Binding of the 68-Kilodalton Protein of *Mycobacterium avium* to $\alpha_v\beta_3$ on Human Monocyte-Derived Macrophages Enhances Complement Receptor Type 3 Expression

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Attachment to and uptake by host cells are important early events in the pathogenesis of intracellular organisms such as *Mycobacterium avium*. Monocyte-derived macrophages (MDM) are known to express multiple surface receptors that play a role in binding to and uptake of *M. avium*. These include complement receptor type 3 (CR3), fibronectin receptor, mannose receptor, and transferrin receptor. In addition to these, we have previously reported that the integrin receptor $\alpha_v\beta_3$ also plays a role in binding to *M. avium* in a nonopsonic environment. Further, we have shown that a 68-kDa surface protein of *M. avium* binds to human monocytes and plays a role in attachment of *M. avium* to MDM. The present study provides direct evidence that this protein mediates attachment of *M. avium* to MDM by binding to $\alpha_v\beta_3$. Using the technique of cell surface enzyme-linked immunosorbent assay, we have shown that the *M. avium* 68-kDa protein inhibits the binding of monoclonal antibodies (MAb) against $\alpha_v\beta_3$ to MDM compared to control proteins such as ovalbumin and laminin ($P < 0.05$). Dual-labeling studies were performed to demonstrate that after phagocytosis, $\alpha_v\beta_3$ is present along with *M. avium* in phagosomes of *M. avium*-infected MDM. In addition, we have demonstrated that this interaction between $\alpha_v\beta_3$ and the *M. avium* 68-kDa protein resulted in enhancement of CR3 expression, which is known to play a role in complement-mediated uptake of *M. avium*. Attachment of MDM to wells coated with the *M. avium* 68-kDa protein resulted in a twofold increase in CR3 expression compared to attachment of MDM to wells coated with ovalbumin. This enhancement was completely inhibited by pretreatment of MDM with MAb against $\alpha_v\beta_3$. In summary, *M. avium* binds to MDM via $\alpha_v\beta_3$ with the help of the *M. avium* 68-kDa protein, and this ligation enhanced the expression of CR3 on MDM. Since CR3 has been known to play a role in *M. avium* uptake, enhanced expression of this receptor mediated by *M. avium*- $\alpha_v\beta_3$ interaction indicates a complex mechanism of communication among different receptors that participate in *M. avium* attachment and uptake. These findings add to current understanding of the roles played by multiple receptor-ligand systems in uptake and pathogenesis of intracellular pathogens such as *M. avium*.

Attachment of bacteria to host cells is the first step in the establishment of intracellular infections. Monocytes and monocyte-derived macrophages (MDM) play an important role in the phagocytosis of bacterial pathogens. These cells express many receptors, including integrins, which mediate cell-cell binding, uptake of pathogens, and antigen processing (15). Microbial pathogens are known to bind to integrin receptors on MDM directly or indirectly via serum components.

Mycobacterium avium is an intracellular pathogen which multiplies predominantly within MDM. The mechanism of attachment of *M. avium* to MDM has been investigated by our group (3, 20) as well as other investigators (1, 25). We have previously demonstrated that the integrin receptor $\alpha_v\beta_3$ on MDM binds to *M. avium* (20). Other receptors involved in attachment to *M. avium* are complement receptor type 3 (CR3), fibronectin receptor, and mannose receptor (1, 25). Similarly, pathogens express a number of molecules, termed ligands, which interact directly or indirectly with host cell surface receptors. As with receptors, several types of bacterial ligands that function in attachment to host cells have been identified (8, 15, 16, 24, 28). We have identified nine *M. avium* proteins that bind to monocyte extracts. One of these proteins, with a molecular mass of 68 kDa, was found to be present on the surface of *M. avium* and was shown by inhibition assays to

play a role in the attachment of this organism to MDM (21). In some cases, binding of a bacterial ligand to a host cell surface receptor is known to affect the binding activity of another receptor on the same cell. For instance, a minor fimbrial-subunit protein, FimD, of *Bordetella pertussis* binds to very late antigen type 5 (VLA-5) on monocytes, and this cross-linking enhances the binding activity of CR3, which in turn binds to filamentous hemagglutinin, another bacterial protein of *B. pertussis*, resulting in an overall enhanced attachment of this organism to monocytes (13). In the present study we have demonstrated that the *M. avium* 68-kDa protein mediates attachment of *M. avium* to MDM by interacting with $\alpha_v\beta_3$ on these cells. Further, we have studied the effect of this binding on the expression of CR3, which is another receptor involved in *M. avium* attachment.

