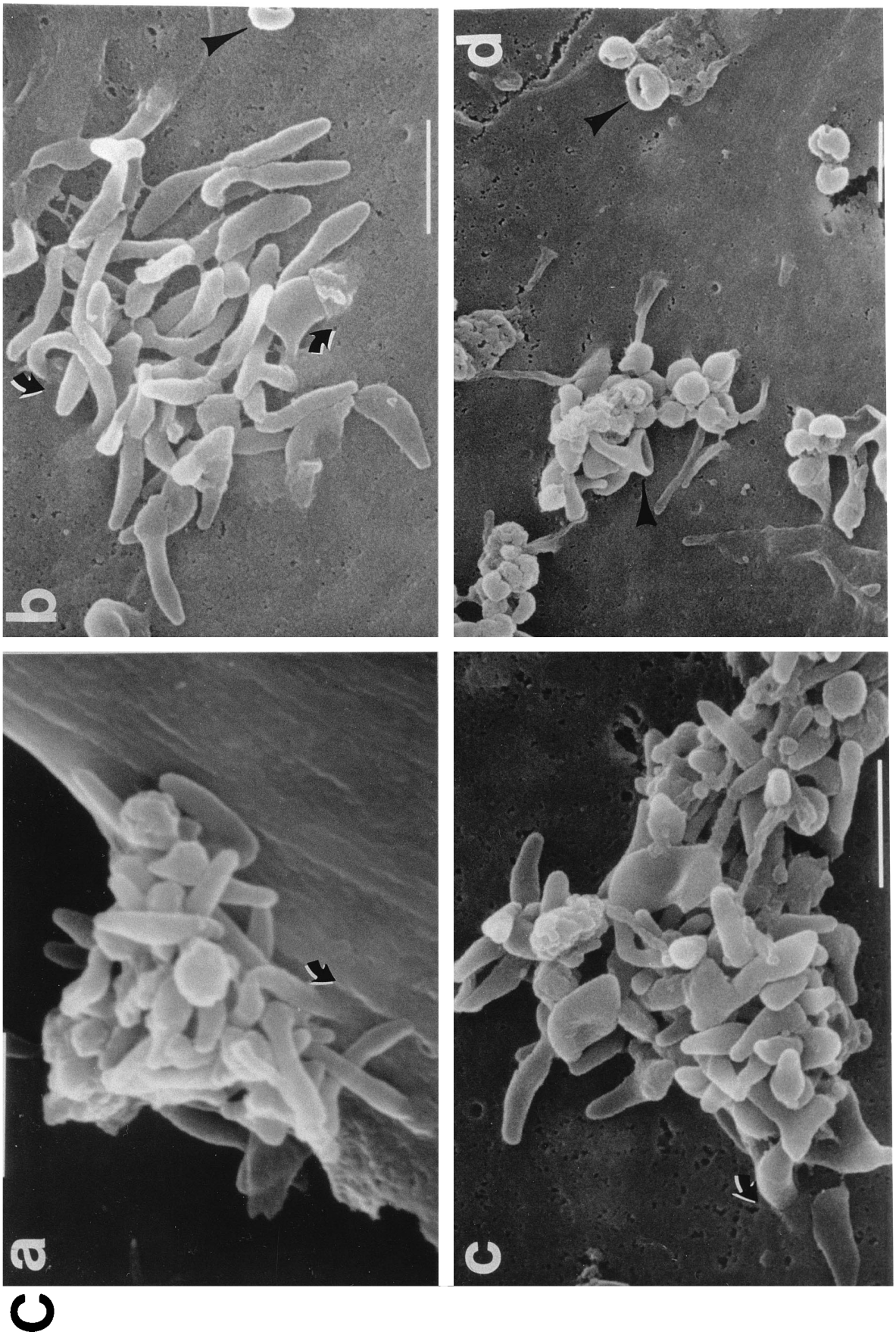

FIG 2—*Continued.*

conjugate (Sigma). After addition of the phosphatase substrate, the plates were read at 405 nm. When radioactively labeled mycoplasmas were used, an inoculum of 100,000 cpm/10^6 CFU/ml per well was added to fibronectin-coated wells, and the mixture was incubated for 1 h at 37°C. Unbound bacteria were washed off, and the radioactivity in the wells was counted in a Beckman scintillation counter.

