

Fibronectin Facilitates *Mycobacterium tuberculosis* Attachment to Murine Alveolar Macrophages

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***Mycobacterium tuberculosis* remains a major cause of pulmonary infection worldwide. Attachment of *M. tuberculosis* organisms to alveolar macrophages (AMs) represents the earliest phase of primary infection in pulmonary tuberculosis. In this study fibronectin (Fn), an adhesive protein, is shown to bind *M. tuberculosis* organisms and facilitates attachment of *M. tuberculosis* to murine AMs. A monoclonal antibody (MAb) specific to the heparin binding domain (HBD) of Fn decreases ^{125}I -Fn binding to *M. tuberculosis*; whereas MAbs specific to either the cell binding domain (CBD) or the gelatin binding domain (GBD) have no effect on Fn binding to *M. tuberculosis*. In the presence of exogenous Fn (10 $\mu\text{g/ml}$) *M. tuberculosis* attachment to AMs increased significantly from control levels (means \pm standard errors of the means) of $11.5\% \pm 1.1\%$ to $44.2\% \pm 4.2\%$ ($P < 0.05$). Fn-enhanced attachment was significantly decreased from $44.2\% \pm 4.2\%$ to $10.8\% \pm 1.2\%$ ($P < 0.05$) in the presence of anti-Fn polyclonal antibodies. The attachment is also inhibited in the presence of MAbs specific for the HBD and CBD, whereas MAbs specific to GBD did not affect the attachment. Further, an Fn cell binding peptide, Arg-Gly-Asp-Ser (RGDS), decreased the attachment from $44.2\% \pm 4.2\%$ to $15.3\% \pm 1.2\%$ ($P < 0.05$), whereas addition of a control peptide, Arg-Gly-Glu-Ser (RGES) did not affect the attachment ($40.5\% \pm 1.8\%$). These results suggest that Fn-mediated attachment of *M. tuberculosis* can occur through the binding of Fn to the AM via the CBD and to *M. tuberculosis* organisms via the HBD.**

Tuberculosis remains a major health problem throughout the world (11, 38). The initial infection with *Mycobacterium tuberculosis* typically occurs in the alveolar spaces of the lung. Attachment of the tubercle bacillus to alveolar macrophages (AMs) is a crucial step in the establishment of infection, as the organism first survives and replicates in AMs as an intracellular pathogen. Previous studies have shown that *M. tuberculosis* organisms can attach and enter macrophages by specific cell surface receptors, including complement receptors (CR1 and CR3), the C2a component of complement, mannose receptor, transferrin receptor, CD14 scavenger receptor, and an unknown receptor that is inhibited by β -glucan (12, 40).

Fibronectin (Fn) is known to mediate attachment of several different microorganisms to host cells (17). Fn is an extracellular matrix protein with two similar subunits joined near the C-terminal end. This heterodimeric glycoprotein contains multiple binding domains and possesses binding properties to different ligands such as heparin, collagen, and fibrin (52). Fn binds to a wide variety of microorganisms in a ligand receptor-mediated manner (17). For instance, previous in vitro studies have shown that Fn binds to *Escherichia coli*, *Streptococcus pyogenes*, *Salmonella enterica* serovar Dublin, and *Candida albicans* (6, 14, 33, 39). The binding sites of Fn play an integral role in the recognition of microorganisms. For example, Fn binds *Staphylococcus aureus* via the N-terminal domain and *Treponema pallidum* via the cell binding domain (CBD) of Fn (36, 47). Similarly, in vitro, Fn facilitates the attachment of *Pneumocystis carinii* to AMs via the CBD (34) that is known to

interact with integrins on the cell surface. Other studies indicate that microbial attachment may occur through different binding sites on Fn, such as the C-terminal domain (24, 45, 51).

Prior studies have shown that mycobacteria interact with Fn. Fn attachment proteins (FAP) are surface proteins that are present in a variety of mycobacterial species, including *Mycobacterium tuberculosis* (1), *Mycobacterium avium* (43), *Mycobacterium leprae* (44), and *Mycobacterium vaccae* (37). The FAP are a family of highly homologous proteins (37). The FAP from *M. leprae* bound to the carboxy-terminal heparin binding chymotryptic fragment of Fn (44). Antibodies directed against FAP significantly inhibit attachment of mycobacteria to host epithelial cells (23). Additionally, *M. tuberculosis* secrete an Fn-binding protein known as the antigen 85 complex (49). This complex is composed of three proteins, Ag85A, Ag85B, and Ag85C. The antigen 85 functions as a mycolyl transferase (3). Disruption of the gene for Ag85A results in diminished growth of *M. tuberculosis* in cultured macrophages (2).