MATERIALS AND METHODS

Bacterial strain and culture. *M. avium* (ATCC 25291, serotype 2), obtained from the American Type Culture Collection, Rockville, Md., was cultured in Middlebrook 7H11 broth (Difco Laboratories, Detroit, Mich.) at 37°C in 5% CO₂ for 7 to 14 days with vigorous agitation once a day. Bacteria were harvested by centrifugation at 900 × g for 20 min.

Labeling of *M. avium*. *M. avium* bacteria were labeled with fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) as described previously (20). Freshly harvested bacteria were resuspended in a 1-mg/ml solution of FITC (Sigma Chemical Co., St. Louis, Mo.) or TRITC (Sigma Chemical Co.) in coupling buffer (50 mM Na₂CO₃, 100 mM NaCl [pH 9.2]) and incubated for 1 h at room temperature (RT) in the dark. The suspension was washed four times with 1× phosphate-buffered saline (PBS) (Gibco BRL, Gaithersburg, Md.) and resuspended in serum-free medium (Cellgro; Mediatech, Herndon, Va.). The suspension was vortexed and then kept station-

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FIG. 1. SDS-PAGE profile of purified *M. avium* 68-kDa protein. The protein was purified by preparative SDS-PAGE, and the purified protein was subjected to electrophoresis on an SDS-10% polyacrylamide gel.

ary for 30 min in order to allow clumps to settle. The optical density (OD) of the supernatant was adjusted to 0.2 at 600 nm, the OD used in the experiments.

Isolation of monocytes. Monocytes were isolated by Ficoll-Hypaque and Percoll gradient centrifugation from human buffy coats obtained from the San Diego Blood Bank as described previously (20). The isolated monocytes were cultured for 5 to 7 days in Iscove's modified Dulbecco's medium supplemented with 10% normal human serum, 2 mM L-glutamine, and 50 U/ml penicillin-streptomycin in Teflon beakers to yield MDM. At this stage, the purity of the cells was greater than 70%. MDM were further enriched by adherence to tissue culture plates or flasks before being used in experiments. The purity of MDM after the second adherence was greater than 95% as assessed by esterase staining as well as Wright-Giemsa staining. The viability of these cells as determined by trypan blue exclusion was greater than 97%.

Antibodies and reagents. Monoclonal antibodies (MAB) LM609 against $\alpha_v\beta_3$ were kindly provided by David Cheresch, Scripps Clinic and Research Foundation, La Jolla, Calif. (5). MAB 23C6 against $\alpha_v\beta_3$ and MAB against CD11a and CD11c were obtained from Pharmingen, San Diego, Calif. MAB L230 (31) was purified from cell culture supernatants of hybridoma cells (ATCC HB8448) grown in our laboratory. IB4 against CD18 (33) was kindly provided by Samuel D. Wright, Rockefeller University, New York, N.Y. MAB against CD11b, CD14, and transferrin receptor (CD71) as well as FITC-conjugated secondary goat anti-mouse antibodies were obtained from Becton-Dickinson, San Jose, Calif. Alkaline phosphatase-conjugated goat anti-mouse antibodies and anti-rabbit antibodies were obtained from Sigma Chemical Co.

Preparation of the *M. avium* 68-kDa protein. The *M. avium* 68-kDa protein was isolated from sonic extracts of *M. avium* by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (22). Briefly, sonic extracts of *M. avium* (6 to 8 mg of protein) were incubated with SDS-PAGE sample buffer for 2 h at 37°C and electrophoresed on 10% polyacrylamide gels under nonreducing conditions with the help of a Prep Cell preparative electrophoresis apparatus (model 491; Bio-Rad, Hercules, Calif.). Proteins were eluted from the gel with Tris-glycine buffer and collected as 8-ml fractions. A 400- μ l aliquot of every third fraction was lyophilized and analyzed by SDS-PAGE to identify fractions containing the 68-kDa protein. Fractions containing this protein were pooled, extensively dialyzed against PBS, and lyophilized. The lyophilized fraction was desalted with Econo-Pac 10DG desalting columns (Bio-Rad), and the purity of the desalted protein was confirmed by SDS-PAGE (Fig. 1). The purified protein was aliquoted and stored at -20°C until further use.