To investigate the nature of the surface component involved in fibronectin recognition, BDSP or biotinylated whole-cell extracts of *M. penetrans* were passed through a Sepharose-fibronectin affinity column (42). The column was washed extensively with PBS containing 0.5% Tween 20 and then with 1 M NaCl until an optical density at 280 nm of zero was achieved. Selectively bound mycoplasma proteins were eluted with 5 M LiCl, and peak fractions were dialyzed against PBS, concentrated by lyophilization, separated by SDS-PAGE (22), blotted, and reacted with strepavidin conjugated to alkaline phosphatase.

RESULTS

Ultrastructural studies of *M. penetrans*. Typically, *M. penetrans* is an elongated flask shape, but it was also seen with blebs located at its tip organelles (Fig. 1A). Furthermore, amorphous shapes, which branch to form long protrusions resembling hyphae, were observed (Fig. 1B). Specific polyclonal anti-*M. penetrans* antibodies reacted with whole cells, blebs, and hyphae, indicating that these structures are of a mycoplasma nature (Fig. 1C). Thin sections of mycoplasmas revealed two distinct compartments divided by a transversal membrane, which are also recognized by anti-*M. penetrans* antibodies (Fig. 1D and E) (26, 27).

Kinetics of adherence to mammalian cells. Regardless of the cell line used, Giemsa-stained *M. penetrans* organisms adhered to the host cell surface as single organisms or formed tight microcolonies that increased in size as the infection proceeded (Fig. 2A). We considered 6 h of infection to be the optimal time point to study the effect of potential inhibitors (see below). Beyond 12 h of infection, cytopathologic effects were more obvious impeding detailed analysis, as a result of cell loss and death. In contrast to an earlier report (27), *M. penetrans* adhered poorly to glass or plastic. The identity of *M. penetrans* organisms in the adhering microcolonies was confirmed by indirect immunofluorescence using anti-*M. penetrans* antibodies (Fig. 2B). A normal rabbit antiserum revealed no fluorescent clusters.

To further confirm the presence of *M. penetrans* organisms in the Giemsa-stained clusters, we analyzed the time course of infection by scanning electron microscopy (Fig. 2C). Consistent with our previous observations, *M. penetrans* organisms initially attached as small clusters, which subsequently grew into larger bacterial aggregates. In some instances, it is apparent that as early as 2 h postinfection, the organisms were in the process of penetrating the cell membrane (Fig. 2C, panels a to c). Interestingly, the mycoplasmas appeared to undergo morphological changes. For example, at 2 h postinfection, typical elongated, flask-shaped organisms are seen on the cell surface. As the infection proceeds, numerous adhering organisms round up into a donut shape, and these spherical forms predominate on the cell surface (Fig. 2C, panel d). Thus, our observations on the adherence of *M. penetrans* to eukaryotic cells as visualized by Giemsa staining, immunofluorescence, and scanning electron microscopy are consistent and reproducible. Furthermore, the addition of ^{35}S-metabolically labeled *M. penetrans* to HEp-2 cells showed a time-dependent binding profile, confirming the selective interaction between *M. penetrans* and eukaryotic cells (Fig. 3).

Effect of anti-*M. penetrans* antibodies and putative inhibitors on adherence. We next investigated the nature of the adhesin(s) and/or the receptor molecules involved in the adhesion process. Toward this aim, a number of potential inhibitors and drugs were tested by the 6-h adherence assay described above (Table 1) (47). Except for trypsin and metaperiodate, which reduced the adherence of *M. penetrans* by 30 and 90%, respectively, we were unable to show any inhibitory dose effect on adherence or cluster formation by *M. penetrans* with other compounds or treatments. Specific anti-*M. penetrans* antibodies blocked mycoplasma adherence to the three cell lines studied and cluster formation in a dose-dependent fashion and without affecting mycoplasma viability, suggesting the involvement of specific adhesin-receptor interactions (data not shown).