In this study, we demonstrated that Fn can mediate attachment of *M. tuberculosis* to murine AMs. The data suggest that Fn interacts with *M. tuberculosis* via the heparin binding domain (HBD) of Fn. Fn-enhanced attachment of *M. tuberculosis* to murine AMs was decreased by the addition of monoclonal antibodies (MAbs) to either HBD or cell binding domain. Further, Fn-mediated attachment of *M. tuberculosis* to AMs was blocked by the tetrapeptide sequence of the CBD, RGDS (Arg-Gly-Glu-Ser), suggesting a possible role for the CBD of Fn in the mediation of Fn attachment to AMs.

MATERIALS AND METHODS

***M. tuberculosis* isolation.** The H37Ra strain of *M. tuberculosis* (American Type Culture Collection, Manassas, Va.) was cultured at 37°C in 5% CO₂ atmosphere in dispersed form in Middlebrook 7H9 broth (Difco Laboratories, Detroit,

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Mich.) containing albumin, dextrose, and catalase as enrichments. Bacterial growth was monitored by a Spectronic 20D spectrophotometer (Milton Roy Company, Rochester, N.Y.) (9). To achieve a single-cell suspension, the bacterial suspension was briefly sonicated (20 W for 5 to 10 s). The suspension was then gently agitated and allowed to settle for 5 min. The top portion of the suspension containing bacteria was used in the assay. Bacterial cultures, 10 to 14 days old, were centrifuged and washed once with normal saline. The final concentration of the bacterial suspension was adjusted to 10^9 organisms/ml. Each batch of bacterial suspension was stained with Kinyoun stain (Midlantic Biomedical Inc., Paulsboro, N.J.) and observed under the microscope to verify the purity of the suspension. Routinely, samples of bacteria were also grown on mycobacterial 7H11 agar (Difco Laboratories) plates as stock.

Isolation and preparation of Fn. Bovine Fn was used for the majority of studies due to its homology with human Fn and because it shares identical binding sites with human Fn (18, 21). Bovine Fn was much less expensive than human Fn and was isolated according to the method of Hynes (19) with modifications (50). Briefly, a gelatin-Sepharose column (4.8 by 30 cm; Pharmacia, LKB Biotechnology, Piscataway, N.J.) was equilibrated with 50 mM Tris-HCl (pH 7.4), and 4.0 liters of serum was applied. The column was washed with 1.0 liter of equilibration buffer (50 mM Tris-HCl [pH 7.4]) followed by 1.0 liter of 50 mM Tris-HCl (pH 7.4) with 0.5 M NaCl. Fn was eluted from the column with an elution buffer (50 mM Tris-HCl [pH 7.4], 4 M urea). The eluted fractions were collected and read in a spectrophotometer at 280 nm. The positive fractions were applied to a DEAE cellulose column (2.5 by 120 cm; Whatman Biosystems Ltd., Maidstone, Kent, United Kingdom) and equilibrated in 50 mM Tris-HCl (pH 7.4)–4 M urea. After washing the DEAE column with 500 ml of 10 mM Tris-HCl (pH 7.4)–4.5 M urea, Fn was eluted with a linear gradient of 500 ml of 10 mM Tris-HCl (pH 7.4)–4.5 M urea and 500 ml of 10 mM Tris-HCl (pH 7.4)–4.5 M urea and 0.5 M NaCl. The fractions were collected and read in a spectrophotometer at 280 nm. The positive fractions were pooled, and the Fn was dialyzed three times in 4 liters of 50 mM NaHCO₃. The concentration of Fn was determined by the Lowry protein assay (28). Purity of the isolated Fn was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (25) and confirmed by Western blotting (48) using an anti-Fn antibody (Gibco-BRL, Life Technologies, Inc., Gaithersburg, Md.). For comparisons, we used commercially available human Fn in selected experiments (Gibco-BRL, Life Technologies).

