INFECTION AND IMMUNITY, Feb. 1993, p. 663–670
0019-9567/93/020663-08$02.00/0

Vol. 61, No. 2

Mycobacterium avium-M. intracellulare Binds to the Integrin Receptor $\alpha_V\beta_3$ on Human Monocytes and Monocyte-Derived Macrophages

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Received 5 October 1992/Accepted 18 November 1992

Mycobacterium avium-M. intracellulare **is an intracellular pathogen responsible for the highest incidence of disseminated bacterial infection in patients with AIDS. Treatment of the infection is difficult and has been of limited efficacy. Attachment of the organism to macrophages is a critical early step in the establishment of the disease. In the present study, we isolated and identified a receptor that mediates attachment of *M. avium-M. intracellulare* to human peripheral blood monocytes and monocyte-derived macrophages. On Western blotting, (immunoblotting), the receptor was found to cross-react with antibodies against a human vitronectin receptor ($\alpha_V\beta_3$). The receptor could be purified from monocyte extracts by using monoclonal antibodies (MAbs) against the α_V subunit of vitronectin receptor coupled to CNBr-Sepharose 4B, as well as with the adhesive tripeptide sequence arginine-glycine-aspartic acid (RGD) coupled to CNBr-Sepharose 4B. Surface-bound MAbs directed against $\alpha_V\beta_3$ were found to inhibit the attachment of *M. avium-M. intracellulare* to monocyte-derived macrophages in an in vitro inhibition assay, while MAbs directed against CD14, CD18, $\alpha_2\beta_1$ and platelet glycoprotein gpIIb/IIIa receptors did not inhibit this attachment. These observations suggest that $\alpha_V\beta_3$ on the surface of human monocytes and monocyte-derived macrophages may function as a receptor for *M. avium-M. intracellulare*. Identification of a receptor for *M. avium-M. intracellulare* on macrophages may offer new approaches to the prevention and control of *M. avium-M. intracellulare* infection at the cellular level.**

Mycobacterium avium-M. intracellulare is the most common cause of disseminated bacterial infection in patients with AIDS (2, 37, 42). In normal or nonimmunocompromised hosts, disease due to this organism is rare, and clinical manifestations, if any, are primarily pulmonary (21). *M. avium-M. intracellulare* is commonly found in fresh water, soil, and air (4). The actual mode of entry of the organism into the host is usually not known, although it is thought to be acquired by ingestion or inhalation because of its ubiquitous distribution in the environment (15). Treatment of *M. avium-M. intracellulare* infection is difficult in patients with normal immune responses and even more difficult in patients with human immunodeficiency virus-induced immunosuppression. This organism has been found to be resistant to many conventional antituberculous agents (2). However, recent clinical trials with amikacin-containing regimens (13) and particularly regimens that contain macrolides (16) offer new hope.

M. avium-M. intracellulare is an intracellular pathogen that proliferates predominantly in the macrophage (14, 41). Adhesion of pathogens to the host cell surface is the first and most critical step in the initiation of infection and is, in fact, a prerequisite for intracellular pathogens to gain entry into the cell. In addition, the macrophage is an important effector cell in host response to intracellular infections. Elimination of pathogens by normal clearance processes involves adhesion of pathogens to macrophages followed by phagocytosis with concomitant removal from circulation. The role of complement in the interaction of *M. avium-M. intracellulare* with human macrophages has been studied (35), and the receptors involved have been identified (5). However, monocytes, macrophages, and stimulated polymorphonuclear cells (but not lymphocytes or unstimulated polymorphonuclear cells) have also been shown to bind *M. avium-M. intracellulare* in the absence of complement, serum, or serum proteins. This binding was shown to be mediated by a proteinaceous receptor on macrophages (8). Here we report that the integrin receptor $\alpha_V\beta_3$ on monocytes and monocyte-derived macrophages (MDM) which is also known as vitronectin receptor (20, 28, 31), binds to *M. avium-M. intracellulare* in the absence of complement.

MATERIALS AND METHODS

Growth and labeling of *M. avium-M. intracellulare*. *M. avium-M. intracellulare* obtained from the American Type Culture Collection (ATCC 25291, Rockville, Md.) was cultured in Middlebrook 7H11 broth (Difco Laboratories, Detroit, Mich.) at 37°C in 5% CO_2 for 7 to 14 days with vigorous agitation once a day. Bacteria were harvested by centrifuging the medium at 800 × *g* for 15 min. In order to label the bacteria, the pellet was resuspended in a 1-mg/ml solution of fluorescein isothiocyanate (FITC) (Sigma Chemical Co., St. Louis, Mo.) in 50 mM Na_2CO_3–100 mM NaCl (pH 9.2) and incubated in the dark for 30 min at room temperature. The suspension was then washed two times with phosphate-buffered saline (PBS) containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (Dulbecco's PBS or DPBS) and resuspended in the same buffer. The final suspension was sonicated for 4 s, and the optical density at 600 nm determined.

Isolation of monocytes and preparation of monocyte extracts. Monocytes were isolated by sequential Ficoll-Hypaque and Percoll gradient centrifugation of human buffy coats obtained from the San Diego Blood Bank (40). Mono-

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cytes thus isolated were found to show less than 10% contamination with platelets and 4 to 5% contamination with lymphocytes. For experiments requiring MDM, the isolated monocytes were cultured in RPMI 1640 medium supplemented with 10% normal human serum, 2 mM L-glutamine, and 50 U of penicillin per ml for 4 to 10 days in Teflon beakers. Detergent extracts of freshly isolated monocytes as well as MDM were prepared in 50 mM Tris-HCl buffer (pH 7.0) containing 100 mM octyl-β-D-glucopyranoside (OG) (Calbiochem, La Jolla, Calif.), 1 mM phenylmethylsulfonyl fluoride, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ (extraction buffer), using an end-over rotator for 1 h at 4°C. The extract was centrifuged, and the supernatant, termed as crude monocyte extract (CME), was used for the isolation of receptors that bind *M. avium-M. intracellulare*. CME was also used to raise polyclonal antibodies in rabbits.