Inhibition cell surface ELISA with MABs against $\alpha_v\beta_3$. Cell surface enzyme-linked immunosorbent assay (ELISA) was carried out as described previously by R. Grunow et al. (10). A total of 10^5 MDM were added to each well of a 96-well microtiter plate (Nunc, Roskilde, Denmark) and allowed to adhere for 2 h. Nonadherent cells were removed by washing the wells twice with 1 $\times$ PBS containing 0.1% fetal bovine serum. Adherent cells were treated with different proteins, such as *M. avium* sonicate and the *M. avium* 68-kDa protein, for 1 h. Proteins, such as vitronectin, fibrinogen, and fibronectin, which are known to bind to $\alpha_v\beta_3$, were used as positive controls, while laminin was used as a negative control. Adherent cells in some wells were treated with ovalbumin instead of the proteins listed above to rule out nonspecific inhibition. After incubation with different proteins, the cells were washed five times and then incubated with LM609 (1:100) or MAB against CD14 (1:50) for 1 h. The cells were washed and blocked with 10% goat serum in PBS for 1 h. Finally, the cells were reacted with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG) (1:1,000) for 1 h. Once again, the cells were washed five times and treated with 100 μ l of a 1.5-mg/ml solution of *p*-nitrophenyl phosphate (Sigma Chemical Co.) for 1 h. All incubations were carried out at 37°C. The reaction was stopped by adding 25 μ l of 1 N NaOH. The OD at 405 nm was measured with the help of an ELISA reader, treating wells incubated with ovalbumin followed by PBS instead of wells incubated with MABs as the reagent blank. The level of binding of LM609 to

MDM treated with ovalbumin was considered 100%. The results were then expressed as percent inhibition of binding of LM609 to cells treated with different proteins compared to cells treated with ovalbumin, according to the following formula: % inhibition = (OD of wells treated with ovalbumin - OD of wells treated with different proteins)/OD of wells treated with ovalbumin.

MDM from five donors were studied. Triplicates were set up for each condition for each donor.

Immunofluorescence staining of $\alpha_v\beta_3$ after phagocytosis of *M. avium* by MDM. A total of 10^5 MDM were allowed to adhere to eight-well Lab-Tek plates (Nunc) for 2 h and washed twice with RPMI 1640 without serum. A suspension of TRITC-labeled *M. avium* bacteria (1:100, MDM to *M. avium* organisms) in serum-free medium (Cellgro) was added. After incubation for 2 h at 37°C, the cells were extensively washed with RPMI 1640 and fixed with a 1:1 mixture of acetone and methanol for 1 min. After blocking with 10% normal human serum, MAB 23C6 against $\alpha_v\beta_3$ was added and incubated at RT for 1 h. MAB against transferrin receptor was used as a positive control since this receptor has previously been reported to be extensively incorporated into mycobacterial phagosomes (6). MAB against CD14 was used as a negative control. Cells were washed three times with Hanks' balanced salt solution (Gibco BRL) containing 0.1% bovine serum albumin. The bound antibodies were visualized after incubation with FITC-conjugated goat anti-mouse IgG for 30 min. Cells were then examined under an Olympus microscope equipped for epifluorescence with filters for FITC as well as a fluorescence set filter for FITC and TRITC. Approximately 25 MDM containing at least one *M. avium* organism were identified. The total number of *M. avium* organisms in these MDM was determined. The number of these TRITC-labeled *M. avium* organisms that colocalized with FITC-stained $\alpha_v\beta_3$, transferrin receptor, or CD14 was determined and expressed as a percentage of the total number of *M. avium* organisms in 25 MDM.