¹²⁵I-Fn binding assay. The binding of ¹²⁵I-Fn (ICN Chemical Co., Irvine, Calif.) to *M. tuberculosis* organisms was quantified as described previously (36, 53). Briefly, *M. tuberculosis* organisms grown in 7H9 Middlebrook broth were isolated, washed by centrifugation and resuspended to a final concentration of 10^9 organisms/ml of Dulbecco's modified Eagle medium (DMEM). The above mixture containing 10^8 *M. tuberculosis* organisms in 100 μ l of DMEM was incubated with ¹²⁵I-Fn (10 μ g/ml) in the presence or absence of 0 to 5 mM Ca²⁺ for 60 min at 37°C in microcentrifuge tubes. For the subsequent reaction conditions the final volume was brought up to 200 μ l. After the incubation, the reaction mixture was centrifuged at $12,000 \times g$ for 20 min in an Eppendorf microcentrifuge. The pellet was washed three times with DMEM to remove free ¹²⁵I-Fn. The pellet containing bound ¹²⁵I-Fn to *M. tuberculosis* organisms was counted in a gamma counter (Beckman 5500; Beckman Instruments Corp., Fullerton, Calif.). The amount of ¹²⁵I-Fn bound was then quantified.

To determine the binding mechanisms, the ¹²⁵I-Fn binding assay was performed in the presence or absence of RGDS (1.0 mM) and a control peptide, RGES (1.0 mM) (Gibco-BRL, Life Technologies, Inc.) or heparin (1.0 μ M) (Sigma Chemical Co., St. Louis, Mo.). The binding assay was also carried out in the presence or absence of anti-Fn MAbs directed against the HBD, CBD, and gelatin binding domain (GBD) of Fn or in the presence or absence of appropriate isotype control MAbs (Chemicon International, Temecula, Calif.). ¹²⁵I-Fn and *M. tuberculosis* organisms were incubated with anti-Fn MAbs for 60 min. After the incubation, the samples were washed by centrifugation in DMEM to remove free ¹²⁵I-Fn and unbound antibody. The fractions were counted in a gamma counter to determine the amount of bound ¹²⁵I-Fn.

AM isolation. Murine AMs were isolated by bronchoalveolar lavage from 10-week-old Swiss Webster pathogen-free mice (Harlan Sprague-Dawley, Inc. Indianapolis, Ind.) as previously described (10). Briefly, the mice were sacrificed by intraperitoneal injection of Beuthanasia-D solution (Schering-Plough Animal Health Corporation, Kenilworth, N.J.). The trachea was cannulated following a midline neck incision, and the lungs were lavaged 10 times with 1.0 ml of 0.9% sodium chloride solution containing 0.6 mM EDTA, penicillin (100 U/ml), streptomycin (100 μ g/ml), gentamicin (40 μ g/ml), and amphotericin B (0.5 μ g/ml). Approximately 7 to 8 ml of lavage fluid was obtained from each mouse. AMs were separated from the lavage fluid by centrifugation at $600 \times g$ for 10 min. Red blood cells were lysed with 10 mM KHCO₃ and 152 mM NH₄Cl. Cells were washed three times with normal saline and resuspended in DMEM (BioWhit-

taker, Inc., Walkersville, Md.) with 10% heat-inactivated fetal bovine serum, glutamine (300 μ g/ml), penicillin (100 U/ml), and 20 mM HEPES (pH 7.2). Cell preparations were demonstrated to be 98% pure AMs by routine Hema 3 staining, a Giemsa-like staining method (Biochemical Sciences, Inc., Bridgeport, N.J.), and to be greater than 95% viable by trypan blue exclusion. AMs were plated at a density of $2.5 \times 10^5/100 \mu$ l/well on rat immunoglobulin G (IgG) (Calbiochem, San Diego, Calif.)-coated 96-well tissue culture plates, incubated for 24 h at 37°C in 5% CO₂, and washed three times with antibiotic-free DMEM (200 μ l/well) to remove unattached cells. The plates were stored at 4°C for 1 h prior to the attachment assay.