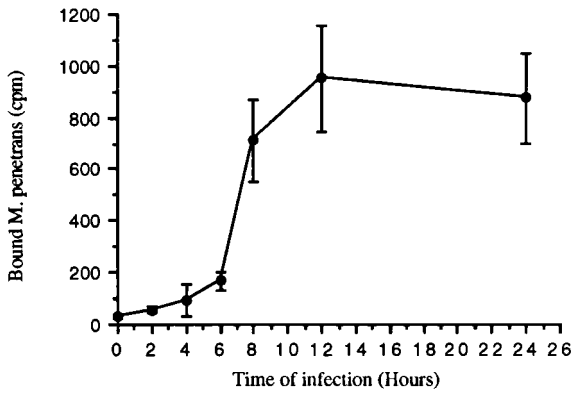

FIG. 3. Kinetics of adherence of ^{35}S-labeled *M. penetrans* to HEp-2 cells. Values are means ± standard errors of the means for four samples. The multiplicity of infection was 10 mycoplasmas per target cell.

Internalization of *M. penetrans* into mammalian cells. We found that as early as 2 h postinfection, *M. penetrans* organisms were internalized or in the process of being internalized without causing obvious damage to the mammalian cell membrane. As the infection proceeded, the number of adherent extracellular clusters of mycoplasmas and intracellular organisms free or within vacuoles in the cytoplasm increased. It was obvious that at late stages of infection, host cell integrity was disrupted and cell death followed (not shown). Intracellular and extracellular *M. penetrans* organisms were further evidenced by immunoelectron microscopy performed on thin sections of infected HEp-2 cells with anti-*M. penetrans* antibodies (Fig. 4). These observations have been reinforced by the direct isolation of viable intracellular *M. penetrans* organisms 96 h postinfection (4).

***M. penetrans* proteins eluted from glutaraldehyde-fixed HEp-2 cells.** In an effort to identify a surface-associated protein(s) involved in attachment of *M. penetrans* to mammalian cells, we reacted biotinylated, detergent-soluble proteins of *M. penetrans* with glutaraldehyde-fixed HEp-2 cells (20, 24). The predominant mycoplasma proteins which bound to HEp-2 cells migrated with apparent masses of 18, 28, 32, 36, 39, and 41 kDa (Fig. 5). Whether all or some of these proteins are involved in the adherence events associated with *M. penetrans* pathogenesis has yet to be determined. Lipid-associated membrane proteins (LAMPs) of 35, 38, 61, and 103 kDa were previously reported to be immunoreactive, to various degrees, with antibodies from humans serologically positive for HIV but not with antibodies from HIV-negative individuals (44). However, no functional correlation of these proteins was implied. In view of these data, HEp-2 cell-eluted *M. penetrans* proteins were reacted in immunoblots with sera from normal and HIV-positive subjects. Two main proteins between 36 and 40 kDa were readily reactive with HIV-positive sera but not with human normal serum (Fig. 5). Reactivity to other polypeptides was less evident. Our data suggest that these 36- to 40-kDa polypeptides may correspond to the previously described LAMPs (44).