Isolation of phagosomes and immunoblotting. *M. avium* phagosomes were isolated according to previously described methods (4). Briefly, 5×10^6 to 10×10^6 MDM were cultured overnight in T-75 flasks. *M. avium* bacteria ($\approx 10^8$) were added and incubated for 1 h. Extracellular bacteria were removed by washing (twice) with RPMI 1640 without serum. The infected cells were collected with the help of a cell scraper and centrifuged at $800 \times g$ for 10 min at 4°C to remove free bacteria. The cells were homogenized on ice in a solution containing 20 mM HEPES, 0.5 mM EGTA, 250 mM sucrose, and 0.5% gelatin (pH 7.0) with protease inhibitors (50 μ g of pepstatin/ml, 100 μ g of leupeptin/ml, 100 μ g of TLCK [*N*- α -*p*-tosyl-L-lysine chloromethyl ketone]/ml, and 50 μ g of E64 [*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane]/ml, all from Sigma Chemical Co.). Unbroken cells and nuclei were pelleted by centrifugation ($200 \times g$, 10 min), and the supernatant was passed through a 3- μ m-pore-size Nucleopore filter (Corning Costar Corporation, Cambridge, Mass.). The flowthrough was overlaid on a 12% sucrose cushion and centrifuged at $1,700 \times g$ for 45 min at 4°C. The pelleted phagosomes were solubilized with 0.05% Nonidet P-40 for 30 min on ice and then centrifuged. The supernatant was subjected to SDS-10% PAGE under nonreducing conditions followed by Western blotting. The filter was incubated with polyclonal antibodies against $\alpha_v\beta_3$ (Chemicon, Temecula, Calif.) followed by incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG. The bound antibodies were detected with a BCIP (5-bromo-4-chloro-3-indolylphosphate toluidinium)-nitroblue tetrazolium alkaline phosphatase substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, Md.). Detergent extracts of noninfected MDM were prepared as described previously (20) and used as a positive control for immunoblotting.

To confirm the presence of *M. avium* in the phagosome preparation, a fraction of the isolated phagosomes was lysed aseptically with 0.25% SDS in 1 $\times$ PBS, and the lysate was plated on Middlebrook 7H11 agar plates. The plates were incubated for 12 to 14 days at 37°C to allow growth of *M. avium*.

Analysis of CR3 expression on MDM. (i) Conjugation of fluoresceinated microspheres with antibodies. Fluoresceinated latex microspheres (MS) (Fluosphere; Molecular Probes Inc., Eugene, Ore.) with a diameter of 0.5 μ m were coated with MAB against CD18, CD11a, CD11b, or CD11c according to the manufacturer's instructions. Briefly, MS were mixed with 20 μ g of MAB in a reaction volume of 400 μ l of MES buffer [2-(*N*-morpholino)ethanesulfonic acid (pH 6.0)]. To this, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Molecular Probes Inc.) was added at a final concentration of 2.5 mg/ml. After incubation overnight at RT, glycine (100 mM) was added, and the MS were incubated for an additional 15 min. The antibody-coated MS were washed three times with 1 $\times$ PBS and resuspended at a density of 10^{10} MS/ml in PBS containing 1% bovine serum albumin.

The specificity of the MAB-coated MS was established by the fact that the level of binding of these MS to MDM was five times greater than the level of binding of MS coated with ovalbumin according to the same protocol. Further, binding of MAB-coated MS was completely inhibited by pretreatment of MDM with the MAB used to coat the MS.

(ii) Binding of MAB-coated MS to adherent MDM. Terasaki plates (Nunc) were precoated with ovalbumin, the *M. avium* 68-kDa protein, or *M. avium* sonicate (100 μ g/ml) for 1 h at 37°C. After two washes with PBS, 5×10^3 MDM in HAP solution (150 mM CaCl₂, 500 mM MgCl₂, and 0.3 U of aprotinin [Sigma Chemical Co.] per ml) was added to each well and allowed to adhere for 1 h. Nonadherent cells were removed by washing three times with PBS. Approximately 10^6 MAB-coated MS were added to each well and incubated for 15 min at 37°C. The plates were then washed five times with PBS and fixed with 2.5%

FIG. 2. Inhibition of binding of LM609 to MDM by the *M. avium* 68-kDa protein. MDM (10^5) were allowed to adhere to each well of a 96-well microtiter plate and incubated with fibrinogen (Fb), the *M. avium* 68-kDa protein (Ma 68 kD), *M. avium* sonicate (Ma son), vitronectin (Vn), laminin (Lm), and fibronectin (Fn) at 100 and/or 300 µg/ml. Ovalbumin at the same concentrations was used as a control for nonspecific binding. LM609 (1:100) was added to the cells, and the bound antibody was detected with an alkaline phosphatase-conjugated secondary antibody. The results are expressed as percent inhibition of binding of LM609 to cells treated with different proteins compared to cells treated with ovalbumin. Means plus standard deviations from three independent experiments with MDM from three different donors are shown.