***M. tuberculosis* attachment assay.** Attachment of *M. tuberculosis* organisms to AMs was determined as described previously by our laboratory (10). Briefly, freshly isolated *M. tuberculosis* organisms were incubated for 18 h in 1 ml of serum-free DMEM and 200 μ Ci of sodium chromate [⁵¹Cr] (New England Nuclear, Boston, Mass.). The ⁵¹Cr-labeled *M. tuberculosis* organisms were centrifuged ($3,800 \times g$, 15 min), the supernatant was discarded, and the pellet was washed four times to remove unincorporated ⁵¹Cr. A final concentration of 2.5×10^5 ⁵¹Cr-labeled *M. tuberculosis* organisms in 100 μ l of DMEM was added to each well of a 96-well tissue culture plate containing adherent AMs. After the incubation at 4°C, unbound organisms were removed by washing the monolayer cells three times with 200 μ l of DMEM. The washes from each well were pooled and centrifuged at $3,000 \times g$ for 30 min to separate unattached *M. tuberculosis* organisms from the supernatant. The supernatant was transferred to a separate tube. The adherent AMs in the 96-well plate containing bound *M. tuberculosis* were solubilized by the addition of 200 μ l of 10% Triton X-100 (Sigma Chemical Co.). The radioactivity in each fraction was measured in a gamma counter, and percent attachment was expressed as follows: % attachment = $(A/A + B) \times 100$, where *A* is ⁵¹Cr-labeled *M. tuberculosis* organisms bound to the AMs and *B* is unattached ⁵¹Cr-labeled *M. tuberculosis* organisms free in the medium. To determine percent injury to *M. tuberculosis* organisms, the percent release is expressed as follows: % release = $(A/(A + B + C)) \times 100$, where *B* is the number of counts per minute from ⁵¹Cr-labeled *M. tuberculosis* organisms bound to the AMs, *A* is the number of counts per minute from ⁵¹Cr-released into the medium, and *C* is the number of counts per minute from unattached ⁵¹Cr-labeled *M. tuberculosis* organisms free in the medium.

To determine the possible mechanisms involved in Fn-mediated attachment, ⁵¹Cr-labeled *M. tuberculosis* organisms were added to each well and incubated with the AMs at 4°C for 4 h. At 4°C only attachment (no phagocytosis) occurs. The attachment assay was performed to examine the effect of heparin (1.0 mM; Sigma Chemical Co.), RGDS (1.0 mM), RGES (1.0 mM), polyclonal antibodies to human Fn (100 μ g/ml), and MAbs to human Fn (100 μ g/ml; Chemicon, International) and in the presence or absence of appropriate isotype control MAbs (Chemicon, International) on *M. tuberculosis* attachment to AMs.

Statistical analysis. A minimum of three experiments conducted in triplicate were performed. The results are expressed as means \pm standard errors of the means (SEM). For each experiment the differences between control and experimental data were compared using Student's *t* test or analysis of variance with paired comparisons. Data were accepted as significantly different if *P* was <0.05 (22).

RESULTS

¹²⁵I-Fn binding to *M. tuberculosis*. ¹²⁵I-Fn binding to *M. tuberculosis* was time dependent, and this binding was saturated after 60 min (24.0 ± 2.5 ng). Fn binding was also concentration dependent, and saturation of binding was observed at or above Fn concentrations of 10 μ g/ml. Further, ¹²⁵I-Fn binding to *M. tuberculosis* was calcium concentration dependent, with saturation of binding occurring at or above 2 mM Ca²⁺ (51.3 ± 2.3 ng). These results indicate that ¹²⁵I-Fn binds to *M. tuberculosis* in time-, Fn concentration-, and Ca²⁺ concentration-dependent manners.

To determine the binding mechanism, ¹²⁵I-Fn and *M. tuberculosis* organisms were incubated in the presence or absence of Fn peptide (RGDS), heparin, and various MAbs directed against different sites on Fn (Fig. 1). Heparin and a MAb specific to the HBD of Fn significantly decreased Fn binding to *M. tuberculosis* organisms from 51.3 ± 2.3 ng to 30.9 ± 2.4 ng and to 21.9 ± 1.5 ng, respectively (*P* < 0.05) (Fig. 1). However,