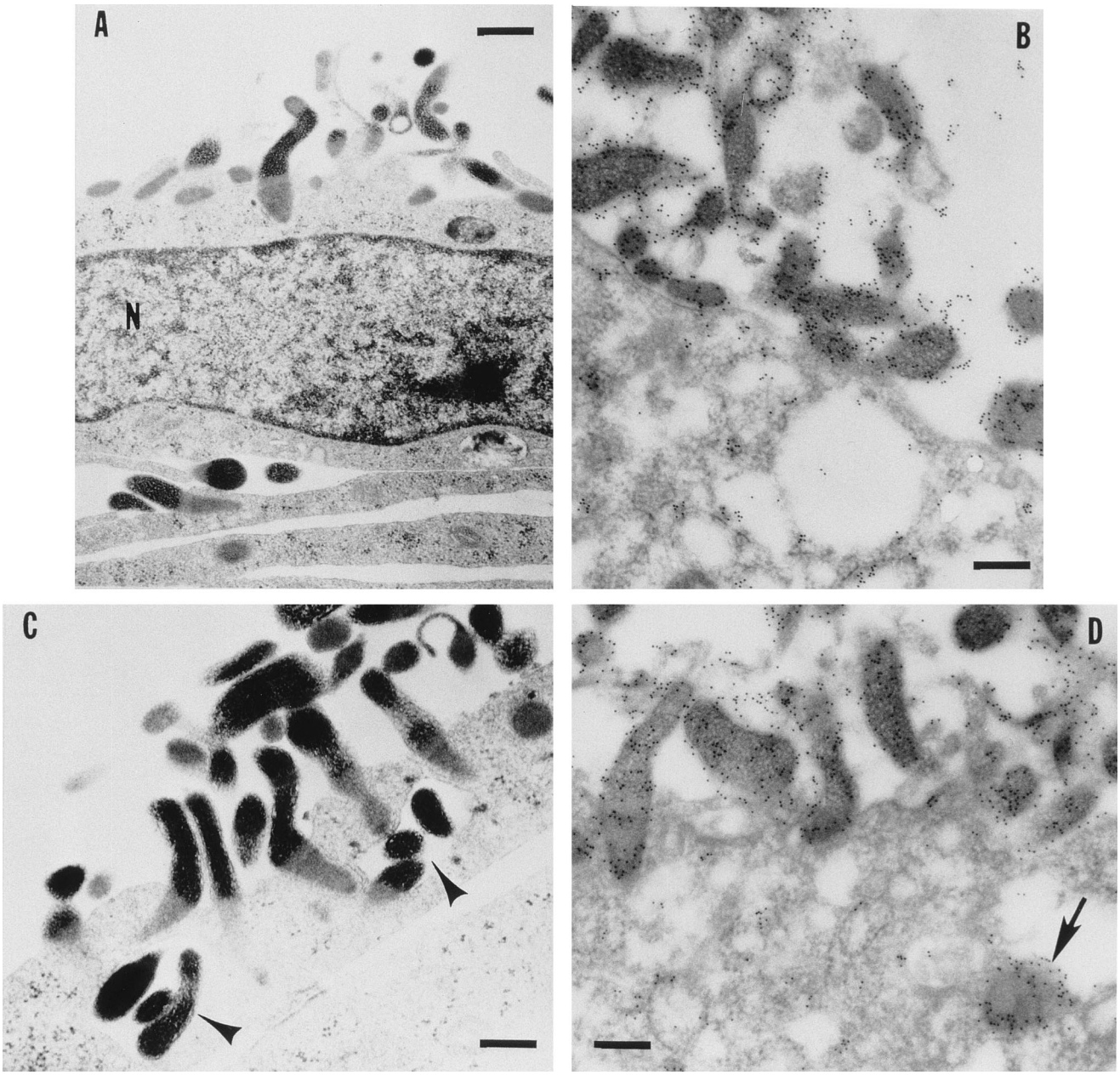

FIG. 4. Transmission electron micrographs of thin sections of HEp-2 cells infected with *M. penetrans*. After 6 h of infection, cells were fixed, sectioned, and negatively stained (A and C) or immunogold labeled with anti-*M. penetrans* antiserum (B and D). Mycoplasmas are seen attached to the cell surface (A to D), and some individual mycoplasmas are seen penetrating the cell membrane (A, C, and D). In some instances intracellular mycoplasmas are surrounded in vacuoles (C, arrowheads). Bars: A, 0.5 μm; B, 0.25 μm; C, 0.3 μm; D, 0.25 μm.

Interaction of *M. penetrans* with fibronectin and identification of a fibronectin-binding protein. We examined the binding properties of *M. penetrans* to extracellular matrix glycoproteins, namely, collagen, fibronectin, laminin, and vitronectin. Among these basement membrane proteins studied, only fibronectin served as a substrate for *M. penetrans* (Fig. 6A). This dose-effect interaction was readily confirmed by an immunoassay with anti-*M. penetrans* antiserum (data not shown). This finding suggests that the recognition of fibronectin by *M. penetrans* in certain biological niches may be relevant for survival and colonization of host tissues.

To begin the characterization of the fibronectin domain recognized by *M. penetrans*, we examined different compounds in the binding assay, including a 70-kDa fibronectin fragment containing the gelatin-heparin-binding domain, an RGD tripeptide, gelatin, laminin, different collagens, vitronectin, and heparan sulfate (30, 33). None of these molecules inhibited the interaction between *M. penetrans* and fibronectin under these experimental conditions, suggesting the existence of an alternate binding site for *M. penetrans* on the fibronectin molecule.