Isolation of a receptor for *M. avium-M. intracellulare* from CME. Freshly harvested *M. avium-M. intracellulare* from 1 liter of 7- to 8-day-old cultures (2×10^8 bacteria per ml) were washed five times with DPBS and incubated with 1 ml of CME (1 mg of protein per ml) for 1 h at room temperature on an end-over rotator. The suspension was centrifuged, and the unbound monocyte extract was collected. The bacterial pellet was washed three times with DPBS, and the bound protein(s) was eluted from *M. avium-M. intracellulare* by suspending the bacterial pellet in 1 ml of extraction buffer containing 20 mM EDTA and 25 mM OG (instead of 100 mM OG). The suspension was rotated for 1 h at room temperature and then centrifuged. The 20 mM EDTA eluate was collected, and the bacterial pellet was further eluted with 1 ml of 8 M urea in extraction buffer. The 20 mM EDTA and 8 M urea eluates were assayed for *M. avium-M. intracellulare*-binding activity in the adherence assay and analyzed by immunoblotting by using antibodies against CME.

SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting (immunoblotting). Samples were electrophoresed on sodium dodecyl sulfate (SDS)-12 or 10% polyacrylamide gels under reduced or nonreduced conditions, respectively, and electrophoretically transferred to nitrocellulose filters by using the Tris-glycine buffer system (pH 8.3). The filters were blocked in a 10% solution of milk proteins in 1× Tris-buffered saline (TBS; 136 mM NaCl, 5 mM KCl, 25 mM Tris) and then incubated with the appropriate antibody solution. The bound antibodies were detected with alkaline phosphatase-conjugated goat antibodies to rabbit immunoglobulin G (IgG) and developed with the 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium (BCIP/NBT) Phosphatase Substrate System (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.).

Adherence assay. The ability of monocyte and MDM extracts to bind *M. avium-M. intracellulare* was assayed as follows. Monocyte extracts were applied on nitrocellulose filters by using a dot-blot apparatus (Bio Dot apparatus; Bio-Rad Laboratories, Richmond, Calif.). The filters were blocked in a 10% solution of milk proteins in DPBS for 1 h at room temperature, washed three times with DPBS, and exposed to FITC-labeled bacteria (1.5 to 2.0 OD_{600}) for 90 min at 37°C. The filters were then washed three times with DPBS, fixed in 3% paraformaldehyde, and observed under UV light. Although good for the determination of the *M. avium-M. intracellulare*-binding ability of a sample, this assay, however, was not found to be sensitive enough to show small differences in the *M. avium-M. intracellulare*-binding ability of two samples.

Biotinylation of surface proteins on monocytes. Freshly isolated intact monocytes were biotinylated by the protocol described by Lisanti and coworkers (24). Briefly, 1.7×10^8 monocytes were suspended in 5 ml of a 1.5-mg/ml solution of *N*-hydrosuccinimide ester biotin (Pierce Chemical Co., Rockford, Ill.) in 1× TBS (pH 8.6) and gently rotated for 45 min at 4°C. At the end of the incubation, the cells were centrifuged at $350 \times g$ and washed seven times with 1× TBS (pH 7.4) to ensure complete removal of unbound biotin. Extracts of the biotinylated monocytes were prepared as described above and used to isolate *M. avium-M. intracellulare*-binding receptor. Biotinylated CME as well as the 20 mM EDTA fraction containing *M. avium-M. intracellulare* binding receptor were analyzed by avidin blots performed as follows. Samples were electrophoresed on SDS-10% polyacrylamide gels under nonreduced conditions, transferred to nitrocellulose filters, and blocked as described above. The filters were incubated with a 1:1,000 dilution of alkaline phosphatase-conjugated avidin (Calbiochem) for 20 min. The biotinylated proteins were then detected by using the BCIP/NBT Phosphatase Substrate System.

Purification of *M. avium-M. intracellulare*-binding receptor by affinity chromatography with Gly-Arg-Gly-Asp-Ser-Pro-Lys (GRGDSPK)-Sepharose and MAb α_V-Sepharose. GRDG SPK coupled to CNBr-Sepharose 4B (10 mg/ml of gel) and monoclonal antibody (MAb) against the α_V subunit of integrin $\alpha_V\beta_3$ coupled to CNBr-Sepharose 4B were kindly provided by Michael D. Pierschbacher (Cancer Research Center, La Jolla Cancer Research Foundation, La Jolla, Calif.). GRGDSPK and MAb directed against α_V were products of Telios Pharmaceutical, Inc., San Diego, Calif.

For chromatography on GRGDSPK-CNBr-Sepharose 4B, monocyte extracts were initially prepared by using extraction buffer without $CaCl_2$ and $MgCl_2$, both of which were subsequently added to the sample at final concentrations of 1 mM before loading on the column. A 1-ml sample of CME (1 mg of protein per ml) was applied to the column (2 ml) and gently rotated for 2 h at room temperature. The column was washed with 8 ml of 1× TBS containing 25 mM OG and 1 mM each phenylmethylsulfonyl fluoride, $CaCl_2$, and $MgCl_2$. Elution was done with 8 ml of the wash buffer containing 20 mM EDTA, and the fractions were analyzed by Western blotting with antibodies against $\alpha_V\beta_3$ and against CME. In addition, the fractions were also analyzed in the adherence assay. As controls, CME was incubated with Sepharose coupled to the GD6 peptide (KQNCLSSRASFRGCVRN LRLSR) of the A chain of laminin (18) as well as with uncoupled CNBr-Sepharose 4B. The fractions collected from these columns were analyzed as described above for the fractions collected from the GRGDSPK-Sepharose column.