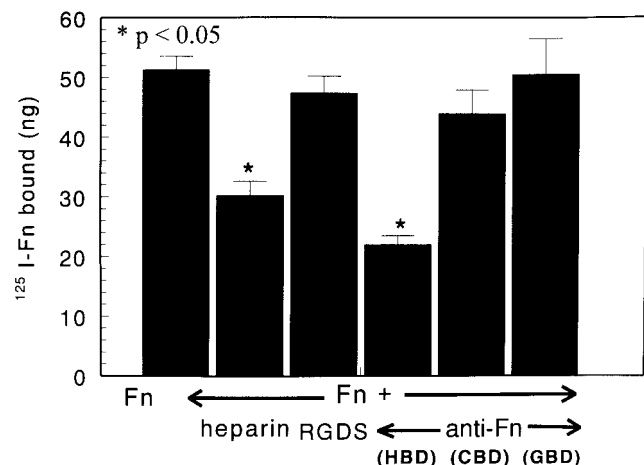


FIG. 1. Binding mechanism of ^{125}I -Fn binding to *M. tuberculosis*. Effect of RGDS, heparin anti-Fn MAbs to specific domains on ^{125}I -Fn binding to *M. tuberculosis*. ^{125}I -Fn was incubated with *M. tuberculosis* organisms in the presence or absence of RGDS or heparin or in the presence or absence of anti-Fn MAbs specific to the HBD, CBD, and GBD. After incubation, the pellet containing bound ^{125}I -Fn was subjected to evaluation in a gamma counter. The results indicate addition of heparin or MAbs to HBD significantly reduces ^{125}I -Fn binding to *M. tuberculosis*; however, RGDS, MAb to CBD, and MAb to GBD did not affect Fn binding. Results are expressed as means + SEMs (error bars) of three experiments performed in triplicate.

the binding of Fn to *M. tuberculosis* (51.3 ± 2.3 ng) in the presence of RGDS (47.4 ± 2.8 ng [$P > 0.05$]) and MAbs specific to either CBD (43.9 ± 3.2 ng [$P > 0.05$]) or GBD (50.5 ± 6.0 ng [$P > 0.05$]) resulted in no significant change in Fn binding (Fig. 1). There was no significant effect on ^{125}I -Fn binding to *M. tuberculosis* organisms in the presence of control isotype antibodies, IgG1 and IgM. Thus, these results suggest that the HBD, but not the CBD or GBD of Fn, is involved in Fn binding to *M. tuberculosis* organisms.

Fn-mediated attachment of *M. tuberculosis* to AMs. The attachment of *M. tuberculosis* to AMs in the absence of Fn increased as a function of time with maximal attachment occurring at 4 h (Fig. 2a). In the presence of Fn, attachment increased in a concentration-dependent manner (Fig. 2b), with maximal binding at approximately $10 \mu\text{g/ml}$ ($33.5\% \pm 3.4\%$). The binding pattern of *M. tuberculosis* to AMs in the presence of Fn appears to be second order. There was no significant killing of *M. tuberculosis* as a result of Fn-mediated attachment to AMs, as measured by ^{51}Cr release from *M. tuberculosis* (data not shown). Therefore, an Fn concentration of $10 \mu\text{g/ml}$ was used for all subsequent experiments.

The specificity of Fn-enhanced attachment of *M. tuberculosis* to AMs was examined in the presence of Fn and polyclonal antibodies to Fn. Addition of anti-Fn polyclonal antibodies to the attachment assay significantly decreased the Fn-enhanced attachment from $44.2\% \pm 4.2\%$ to $10.8\% \pm 1.2\%$ ($P < 0.05$) (Fig. 3). Use of nonspecific IgG or control isotype antibodies IgG1 and IgM did not affect *M. tuberculosis* attachment to AMs (data not shown).

To determine whether the attachment of *M. tuberculosis* to AMs is Ca^{2+} dependent, the attachment assay of *M. tuberculosis* to AMs was repeated in the presence or absence of EDTA and EGTA. Both EDTA and EGTA significantly decreased

Fn-enhanced attachment of *M. tuberculosis* to AMs from $44.2\% \pm 4.2\%$ to $8.4\% \pm 1.2\%$ and $9.7\% \pm 0.6\%$ ($P < 0.05$, both comparisons), respectively, indicating the requirement of Ca^{2+} for the Fn-enhanced attachment of *M. tuberculosis* to murine AMs. As the CBD and HBD sites for Fn are known to be Ca^{2+} dependent (15, 34), the Ca^{2+} dependency of *M. tuberculosis* binding to AMs is clearly multifactorial.