To identify the fibronectin-binding protein (FnBP) of *M. penetrans*, the BDSP preparation obtained from biotinylated intact mycoplasmas was subsequently electrophoresed, transferred to nitrocellulose membranes, and reacted with human

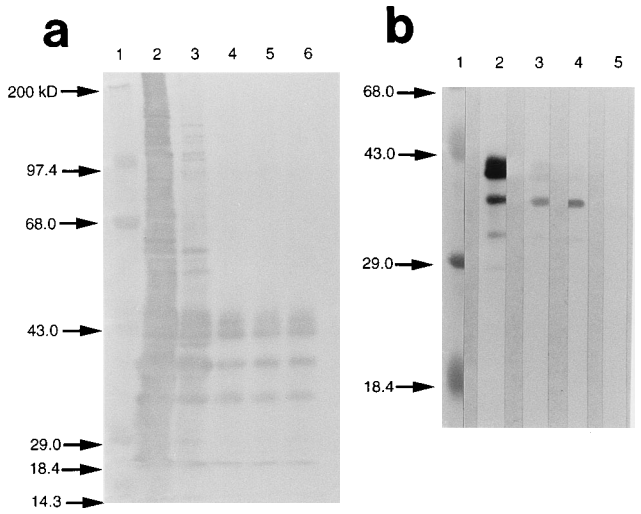

FIG. 5. (A) Profile of biotinylated *M. penetrans* proteins eluted from glutaraldehyde-fixed cultured human cells. Lanes: 1, prestained molecular mass standards; 2, whole-cell lysate of biotinylated mycoplasmas; 3, biotinylated, detergent-soluble mycoplasma proteins; 4, 5, and 6, biotinylated proteins eluted from fixed HEp-2, WI38-VA13, and NCM/SM cells, respectively. Six proteins with apparent molecular masses of 18, 28, 32, 36, 39, and 41 kDa were eluted from the three human cell lines. (B) Immunoblot of eluted mycoplasma proteins with sera from human AIDS patients. Lanes: 1, molecular mass standard markers; 2, biotinylated proteins eluted from glutaraldehyde-fixed HEp-2 cells; 3 and 4, eluted mycoplasma proteins reacted with sera from individual AIDS patients; 5, eluted mycoplasma proteins reacted with pooled normal human sera.

fibronectin (1 μg/ml). After being washed, the membrane was reacted with anti-human fibronectin MAb diluted 1:1,000 and species-specific alkaline phosphatase conjugate. A 65-kDa FnBP was identified by this approach (Fig. 6B). By using a different experimental design, whole-cell extracts of ^{35}S-biosynthetically labeled mycoplasmas were passed through a Sepharose-fibronectin affinity column and a highly enriched FnBP of ca. 65 kDa was eluted. Several other mycoplasma proteins exhibited varying degrees of fibronectin binding (data not shown).

Cytoskeletal rearrangements induced by *M. penetrans* interaction with host cells. The recruitment of microtubule and microfilament proteins following adherence to the cell surface is characteristic of several pathogenic invasive bacteria (11a, 13, 36, 38, 40, 48). Similarly, the binding of *M. penetrans* to HEp-2 cells triggers a signal that induces cytoskeletal changes revealed by the aggregation of tubulin at the sites of bacterial clustering, as demonstrated by immunofluorescence and confocal microscopy (Fig. 7). In addition, the aggregation of α-actinin and the accumulation of phosphorylated proteins were observed by using MAbs reactive against α-actinin and phosphotyrosine (data not shown). These rearrangements appeared to be time dependent, since aggregation was more apparent after 6 h of infection. It is possible that this reorganization of the mammalian cell cytoskeleton promotes mycoplasma uptake. None of the MAbs reacted directly with *M. penetrans* on the basis of dot blots with whole mycoplasmas or SDS-PAGE immunoblots of total mycoplasma proteins.