A 3-ml sample of CME (1 mg of protein per ml) was applied to the MAb α_V-CNBr-Sepharose 4B column (3 ml). The flowthrough was passed over the column five times, and the column was ultimately washed with 30 ml of 1× TBS containing 100 mM OG and 1 mM each phenylmethylsulfonyl fluoride, $CaCl_2$, and $MgCl_2$. Fractions were eluted with 12 ml of the wash buffer containing 8 M urea, 50 mM Tris, and 50 mM OG, dialyzed immediately against wash buffer, and analyzed by Western blotting with anti-$\alpha_V\beta_3$ and anti-CME, as well as in the adherence assay. As controls, CME was incubated with CNBr-Sepharose coupled to purified IgG against bovine serum albumin (BSA), and with uncoupled CNBr-Sepharose 4B. Samples from the control columns were also analyzed in a similar manner.

Antibodies. Rabbit antibodies against human vibronectin receptor ($\alpha_V\beta_3$) (34) and human fibronectin receptor (FnR, $\alpha_5\beta_1$) (7) were obtained from Telios Pharmaceutical, Inc.

Antibodies against human laminin receptor (LmR, $\alpha_3\beta_1$) were obtained as a gift from Kurt R. Gehlsen (17) (California Institute of Biological Research, La Jolla). Rabbit antibodies against CME (anti-CME) were prepared in our laboratory and showed no cross-reactivity with extracts of *M. avium-M. intracellulare* on Western blotting. Two MAbs against different epitopes of the platelet glycoprotein gpIIb/IIIa (38) and MAb against $\alpha_2\beta_1$ (27) were kindly provided by Virgil L. Woods, Jr. (UCSD Medical Center, San Diego, Calif.). MAbs IB4 directed against CD18 (39) and LM609 directed against $\alpha_V\beta_3$ (12) were obtained as generous gifts from Samuel D. Wright (The Rockefeller University, New York, N.Y.) and David A. Cheresh (Scripps Clinic and Research Foundation, La Jolla, Calif.), respectively. MAb against human CD14 and mouse IgG were from Sigma Chemical Co. All MAbs used were purified IgGs.

Inhibition of attachment of *M. avium-M. intracellulare* to MDM. Inhibitors used in this assay were LM609, IB4, mannan (Sigma Chemical Co.), and mannosylated-BSA (E-Y Laboratories, San Mateo, Calif.). A suspension (5 μl) of MDM (10^6 cells per ml) in HAP (DPBS containing 3 mM glucose, 0.5 mg of human serum albumin (HSA) [Armor Pharmaceuticals, Kankakee, Ill.] per ml, and 0.3 U of aprotinin [Sigma Chemical Co.] per ml) was added to each well of a Terasaki plate. This was followed by the addition of another 10 μl of HAP. The plates were incubated at 37°C for 1 h and washed two times with DPBS. The inhibitor (5 μl) (or DPBS for the control) was then added to each well and incubated at 37°C for 1 h. To this, 5 μl of *M. avium-M. intracellulare* suspension containing FITC-labeled and unlabeled bacteria in the ratio of 1:7 was added. The plate was centrifuged at 200 × *g* for 5 min and further incubated at 37°C for 5 min. At the end of the incubation period, the plate was washed five times with DPBS, fixed with 2% glutaraldehyde for 3 min, and then washed two times. The attachment of *M. avium-M. intracellulare* to MDM was scored by fluorescence microscopy. MDM in a field were identified and counted, and the number of MDM with fluorescent *M. avium-M. intracellulare* was determined. The number of *M. avium-M. intracellulare* attached was calculated in terms of attachment index (AI), defined as the number of *M. avium-M. intracellulare* (labeled and unlabeled) that were bound per hundred macrophages (8). Since each macrophage binds more than 30 bacteria, enumeration was difficult. To facilitate accurate enumeration, a mixture of labeled and unlabeled *M. avium-M. intracellulare* was used. The AI determined for fluorescently labeled *M. avium-M. intracellulare* was then multiplied by the ratio of labeled to unlabeled bacteria to yield the true AI. The results of this experiment were expressed as a percent inhibition of the attachment observed for MDM treated with DPBS instead of the inhibitor.

Indirect inhibition of attachment of *M. avium-M. intracellulare* to MDM by MAbs against $\alpha_V\beta_3$. MDM were harvested from Teflon beakers after 4 to 7 days, washed, and suspended at 0.5 × 10^6 to 1.0 × 10^6/ml in HAP. A 5-μl sample of the macrophage solution was added to each well of a Terasaki plate (Miles Laboratories, Inc., Naperville, Ill.) previously coated for 2 h at room temperature with DPBS, normal mouse IgG, or the indicated MAbs and incubated for 1 h at 37°C. The plate was washed two times with DPBS. A 5-μl sample of *M. avium-M. intracellulare* suspension containing FITC-labeled and unlabeled bacteria in a ratio of 1:5 was added to each well, centrifuged at 200 × *g* for 5 min, and incubated at 37°C for 5 min. The plate was then washed and fixed as described above. The AI was calculated, and the results were expressed as a percent inhibition of the attachment observed in wells that were coated with DPBS instead of the MAb.