glutaraldehyde. The number of adhered MS was determined manually with the help of an epifluorescence microscope and expressed as the attachment index (AI) (number of MS bound to 100 MDM). Results are expressed as percent enhancement of attachment of MAb-coated MS to MDM in wells coated with test protein compared to attachment to MDM in wells coated with ovalbumin. There was no significant difference in AI between MAB-coated MS bound to MDM added to wells coated with 100 µg of the *M. avium* 68-kDa protein/ml and those bound to MDM added to wells coated with 300 µg of the protein/ml ($P > 0.05$). Therefore, only data obtained with 100 µg of the *M. avium* 68-kDa protein/ml is shown.

An analysis of the time course of enhancement of attachment of anti-CD18- and anti-CD11b-coated MS indicated that maximum enhancement was observed at 1 h. Incubation for longer periods showed no further increase in enhancement of attachment of anti-CD18- and anti-CD11b-coated MS.

For the inhibition studies, MDM were preincubated with specific MAb, control ascites, or normal mouse IgG at 4°C for 1 h on an end-over-end rotator before being added to wells.

Statistical analysis. The Student *t* test was used to make comparisons between groups.

RESULTS

The *M. avium* 68-kDa protein binds to $\alpha_v\beta_3$ on MDM. Cell surface ELISA was used to determine whether the *M. avium* 68-kDa protein binds to $\alpha_v\beta_3$ on MDM. Adhered MDM were treated with the *M. avium* 68-kDa protein, *M. avium* sonicate, and various extracellular matrix proteins before incubation with MAb against $\alpha_v\beta_3$ (LM609). The *M. avium* 68-kDa protein inhibited binding of LM609 to MDM by $34.7\% \pm 5.6\%$ and $59.8\% \pm 19.8\%$ at 100 and 300 µg/ml, respectively (Fig. 2). Further, inhibition at 300 µg/ml was significantly higher than inhibition at 100 µg/ml ($P = 0.022$), suggesting that the *M. avium* 68-kDa protein inhibits attachment of LM609 to MDM in a dose-dependent manner. In addition, inhibition by the *M. avium* 68-kDa protein was found to be significantly higher ($P = 0.025$ at 300 µg/ml) than inhibition with laminin, which was used as the negative control. Vitronectin, which is one of the ligands for $\alpha_v\beta_3$, also inhibited this binding by $55.8\% \pm 11.4\%$ ($P = 0.009$) at 300 µg/ml, and maximum inhibition was observed with fibrinogen ($85.8\% \pm 19.8\%$ [$P = 0.0001$] at 300

µg/ml). The *M. avium* 68-kDa protein did not inhibit binding of the control antibody (MAb against CD14) to MDM. These results indicate that this protein binds to $\alpha_v\beta_3$ on MDM, thereby inhibiting the binding of LM609 to this receptor.

Effect of the *M. avium* 68-kDa protein on expression of CR3 on MDM. Since CR3 has been previously shown to function as a receptor for *M. avium*, it was of interest to study whether the binding of the *M. avium* 68-kDa protein to MDM in any way influenced the expression of CR3 (which consists of the subunits CD11b and CD18) on these cells. To determine this, the expression of CR3 on MDM after adherence to wells pre-coated with the *M. avium* 68-kDa protein was examined with fluorescent MS coated with MAbs against CR3.

An enhancement of the attachment of anti-CD11b- and anti-CD18-coated MS in wells coated with the *M. avium* 68-kDa protein compared to attachment in wells coated with ovalbumin was observed (Fig. 3). The *M. avium* 68-kDa protein, at 100 and 300 µg/ml, significantly increased the AI for anti-CD11b-coated MS bound to MDM by $179.1\% \pm 26.2\%$ ($P = 0.002$) and $184.6\% \pm 38.6\%$ ($P = 0.01$), respectively, compared to ovalbumin at the same concentrations (Fig. 3B). Similarly, a significant increase in AI for anti-CD18-coated MS in wells coated with the *M. avium* 68-kDa protein compared to ovalbumin-coated wells was observed. At 100 and 300 µg/ml, this protein enhanced attachment of anti-CD18-coated MS by $253.1\% \pm 34.6\%$ ($P = 0.0004$) and $277.6\% \pm 56.2\%$ ($P = 0.0016$), respectively (Fig. 3D). There was no increase in the attachment of anti-CD11a- and anti-CD11c-coated MS to MDM adhering to the *M. avium* 68-kDa protein compared to MDM adhering to ovalbumin (Fig. 3A and C, respectively). These results clearly indicate that attachment of MDM to the *M. avium* 68-kDa protein specifically increases the expression of the CD11b/CD18 molecule.