DISCUSSION

The interaction between *M. penetrans* and the host target cell is characterized by mycoplasma attachment to the mammalian cell surface in a highly oriented, tip-mediated process which leads to surface parasitism and intracellular penetration (4, 26, 27, 41). However, the molecular basis of the pathogenic scheme of *M. penetrans* remains largely unknown. In this study, the dynamics of *M. penetrans* adherence to several cell lines was investigated. Independent of the cell line used, this organism adheres and forms large aggregates that localize to host cell surfaces. These adhering microcolonies were readily visualized by Giemsa staining, and the identity of mycoplasmas within the clusters was confirmed by immunofluorescence and scanning electron microscopy. The formation and the size of bacterial clusters were time-dependent events, which were inhibited only by anti-*M. penetrans* antibodies. Surprisingly, drugs that affect microtubule and microfilament function (37), did not show any apparent inhibitory effect under the experimental conditions used. Among several putative inhibitors tested, only metaperiodate and trypsin treatment reduced adherence, suggesting the involvement of carbohydrate and protein moieties in this interaction.

Ultrastructural studies of HEp-2 cells infected with mycoplasmas revealed that as early as 2 h postinfection, the organisms were invaginated in the cell membrane or internalized in the cytoplasm, free or inside vesicles. Within 24 h postinfection, the cells underwent obvious cytopathic changes, resulting

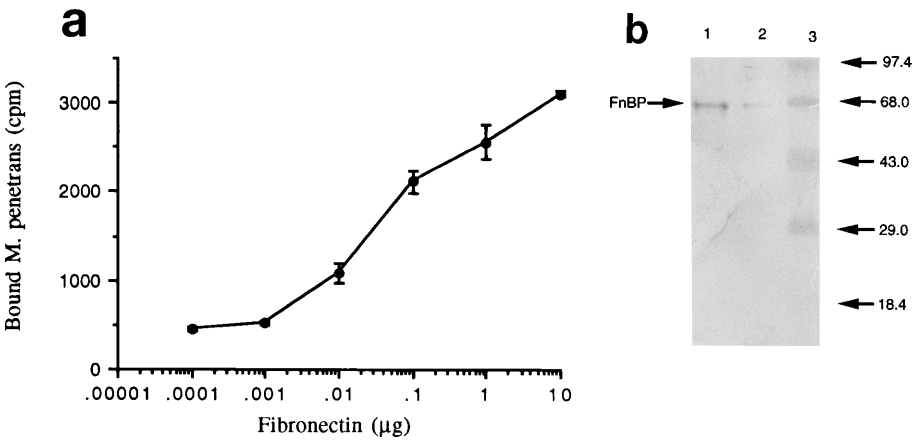

FIG. 6. (A) Binding of ^{35}S-labeled *M. penetrans* to immobilized fibronectin. Values are means ± standard errors of the means for four samples. (B) Identification of a ca. 65-kDa mycoplasma FnBP by immunoblotting with mouse anti-fibronectin MAb. Mycoplasma proteins were separated by electrophoresis, transferred to nitrocellulose, and reacted with human fibronectin prior to the addition of anti-fibronectin MAb (see Results). Lanes: 1, total detergent-soluble *M. penetrans* proteins; 2, mycoplasma proteins eluted from fixed HEp-2; 3, molecular mass standard markers.