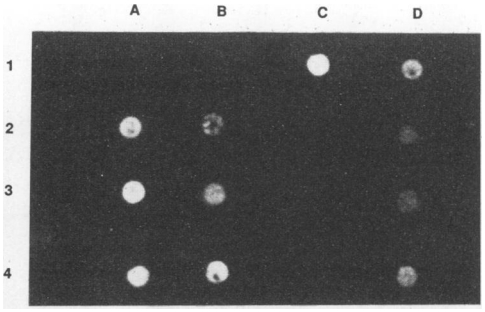

FIG. 1. *M. avium-M. intracellulare*-binding activity of monocyte extracts and *M. avium-M. intracellulare*-binding receptor isolated by different methods. A1, normal human serum (20 μg); B1, fibronectin (20 μg); C1, THP-1 extract (25 μg); D1, $\alpha_V\beta_3$ from placenta (25 μg); A2, CME (25 μg); B2, depleted monocyte extract after incubation with *M. avium-M. intracellulare* (25 μg); C2, last buffer wash of *M. avium-M. intracellulare*; D2, 20 mM EDTA eluate of *M. avium-M. intracellulare* (25 μg); A3: CME (25 μg); B3, depleted monocyte extract after incubation with GRGDSPK-CNBr-Sepharose 4B (25 μg); C3, buffer wash of GRGDSPK-CNBr-Sepharose 4B column; D3, 20 mM EDTA eluate of the GRGDSPK-CNBr-Sepharose 4B column (25 μg); A4, CME (25 μg); B4, depleted monocyte extract after incubation with MAb α_V-CNBr-Sepharose 4B (25 μg); C4, buffer wash of the MAb α_V-CNBr-Sepharose 4B column; D4, 8 M urea eluate of MAb α_V-CNBr-Sepharose 4B (25 μg).

In another set of experiments performed under similar conditions, a sonicate of *M. avium-M. intracellulare* prepared by the protocol of Lamb and coworkers (22) was used as the surface-bound ligand. A sonicate of *Escherichia coli* was identically prepared, and HSA were used as controls.

RESULTS

***M. avium-M. intracellulare*-binding receptor isolated from CME is active in adherence assay.** Adherence to FITC-labeled *M. avium-M. intracellulare* as described in Materials and Methods was used to identify *M. avium-M. intracellulare*-binding receptor. CME was incubated with *M. avium-M. intracellulare* as described above, and the unbound monocyte extract as well as the EDTA- and urea-eluted fractions containing *M. avium-M. intracellulare*-bound protein(s) were analyzed in the adherence assay (Fig. 1). The FITC-labeled *M. avium-M. intracellulare* bound specifically to monocyte extracts (Fig. 1, A2) but not to normal human serum (Fig. 1, A1) or fibronectin (Fig. 1, B1). A detergent extract of erythrocytes did not bind labeled *M. avium-M. intracellulare* (data not shown). On the other hand, an extract of THP-1 cells, which is a macrophagelike cell line, was found to bind the labeled bacteria (Fig. 1, C1). The monocyte extract depleted of receptor for *M. avium-M. intracellulare* by incubation with *M. avium-M. intracellulare* showed significant decrease in activity (Fig. 1, B2) compared to CME (Fig. 1, A2). While the DPBS wash (third) of the bacteria showed no binding to labeled *M. avium-M. intracellulare* (Fig. 1, C2), the EDTA-eluted fraction bound *M. avium-M. intracellulare* (Fig. 1, D2). The 8 M urea eluate showed no activity (data not shown), indicating that all *M. avium-M. intracellulare*-bound protein was eluted with 20 mM EDTA.

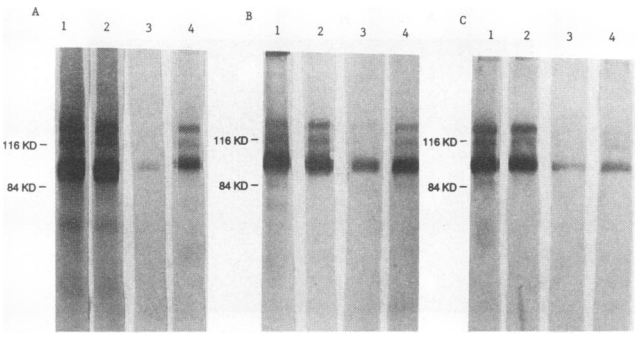

FIG. 2. Western blot analysis of CME and *M. avium-M. intracellulare*-binding receptor isolated based on its affinity for *M. avium-M. intracellulare* (A), GRGDSPK-CNBr-Sepharose 4B (B), and MAb α_V-CNBr-Sepharose (C). Lanes: 1, CME; 2, depleted monocyte extract; 3, last fraction of the buffer wash of *M. avium-M. intracellulare* (in panel A) or of the affinity columns (panels B and C); 4, 20 mM EDTA-eluted fraction (in panels A and B) and 8 M urea-eluted fraction (in panel C). The antibody used was anti-CME (1:500 dilution). KD, kilodaltons.

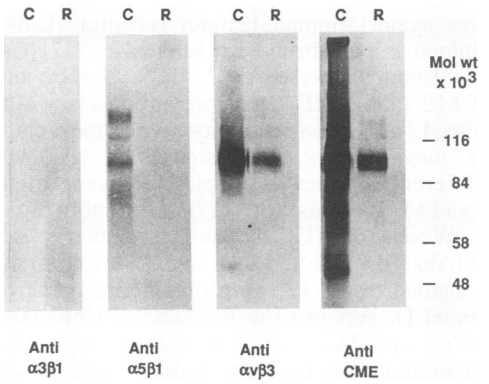

FIG. 3. Western blot analysis of CME and *M. avium-M. intracellulare*-binding receptor with antisera against different integrin receptors. CME (lanes C) and *M. avium-M. intracellulare*-binding receptor (lanes R) isolated from CME based on its affinity for *M. avium-M. intracellulare* were electrophoresed on SDS-10% polyacrylamide gels, transferred to nitrocellulose filters, and incubated with different integrin receptor antisera (1:500 dilution).

With a view to identify the proteins in CME which bind *M. avium-M. intracellulare*, CME, monocyte extract depleted of *M. avium-M. intracellulare*-binding receptor by incubation with *M. avium-M. intracellulare* and the 20 mM EDTA-eluted fraction were analyzed by Western blotting with rabbit antiserum against CME (anti-CME) (Fig. 2). Antibodies against a whole monocyte extract were used to detect all (or any) monocyte proteins that bind *M. avium-M. intracellulare*. Elution of specifically bound protein from the bacterial pellet with 20 mM EDTA yielded three proteins with molecular masses of 138, 120, and 95 kDa under nonreduced conditions (Fig. 2A, lane 4). The 8 M urea eluate showed no bands (data not shown). Extracts of MDM were also found to bind labeled *M. avium-M. intracellulare* in the adherence assay and showed identical profiles on Western blots (data not shown).