Enhancement of expression of CR3 requires binding of the *M. avium* 68-kDa protein to $\alpha_v\beta_3$. As described above, we have demonstrated that the *M. avium* 68-kDa protein binds to $\alpha_v\beta_3$ and that binding of MDM to surfaces coated with the *M. avium* 68-kDa protein results in increased expression of CR3. In order to determine whether this enhancement is mediated by binding of the *M. avium* 68-kDa protein to $\alpha_v\beta_3$, MDM were pretreated with MAb against $\alpha_v\beta_3$ (L230) before being added to wells coated with the 68-kDa protein in the experiment described in the legend to Fig. 4. Although binding of MDM to the wells was unaffected, L230 significantly inhibited enhancement of CR3 expression ($P < 0.02$). CR3 expression on MDM after treatment of these cells with MAb against $\alpha_v\beta_3$ was similar to that observed in the case of cells added to wells coated with ovalbumin instead of the *M. avium* 68-kDa protein (Fig. 4). Pretreatment of MDM with normal mouse IgG instead of $\alpha_v\beta_3$ -specific MAb did not inhibit enhancement of CR3 expression. These results indicate that enhanced CR3 expression on MDM caused by binding of MDM to the *M. avium* 68-kDa protein is mediated via $\alpha_v\beta_3$.

Intracellular movement of $\alpha_v\beta_3$ after infection of MDM with *M. avium*. To study the intracellular fate of $\alpha_v\beta_3$ after phagocytosis of *M. avium* by MDM, MDM were infected with TRITC-labeled *M. avium* and then incubated with MAb against $\alpha_v\beta_3$. FITC-labeled secondary antibodies were used to localize the receptor. The early endosomal marker, transferrin receptor, was used as a positive control since it has been reported that this receptor is efficiently incorporated into mycobacterial phagosomes (6).

Sixty minutes after infection, $8.4\% \pm 4.4\%$ of the phagocytosed *M. avium* bacteria were found to colocalize with $\alpha_v\beta_3$, while $68.4\% \pm 8.4\%$ of phagocytosed *M. avium* bacteria colocalized with transferrin receptor. Only $4.5\% \pm 1.5\%$ of phago-

FIG. 3. Effect of binding of MDM to the *M. avium* 68-kDa protein on CD11a (A), CD11b (B), CD11c (C), and CD18 (D) expression. MDM were added to Terasaki plates precoated with the *M. avium* 68-kDa protein (Ma 68 kD), *M. avium* sonicate (Ma son), or ovalbumin (Ova) at 100 μ g/ml and incubated for 1 h at 37°C. Anti-CD18-, anti-CD11a-, anti-CD11b-, or anti-CD11c-coated MS were added to the wells and incubated for 15 min. The plates were washed, and the numbers of MAb-coated MS attached to MDM in wells coated with the *M. avium* 68-kDa protein, *M. avium* sonicate, or ovalbumin and the respective AIs were determined. Means plus standard deviations of five experiments with MDM from three different donors are shown.

cytosed *M. avium* bacteria were found to colocalize with CD14, which served as a negative control. The percent colocalization with transferrin receptor and $\alpha_v\beta_3$ was significantly higher ($P = 0.0001$ and 0.025 , respectively) than the percent colocalization with CD14. These results suggest that $\alpha_v\beta_3$ is internalized along with *M. avium*, although incorporation is modest compared to incorporation of transferrin receptor.