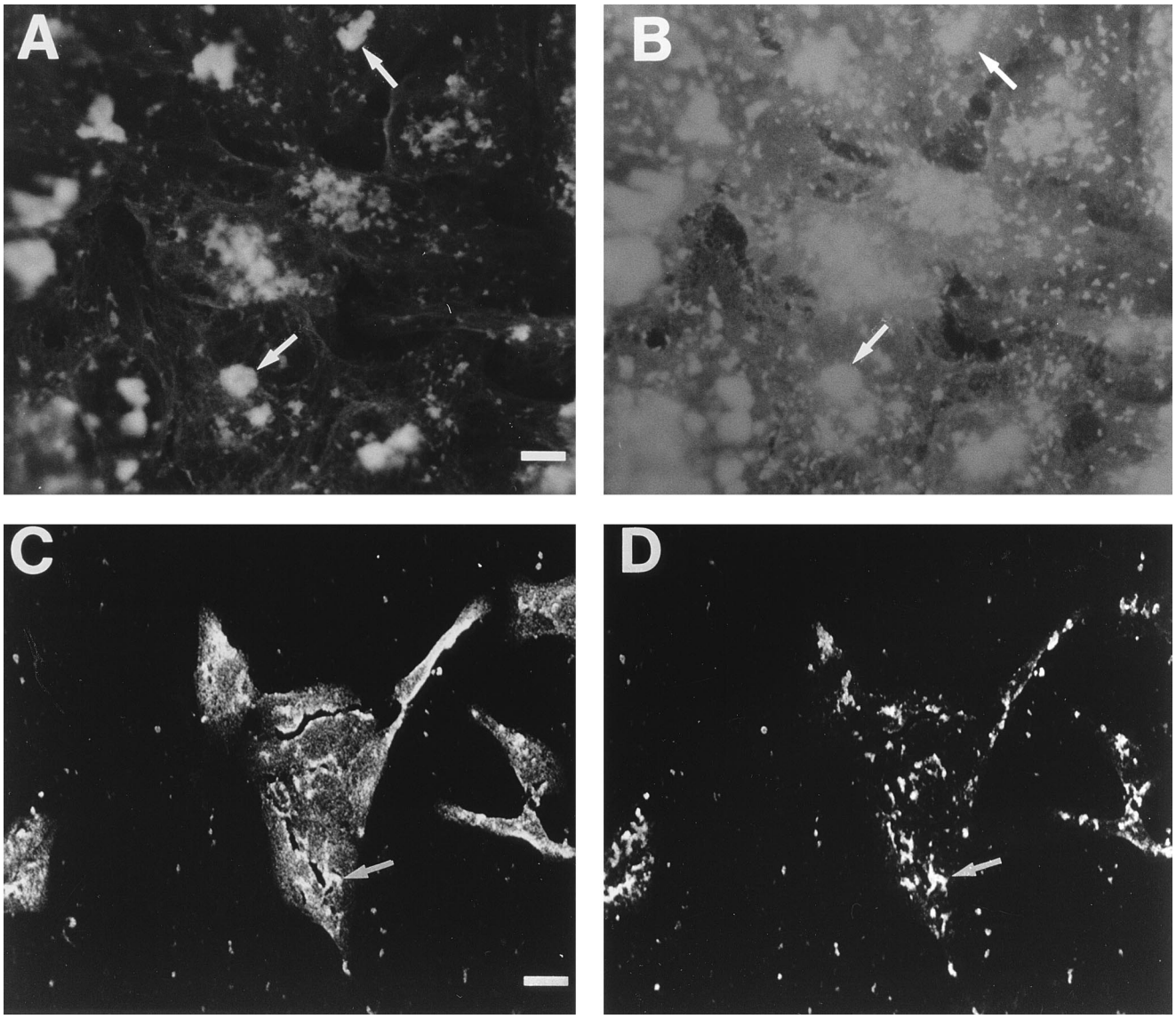

FIG. 7. Double exposure of indirect immunofluorescence and confocal micrographs of HEp-2 cells after 24 h of infection with *M. penetrans* (labeled with rhodamine). (A) Anti-tubulin MAb and anti-mouse FITC conjugate were used to stain tubulin (arrows point at aggregated tubulin). (B) Same field as panel A, except that mycoplasmas were labeled with anti-*M. penetrans* antibody and anti-rabbit rhodamine conjugate (arrows). The sites of large accumulations of tubulin correspond to the locations of mycoplasma clusters (arrows). (C) Confocal micrograph showing tubulin accumulation detected with anti-tubulin MAb and FITC (arrow). (D) Same field as panel C, except that the location of bacterial aggregates (arrow) is detected with anti-*M. penetrans* antibody labeled with rhodamine.

in destruction of the cell monolayer. In contrast, cytopathic effects on fibroblastic, epithelial, endothelial, and lymphocytic mammalian cells were previously reported to occur within 2 to 5 days (26, 27). These discrepancies could be explained by the type of cell line used, the infecting dose of mycoplasmas, or differences in the assays performed.