Receptor for *M. avium-M. intracellulare* cross-reacts with antibodies against $\alpha_V\beta_3$. The possible involvement of integrin receptors in the adherence of *M. avium-M. intracellulare* to monocytes was investigated since the use of integrins for invasion of host cells appears particularly prevalent among intracellular parasites of macrophages (6, 29, 36). Further, the role of these cell surface molecules in cell-cell interactions is well documented (1, 31). In the present study, CME and the fraction containing the *M. avium-M. intracellulare*-binding receptor were analyzed for the presence of integrin receptors by Western blotting with characterized antisera against different integrin receptors (Fig. 3). CME was found to contain fibronectin receptor ($\alpha_5\beta_1$) and vitronectin receptor ($\alpha_V\beta_3$), but no detectable receptor for laminin ($\alpha_3\beta_1$). The fraction containing the receptor for *M. avium-M. intracellulare*, on the other hand, was found to cross-react only with antibodies against $\alpha_V\beta_3$, but not with antisera against either $\alpha_5\beta_1$ or $\alpha_3\beta_1$. Further, anti-CME and anti-$\alpha_V\beta_3$ cross-reacted with the same bands in *M. avium-M. intracellulare*-binding receptor preparations. The α chain of $\alpha_V\beta_3$ stains much more poorly than the β chain in Western blots and is therefore not clearly visible in the blot probed with anti-$\alpha_V\beta_3$ (Fig. 3). However, when subjected to Western blotting under reduced conditions, the α chain is more clearly visible (see Fig. 5B). These observations suggest that $\alpha_V\beta_3$ on monocytes and MDM may function as a receptor for *M. avium-M. intracellulare*.

Receptor for *M. avium-M. intracellulare* is present on the surface of monocytes. Cell surface labeling with *N*-hydrosuccinimide ester biotin has been previously used to study molecules present on the surface of cells (19, 24). This procedure is well established for epithelial cells (24) and human umbilical vein endothelial cells (19). In the present study, this method was used to demonstrate that the *M. avium-M. intracellulare*-binding receptor is present on the surface of monocytes. Extracts of surface biotinylated monocytes were incubated with *M. avium-M. intracellulare*, and the bound protein was eluted with 20 mM EDTA as described above. Biotinylated CME and the EDTA-eluted fraction were subjected to SDS-PAGE, transferred to nitrocellulose, and detected with avidin-alkaline phosphatase (Fig. 4). The 20 mM EDTA-eluted material showed three bands with molecular masses of 138, 120, and 95 kDa, which correspond to the molecular mass of the *M. avium-M.*

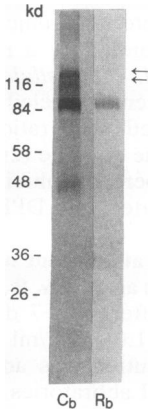

FIG. 4. Receptor for *M. avium-M. intracellulare* is present on the surface of monocytes. Biotinylated monocyte extract (C_b) was incubated with *M. avium-M. intracellulare*, and the *M. avium-M. intracellulare*-binding receptor (R_b) was eluted with 20 mM EDTA. Both samples were subjected to SDS-10% PAGE under nonreduced conditions, transferred to nitrocellulose, and detected with avidin-alkaline phosphatase. kd, kilodaltons. Arrows indicate bands corresponding to molecular masses of 138 and 120 kDa.

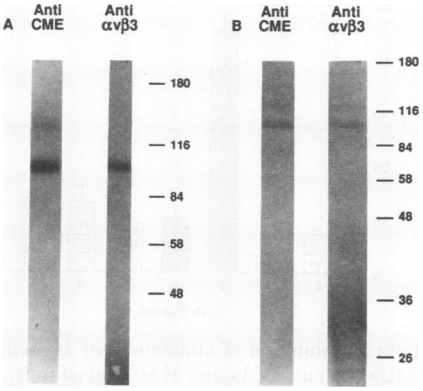

FIG. 5. Molecular weight of the receptor for *M. avium-M. intracellulare*. *M. avium-M. intracellulare*-binding receptor was electrophoresed on an SDS-10% polyacrylamide gel under nonreduced conditions (A) and on an SDS-12% polyacrylamide gel under reduced conditions (B). Western blots with anti-CME (left lanes of panels A and B) and anti-$\alpha_V\beta_3$ (right lanes of panels A and B) are illustrated. Numbers on right show molecular weight ($\times 10^3$).

intracellulare-binding receptor isolated from nonbiotinylated monocyte extracts (Fig. 2, A4). Since only surface proteins which are biotinylated are detected by this method, these observations demonstrate that *M. avium-M. intracellulare*-binding protein is present on the surface of monocytes.

Molecular mass of *M. avium-M. intracellulare*-binding receptor. The molecular mass of the protein isolated in the present study was determined by Western blotting with anti-CME and anti-$\alpha_V\beta_3$ (Fig. 5). The molecular mass under nonreduced conditions (Fig. 5A) was found to be 138 and 95 kDa. Under reduced conditions (Fig. 5B), the molecular mass was 122 and 103 kDa. The α chain of $\alpha_V\beta_3$ has previously been shown to decrease in molecular weight under reduced conditions, while the β chain shows an increase (20). Our results were consistent with the properties described for $\alpha_V\beta_3$.