To further confirm the presence of $\alpha_v\beta_3$ in phagosomes after infection with *M. avium*, phagosomes were isolated from 3×10^7 to 4×10^7 infected MDM from each of four different donors. Extracts of these phagosomes were pooled and then subjected to immunoblotting with polyclonal antibodies against $\alpha_v\beta_3$. Phagosome extracts were found to contain a

FIG. 4. Effect of MAb against $\alpha_v\beta_3$ on the enhancement of CR3 expression on MDM bound to the *M. avium* 68-kDa protein. MDM were pretreated with 25 μ g of normal mouse IgG (MDM/cIgG)/ml or 25 μ g of L230 (MDM/L230)/ml at 4°C for 1 h and then added to wells precoated with 100 μ g of ovalbumin/ml or 100 μ g of the *M. avium* 68-kDa protein/ml. Anti-CD11b- or anti-CD18-coated MS were added to the adhered MDM and AIs were determined as described in Materials and Methods. Means plus standard deviations of three experiments with MDM from three different donors are shown. $P = 0.015$ for MDM/L230 values compared with 68-kDa-MDM/cIgG values.

95-kDa protein, which corresponds to the β_3 chain of $\alpha_v\beta_3$ (20). A protein of similar molecular mass was also present in extracts of noninfected MDM, which were used as a positive control (Fig. 5). The α chain of $\alpha_v\beta_3$, however, was not detectable or was below the level of detection under these conditions. This data is consistent with our previous report (20) for this receptor. These findings further substantiate the results of the immunofluorescence studies described above.

The presence of *M. avium* in phagosomes was confirmed by plating lysates of freshly isolated phagosomes on Middlebrook 7H11 agar plates. After 12 to 14 days, several bacterial colonies were observed on the plates.

FIG. 5. Immunoblotting of *M. avium*-containing phagosome extracts with polyclonal antibodies against $\alpha_v\beta_3$. Phagosomes were isolated from *M. avium*-infected MDM of four different donors (lane 1) and subjected to electrophoresis on SDS-10% polyacrylamide gels (nonreducing conditions) followed by immunoblotting with polyclonal antibodies against $\alpha_v\beta_3$. An extract of intact noninfected MDM (lane 2) was used as a positive control.

DISCUSSION

The entry of intracellular pathogens into host cells is initiated by binding of the pathogen to the host cell surface. Several pathogens are known to bind to integrin receptors on macrophages as well as other host cells (15). We have previously shown that *M. avium* binds to MDM with the help of the integrin receptor $\alpha_v\beta_3$ (20). *M. avium* attachment is also known to be mediated by CR3, mannose receptor, and fibronectin receptor (1, 25). Multiple receptors for a single pathogen have been reported in the cases of *B. pertussis* (12) and *Histoplasma capsulatum* (2). *B. pertussis* binds to two receptors on macrophages with the help of two different ligands. FimD binds to VLA-5, and filamentous hemagglutinin binds to CR3 (12). *H. capsulatum* binds to three different receptors on macrophages, LFA-1 (CD11a/CD18, $\alpha_L\beta_2$), CR3 (CD11b/CD18, $\alpha_M\beta_2$), and P150,95 (CD11c/CD18, $\alpha_X\beta_2$), which share a β_2 chain (β_2). Blocking of the β_2 chain results in complete inhibition of attachment of *H. capsulatum* to MDM, while blocking of each α chain results only in partial inhibition (2). It is possible that different receptors play the same role at different sites in the body.

We have previously identified two *M. avium* ligands that play a role in attachment to MDM by an inhibition assay where pretreatment of MDM with these ligands resulted in the inhibition of binding of intact *M. avium* to MDM (21, 23). The 68-kDa surface protein of *M. avium* is one such ligand. However, the receptor(s) to which these ligands bind was not investigated. In this study, we have shown that the 68-kDa protein binds to $\alpha_v\beta_3$ on MDM. Furthermore, the binding of $\alpha_v\beta_3$ on MDM to the 68-kDa protein resulted in enhanced expression of CR3 on the apical surface of MDM. A similar phenomenon has been shown to occur during the interaction of *B. pertussis* with MDM (13).