Morphologically, *M. penetrans* was reported as an elongated, flask-shaped organism characterized by the presence of two distinct compartments (26–28). Densely packed fine granules are contained in the narrow end of the cell, whereas loosely packed coarse granules, consistent with bacterial ribosomal structures, are localized to the broader body (27, 28). Our electron-microscopic observations are consistent with this morphological description. However, we also found that *M. penetrans* is a polymorphic organism which is able to form long filamentous and branching structures resembling hyphae, a morphological feature reminiscent of the hyphal growth of *Candida albicans* (47). Polar knob-like structures have been described for *M. penetrans*, and a role in mycoplasma replication was suggested (34, 35). Furthermore, the presence of blebs located at the tips of the organisms was noted in our negative-stain preparations of *M. penetrans*. The nature and function, if any, of this bleb formation in the life cycle or pathogenesis of this organism have yet to be determined. Similar polar bleb formation has been reported during apical growth in *C. albicans* (1). Interestingly, when the course of infection was monitored by scanning electron microscopy, the adhering organisms appeared to undergo obvious morphological changes. For example, at early stages of infection, the adhering mycoplasmas exhibited a typical elongated morphology, but as the infection proceeded, the mycoplasmas rounded up to spherical forms. The analysis of the basic mechanism of morphogenesis

and the relationship between the polymorphism of *M. penetrans* and infection require further investigation.

LAMPs of 35, 38, 61, and 103 kDa extracted from *M. penetrans* were previously reported to be immunoreactive with antibodies from humans serologically positive for HIV but not with antibodies from HIV-negative individuals (43). However, the function of these proteins was not explored (43). Toward this end, we extracted surface proteins from mycoplasmas and reacted them with glutaraldehyde-fixed HEp-2 cells (19, 23). Among six adhesive proteins eluted from these cells, three polypeptides which migrated with apparent masses of 36, 39, and 41 kDa reacted with different intensities with HIV-positive sera but not with human normal serum (Fig. 5). These "adhesive" polypeptides may correspond to the LAMPs previously described (44).

The interaction of basement membrane glycoproteins such as fibronectin, collagen, vitronectin, and laminin with different bacterial, parasitic, and viral pathogens and the implication of this relationship in colonization and disease have been described previously (2, 5, 6, 8, 16, 30, 40, 46, 48). These interactions serve as models to study putative adhesins and receptor-containing molecules within the context of bacterium-host interplay (6, 17, 19, 30). Among these matrix glycoproteins, *M. penetrans* selectively bound to immobilized fibronectin in a dose-dependent fashion, suggesting a role of this interaction in mycoplasma survival and colonization of human tissues. To clarify the interaction of fibronectin with *M. penetrans*, different molecules were examined for inhibition. A number of compounds were tested, including extracellular matrix glycoproteins, a 70-kDa fibronectin fragment containing the gelatin-heparin-binding domain, gelatin, and an RGD tripeptide, and none was inhibitory under the experimental conditions used. These data suggest the existence of a selective binding site on the fibronectin molecule for *M. penetrans*. We identified a FnBP of 65 kDa in whole-cell extracts of mycoplasmas that bound selectively to soluble fibronectin or to immobilized fibronectin on a Sepharose column. The genetic determinant(s) and role of this protein in the interaction of *M. penetrans* with the host cell are currently under study.

The recruitment of proteins associated with microtubule and microfilament function following adherence to the cell surface is a feature of several pathogenic invasive bacteria (7, 11a, 13, 36, 40, 48). Similarly, adherence of *M. penetrans* to cultured human cells triggers a signal that promotes cytoskeletal changes, namely, aggregation of tubulin and α-actinin and condensation of phosphorylated proteins. Other cytoskeletal components such as talin, tropomyosin, and vinculin, as well as surface receptors like CD44, β_2-microglobulin, and epidermal growth factor receptor (11a), did not appear to accumulate at the sites of bacterial clustering, suggesting that *M. penetrans* selectively utilizes signals to induce specific cytoskeletal rearrangements.

The pathogenic scheme of many adherent and invasive organisms is comprised of a multifactorial scenario (12, 31, 39). Thus, it is possible that *M. penetrans* utilizes different surface components to adhere to and penetrate mammalian cells (3, 23). This hypothesis is supported by the fact that hemadsorption and cytadherence are two independent virulence properties of mycoplasmas (23). *M. penetrans* adheres to and penetrates mammalian cells, binds fibronectin, and triggers reorganization of cytoskeletal proteins. The significance of these properties within the context of the interaction of *M. penetrans* and the host target cell in natural infections in humans is an important question. Our findings open new avenues to study and understand the pathogenic strategies of this unique microorganism.

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