Affinity purification of a receptor for *M. avium-M. intracellulare*. The nature of the protein(s) in CME that binds *M. avium-M. intracellulare* was further confirmed by using an alternate approach in which it was isolated from CME by affinity chromatography with MAb against the α_V chain of $\alpha_V\beta_3$ coupled to CNBr-Sepharose 4B. The unbound monocyte extract or flowthrough from the column showed a very slight decrease in its ability to bind FITC-labeled *M. avium-M. intracellulare* on nitrocellulose (Fig. 1, B4) compared to CME (Fig. 1, A4), although this decrease cannot be clearly seen since the assay is not designed to detect small differences in the binding of FITC-labeled *M. avium-M. intracellulare*. The last fraction of the buffer wash did not show any activity (Fig. 1, C4), while the 8 M urea eluate containing the bound protein was found to bind labeled *M. avium-M. intracellulare* (Fig. 1, D4). These results indicate that the receptor for *M. avium-M. intracellulare* binds to MAbs directed against the α_V subunit of $\alpha_V\beta_3$. As a control, CME was subjected to affinity chromatography under identical conditions using anti-BSA rabbit IgG coupled to CNBr-Sepharose 4B as well as uncoupled CNBr-Sepharose. There was no depletion of *M. avium-M. intracellulare*-binding activity in the CME after passing through these columns, and the 8 M urea eluates did not bind labeled *M. avium-M. intracellulare* in either case (data not shown). Western blot analysis of the EDTA eluates from the control columns with anti-CME and anti-$\alpha_V\beta_3$ did not show any cross-reacting bands (data not shown). These results suggest that this receptor specifically binds to MAb against $\alpha_V\beta_3$. To further confirm our findings, we analyzed $\alpha_V\beta_3$ purified from a different source (human placenta) (28) in the adherence assay and found that it also binds labeled *M. avium-M. intracellulare* (Fig. 1, D1).

Binding of integrin receptors to their ligands has often been shown to be mediated by the tripeptide sequence Arg-Gly-Asp (RGD), a sequence which is common to many extracellular proteins and which is though to play a key role in cell adhesion (30, 31). Since the receptor isolated in the present study appeared to be an integrin, we exploited this property to isolate the protein from CME. CME was incubated with GRGDSPK-CNBr-Sepharose 4B for 2 h at room temperature to achieve optimal binding, and the unbound monocyte extract was collected. The CME depleted of RGD-binding protein(s) showed a substantial decrease in its ability to bind labeled *M. avium-M. intracellulare* (Fig. 1, B3) when compared with CME (Fig. 1, A3). The column was extensively washed. The last fraction of the buffer wash showed no activity (Fig. 1, C3), while the bound protein eluted with 20 mM EDTA was found to bind labeled *M. avium-M. intracellulare* (Fig. 1, D3). As a control, CME was incubated with CNBr-Sepharose coupled to the GD6 peptide of laminin as well as with uncoupled CNBr-Sepharose 4B. The 20 mM EDTA eluate from both these columns did not bind FITC-labeled *M. avium-M. intracellulare* in the adherence assay (data not shown). In addition, Western blot analysis of the EDTA-eluted fractions with anti-CME and anti-$\alpha_V\beta_3$ did not show any bands (data not shown). Although monocyte proteins that bind *M. avium-M. intracellulare* bound to an RGD-containing peptide sequence in the present study, binding to the bacterial ligand itself may or may not be RGD mediated.

Isolation of a receptor for *M. avium-M. intracellulare* from CME based on its affinity for *M. avium-M. intracellulare*, RGD, or MAb against α_V yielded the same molecule (Fig. 2A, lane 4, B, lane 4, and C, lane 4, respectively). The double band of the α chain of the receptor may be due to isoforms of this subunit. Further, the receptor isolated by all three methods was found to bind FITC-labeled *M. avium-M. intracellulare* in the adherence assay (Fig. 1, D2, D3, and D4, respectively).

Direct inhibition of attachment of *M. avium-M. intracellulare* to MDM. MDM preincubated with increasing concentrations of LM609 was found to inhibit the attachment of *M. avium-M. intracellulare* to MDM in a dose-dependent manner (Fig. 6). Maximum inhibition of 37% was observed with 200 μg of the antibody per ml. At 400 μg/ml, there was no further inhibition of attachment. On the other hand, with MAb IB4 against the CD18 receptor, this attachment was inhibited only by 7.8% even at 400 μg/ml. The effect of mannan and mannosylated-BSA on the attachment of *M. avium-M. intracellulare* to MDM was tested at different concentrations up to 1 mg/ml. Both these compounds are known to inhibit the mannosyl-fucosyl receptor, another receptor implicated in binding to *M. avium-M. intracellulare* (5). At the lower concentrations (100 and 200 μg/ml), neither compound inhibited *M. avium-M. intracellulare*–MDM attachment. However, at concentrations of 1 mg/ml, mannan as well as mannosylated-BSA inhibited attachment up to 15%. These results suggest that $\alpha_V\beta_3$ is involved in attachment of MDM to *M. avium-M. intracellulare*, while CD18 does not appear to be involved in this attachment under similar conditions. Since intracellular pathogens are known

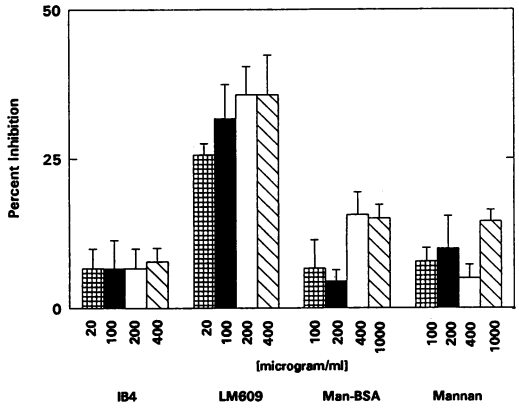

FIG. 6. Direct inhibition of attachment of *M. avium-M. intracellulare* to MDM by MAb against $\alpha_V\beta_3$. MDM were allowed to adhere to wells of a Terasaki plate for 1 h and then treated with different concentrations of IB4 (MAb to CD18), LM609 (MAb to $\alpha_V\beta_3$), mannosylated-BSA (Man-BSA), or mannan. *M. avium-M. intracellulare* was then added and incubated for 5 min. The plates were washed to remove unbound *M. avium-M. intracellulare* and fixed. The AI of *M. avium-M. intracellulare* in the presence of each inhibitor was determined, and the results are expressed as percent inhibition of the attachment observed in the presence of DPBS instead of the inhibitor. Data shown are representative of three experiments.