Inhibition of binding of MAb LM609 to MDM by the 68-kDa protein, shown in Fig. 2, indicates that this protein competes with the MAb for binding to $\alpha_v\beta_3$. The 68-kDa protein inhibited the binding of LM609 to $\alpha_v\beta_3$ on MDM by 59% $\pm$ 19.8%. Vitronectin, which is a well-known ligand for $\alpha_v\beta_3$, also inhibited this binding to a similar extent (55% $\pm$ 11.4%). Fibrinogen, which is also a ligand for $\alpha_v\beta_3$, was found to inhibit binding of LM609 to $\alpha_v\beta_3$ by 85% $\pm$ 8.3%. These findings are consistent with previous studies by Cheresh and coworkers, who reported that LM609 inhibited attachment of endothelial cells to surfaces precoated with vitronectin only up to 50%, while attachment to fibrinogen, von Willebrand factor, and RGD peptide was inhibited by 75 to 80% (5). It is possible that LM609 and the *M. avium* 68-kDa protein bind to different sites on $\alpha_v\beta_3$, accounting for the partial inhibition observed. The presence of distinct ligand binding sites has been reported for $\alpha_v\beta_5$ and $\alpha_4\beta_1$. Human immunodeficiency virus Tat protein and RGD peptide are known to bind to different sites on $\alpha_v\beta_5$ (30), and VCAM-1 and fibronectin bind to distinct sites on VLA-4 ($\alpha_4\beta_1$) on endothelial cells (7).

Internalization and intracellular movement of pathogen-binding receptors on the host cell surface have been investigated in the case of several pathogens, including *Mycobacterium tuberculosis* (6, 19). However, this process is poorly understood in the case of *M. avium*. Host cell membrane components and receptors which are internalized into phagosomes vary from pathogen to pathogen (11, 19). In the present study we have shown that $\alpha_v\beta_3$ is internalized along with *M. avium* into phagosomes after infection. However, compared to receptors such as the transferrin receptor, the number of *M. avium* bacteria that colocalized with this receptor was low. It is possible that the bulk of the receptor may be recycled to the cell

surface after the organism is taken up. It is also possible that the receptor is degraded by intracellular enzymes or proteases.

Attachment of *M. avium* to host phagocytes is an important initial event in the pathogenesis of mycobacterial infection. The phagocytosis and survival mechanism of *M. avium*, involving many host receptor-bacterial ligand interactions, is a complex phenomenon and one which is not clearly understood. It is not known whether these interactions act independently or in association with other cellular events to facilitate phagocytosis of this organism. Our results show that binding of $\alpha_v\beta_3$ to the *M. avium* 68-kDa protein enhances the expression of CR3, another macrophage receptor for *M. avium* as well as several other pathogens (2, 14, 18, 25–27). This receptor is known to be up-regulated by fibronectin receptor (32) as well as integrin associated protein (29) and bacterial antigens (13). Enhancement of receptor expression often involves a conformational change leading to increased receptor binding affinity as well as an increase in the number of receptor molecules expressed on the cell surface. In this study, attachment assays with fluoresceinated MS coated with MAb against CD18 and CD11b were employed to demonstrate that attachment of MDM to surfaces coated with the *M. avium* 68-kDa protein increases the number of CR3 molecules expressed on the surface of MDM. Further, the enhanced expression of CR3 was dependent on the binding of the *M. avium* 68-kDa protein to $\alpha_v\beta_3$ on MDM. Since CR3 is known to play a role in uptake of *M. avium*, it is likely that binding of *M. avium* to MDM acts as a signal, increasing CR3 expression, which then helps in enhanced attachment to *M. avium*. CR3 is known to mediate the attachment of several pathogens to macrophages, mainly under opsonic conditions. However, it is also known to function under nonopsonic conditions (17). Nonopsonic phagocytosis is important in pathogen uptake in areas such as the lung, where the concentration of opsonins might be expected to be low (9).

As described earlier, pathogens are known to use multiple ligands to bind to host cells, often interacting with multiple receptors on these cells. Here we have shown that a 68-kDa protein of *M. avium* binds to $\alpha_v\beta_3$ and have analyzed the intracellular movement of this receptor after phagocytosis. Further, we have identified the manner in which binding of the 68-kDa protein can affect the expression of other receptors (e.g., CR3) involved in *M. avium* uptake. This data helps in further understanding *M. avium*-host cell interactions.

Exposure to *M. avium* is common in the environment. It is likely that exposure to *M. avium* may result in enhancement of expression of *M. avium*-binding receptors on the surface of host cells, which in turn may help in efficient uptake of this organism by these cells. Cooperation among receptors that participate in binding to a particular organism, as shown in the present study, may promote efficient adherence and phagocytosis of the organism, favoring the host's defense mechanism against this organism.

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