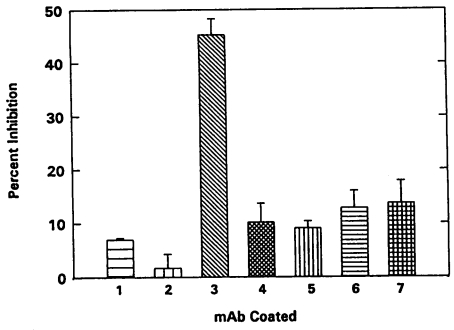

FIG. 7. Indirect inhibition of attachment of *M. avium-M. intracellulare* to MDM by surface-bound MAb against $\alpha_V\beta_3$. MDM were added to plates previously coated with different MAbs and incubated for 1 h. *M. avium-M. intracellulare* was then added to the plates and further incubated for 5 min. The plates were then washed and fixed, and the number of *M. avium-M. intracellulare* bound (AI) in each case was determined. Results are expressed as percent inhibition of the attachment obtained in wells coated with DPBS instead of the MAb. 1, mouse IgG; 2, MAb to CD14; 3, MAb to $\alpha_V\beta_3$ (LM609); 4, MAb to CD18 (IB4); 5, MAb to gpIIb/IIIa (3F5); 6, MAb to gpIIb/IIIa (2A9); 7, MAb to $\alpha_2\beta_1$ (12F1). All MAbs and normal mouse IgG were used at a concentration of 20 μg/ml. Data shown are representative of four experiments. The two MAbs against gpIIb/IIIa are directed against different epitopes.

to utilize more than one receptor on host cell surfaces to gain entry into the cell (6, 36), the mannosyl-fucosyl receptor may also be involved to some extent in the attachment of *M. avium-M. intracellulare* to MDM under the experimental conditions used in our studies.

Indirect inhibition of attachment of *M. avium-M. intracellulare* to MDM by MAbs against $\alpha_V\beta_3$. Phagocytosis-promoting receptors are known to diffuse freely in the plane of the macrophage plasma membrane (26). When cells spread on a surface coated with anti-receptor MAbs, the receptors diffuse to the antibody-adherent domain of the cell, leaving the apical surface of the cell depleted of the receptor. Several receptors are known to behave in this manner, for example, the CR3/LFA-1/p150,95 family (36). We used this property to remove specifically $\alpha_V\beta_3$ from macrophages using MAbs against this integrin receptor (LM609). In the present experiment, the binding of *M. avium-M. intracellulare* to MDM was inhibited by 45% with surface-bound MAb LM609 (Fig. 7). MAbs against several other macrophage receptors such as CD14, CD18, $\alpha_2\beta_1$, and gpIIb/IIIa, as well as normal mouse IgG, which was used as a control, showed extremely little or no inhibition of the attachment. These findings suggest that $\alpha_V\beta_3$ on MDM mediates attachment to *M. avium-M. intracellulare*, while other macrophage receptors do not appear to play a role in this interaction under these assay conditions. Although in the direct inhibition assay described earlier (Fig. 6), a maximum inhibition of 37% was achieved with 200 μg of LM609 per ml, in the indirect inhibition assay described here, attachment of *M. avium-M. intracellulare* to MDM was inhibited by 45% with 20 μg of this antibody per ml. No further increase in inhibition was observed with increasing concentrations of LM609 (data not shown). Both assays clearly show that $\alpha_V\beta_3$ is involved in the attachment of *M. avium-M. intracellulare* to MDM. The two assays differ with respect to the manner in which the antibody is presented to the MDM (surface bound in the indirect inhibition assay, versus in solution in the direct inhibition assay), which may account for one assay being more sensitive than the other.

In another set of experiments in which a sonicate of *M. avium-M. intracellulare* was used as the surface-bound ligand, attachment of *M. avium-M. intracellulare* to MDM was inhibited in a dose-dependent manner (Table 1). A 42% inhibition of attachment was observed at 400 μg of surface-bound *M. avium-M. intracellulare* sonicate per ml. However, *E. coli* sonicate showed only a 7% inhibition at the same concentration. These results confirm the specificity of the *M. avium-M. intracellulare*–MDM interaction.

DISCUSSION

Despite the fact that *M. avium-M. intracellulare* is widely distributed in the environment, being present even in tap water, disease due to this organism is rare and, when it does occur, is usually confined to the lungs of patients who have structural abnormalities, such as chronic obstructive pulmo-

TABLE 1. Indirect inhibition of attachment of *M. avium-M. intracellulare* to MDM by surface-bound ligand

Surface-bound ligand	Concn (μg/ml)	% Inhibition of attachment observed in control (DPBS) wells[a]
M. avium-M. intracellulare sonicate	100	33 ± 2.9
	200	40 ± 1.4
	400	42 ± 1.3[b]
E. coli	100	0
	200	9 ± 4.1
	400	11.5 ± 3.0
HSA	100	6.8 ± 0.1
	200	7.3 ± 2.8
	400	8 ± 2.7

[a] Data are representative of three experiments.
[b] $P \leq 0.0002$ (analysis of variance).

nary disease, bronchitis, and healed tuberculosis. However, with the spread of human immunodeficiency virus infection, *M. avium-M. intracellulare* is now responsible for the highest incidence of disseminated bacterial infection in individuals suffering from this infection. *M. avium-M. intracellulare* is an intracellular pathogen. Large numbers of the organism are found within the macrophages of patients with AIDS (41). Recent work with macrophages demonstrated the role played by serum components in phagocytosis of *M. tuberculosis*, in which CR3 and CR1 were implicated (33). CR3, fibronectin, and mannosyl-fucosyl receptors on human macrophages have been shown to play a role in the uptake of *M. avium-M. intracellulare* (5). However, *M. avium-M. intracellulare* has been shown to bind to monocytes and MDM, even in the absence of complement, serum, or serum components (8). The primary phase of macrophage–*M. avium-M. intracellulare* interaction is critical for successful parasitization of macrophages. We therefore examined this interaction in vitro and demonstrated that attachment of *M. avium-M. intracellulare* to macrophages is mediated by $\alpha_V\beta_3$. This molecule belongs to the integrin family of cell surface receptors, which are known to mediate cell-substratum and cell-cell adhesion (20, 28, 31).

M. avium-M. intracellulare-binding receptor isolated in the present study was found to be present on the surface of monocytes as well as MDM and to contain two subunits which cross-react with antibodies against $\alpha_V\beta_3$, but do not cross-react with antisera against $\alpha_5\beta_1$ or $\alpha_3\beta_1$. Alternate approaches used to isolate the *M. avium-M. intracellulare*-binding receptor from detergent extracts of monocytes by using MAb against the α_V subunit of $\alpha_V\beta_3$ coupled to CNBr-Sepharose 4B and GRGDSPK coupled to CNBr-Sepharose 4B yielded a molecule which was active in the adherence assay (Fig. 1) and had a Western blot profile identical to that of the receptor isolated by coupling CME to *M. avium-M. intracellulare* (Fig. 2). THP-1 cells, which are cells of monocyte lineage, were also found to bind *M. avium-M. intracellulare*, ruling out the possibility that platelet integrin contamination of CME may account for the *M. avium-M. intracellulare*-binding activity of monocytes. Further, $\alpha_V\beta_3$ purified from an alternate source (human placenta) was also found to bind FITC-labeled *M. avium-M. intracellulare* in the adherence assay, strongly suggesting the involvement of vitronectin receptor in binding *M. avium-M. intracellulare* (Fig. 1, D1).

Several intracellular parasites of macrophages are known to invade host cells with the help of integrin receptors (6, 29, 36). Integrin-ligand interactions have been shown to be predominantly mediated through RGD sequences found in many adhesive proteins present in extracellular matrices (31). RGD sequences have also been identified in bacterial proteins and have been shown to interact with host receptors. Macrophage CR3 has been shown to bind a filamentous hemagglutinin of *Bordetella pertussis*, the causative agent of whooping cough, and this interaction involves recognition of an RGD sequence in the bacterial protein (29). On the other hand, non-RGD-mediated interactions between bacterial ligands and their host cell integrin receptors have also been reported (23). In our studies, *M. avium-M. intracellulare*-binding receptor was found to bind to RGD coupled to Sepharose. However, preliminary experiments showed no inhibition of the *M. avium-M. intracellulare*–MDM interaction by the peptide in solution (data not shown), suggesting that the binding of the *M. avium-M. intracellulare* receptor on MDM to the ligand on *M. avium-M. intracellulare* may involve a site other than RGD. Identification and characterization of the ligand(s) on *M. avium-M. intracellulare* would enable a better understanding of the macrophage–*M. avium-M. intracellulare* interaction.

We have previously shown that the receptor for *M. avium-M. intracellulare* on MDM is mobile in the plane of the membrane (8). Removal of $\alpha_V\beta_3$ physically from the apical surface of intact MDM by using a specific surface-bound MAb directed against $\alpha_V\beta_3$ significantly inhibited the binding of *M. avium-M. intracellulare* to macrophages (Fig. 7). Surface-bound *M. avium-M. intracellulare* sonicate was also found to reduce this attachment (Table 1). These observations demonstrate that the interaction between $\alpha_V\beta_3$ and *M. avium-M. intracellulare* occurs on the surface of the macrophages and that it is specific for *M. avium-M. intracellulare*. No inhibition was observed with several other MAbs tested (Fig. 7). Receptors such as CD18 (Fig. 6) and gpIIb/IIIA (Fig. 6) were not found to play a role in the attachment of *M. avium-M. intracellulare* to MDM in the inhibition assays. However, intracellular pathogens are often known to have more than one receptor on the host cell surface for the ligands they express, forming a complex repertoire of receptor-ligand interactions. It is quite possible that even for *M. avium-M. intracellulare*, $\alpha_V\beta_3$ may be just one of the receptors mediating attachment of the organism to macrophages. Identification of the other monocyte receptors involved in attachment to *M. avium-M. intracellulare* would be required to determine whether $\alpha_V\beta_3$ is a major or minor *M. avium-M. intracellulare*-binding constituent of CME.

The functions of $\alpha_V\beta_3$ were believed to be limited to cell anchorage (9–11). However, it has recently been reported that this receptor has a role in phagocytosis of cells undergoing apoptosis by macrophages (32). Binding of *M. avium-M. intracellulare* to $\alpha_V\beta_3$ on macrophages described here may serve as the first step in phagocytosis of this pathogen. $\alpha_V\beta_3$ has been shown to be present on several other cell types, including endothelial cells, smooth muscle cells, and epithelial cells (1, 3). Host response to *M. avium-M. intracellulare* may also involve these cells. Human intestinal epithelial cell monolayers have been shown to bind *M. avium-M. intracellulare* (25), which may explain the common intestinal infestation of AIDS patients with *M. avium-M. intracellulare* (41). Identification of a receptor for *M. avium-M. intracellulare* offers additional avenues by which infection due to *M. avium-M. intracellulare* could be treated or prevented by developing drugs which would compete with the bacterial ligand for the receptor or prevent adhesion to host tissue.

ACKNOWLEDGMENTS

We thank John Grzesiak, Cancer Research Center, La Jolla Cancer Research Foundation, La Jolla, Calif., for preparing the vitronectin receptor. We are grateful to Samuel D. Wright and Michael D. Pierschbacher for experimental advice. Experimental advice given by P. Sriramarao and David A. Cheresh and helpful suggestions from Kurt R. Gehlsen are greatly appreciated.

This work was supported by the University of California Universitywide AIDS Research Program.

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