MAbs directed against the HBD, CBD, and GBD were added to the assay to determine the site(s) on the Fn molecule responsible for the Fn-mediated attachment of *M. tuberculosis* to AMs. Addition of a MAb specific to HBD significantly decreased the attachment of *M. tuberculosis* to AMs from $44.2\% \pm 4.2\%$ to $19.5\% \pm 1.2\%$ ($P < 0.05$). In addition, the MAb to CBD of Fn decreased attachment of *M. tuberculosis* to AMs from $44.2\% \pm 4.2\%$ to $22.6\% \pm 2.2\%$ ($P < 0.05$), re-

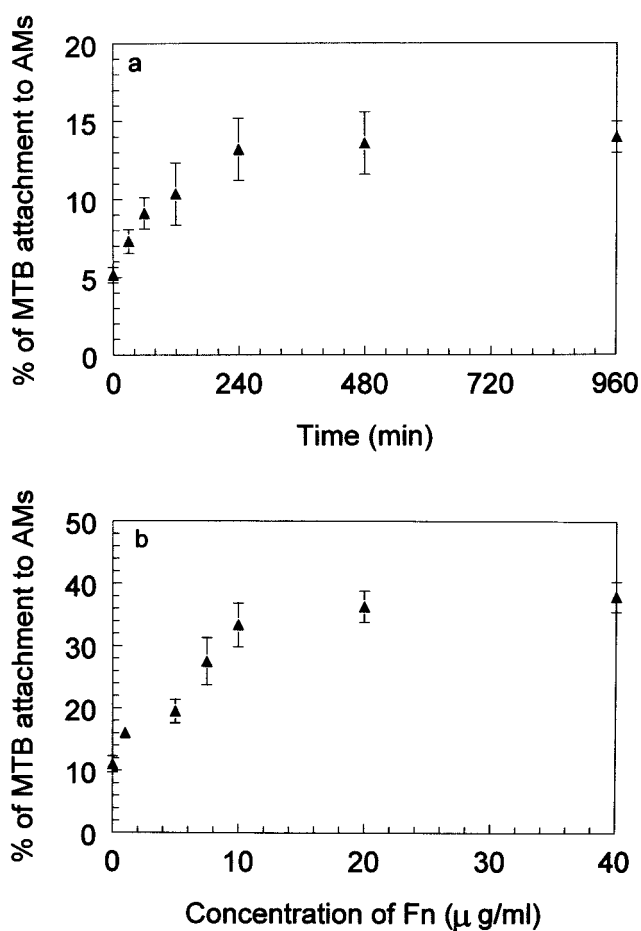


FIG. 2. Effects of time and Fn concentration on attachment of *M. tuberculosis* to AMs. (a) Attachment of ^{51}Cr -labeled *M. tuberculosis* organisms to murine AMs was quantified at different time intervals (0 to 16 h) at 4°C , and at this temperature only attachment occurs but not phagocytosis. (b) The effect of Fn on attachment was examined in the presence of increasing concentrations of human Fn (0 to $40 \mu\text{g/ml}$) at 4°C for 4 h. The attachment increased in the presence of increasing concentrations of Fn, and the maximum binding occurred at $10 \mu\text{g}$ of Fn/ml. The binding pattern of *M. tuberculosis* to AMs in the presence or absence of Fn appears to be second order. Experimental values were significantly different from the control values ($P < 0.05$). Results are expressed as means \pm SEMs (error bars) of three experiments performed in triplicate.

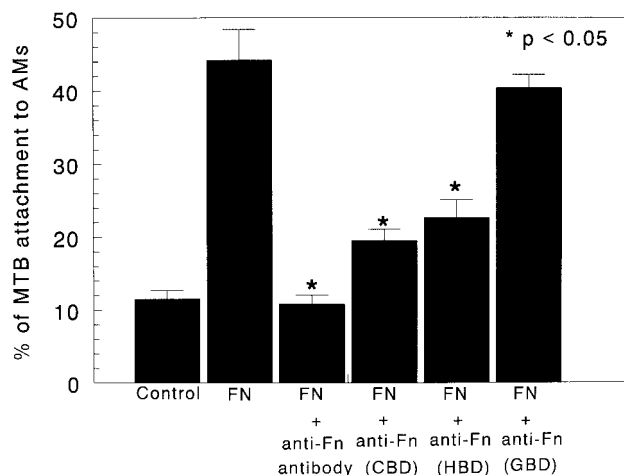


FIG. 3. Effects of anti-Fn polyclonal antibodies and MABs on in vitro attachment of *M. tuberculosis* to AMs. Fn-enhanced attachment of *M. tuberculosis* to AMs was examined in the presence of Fn and in the presence or absence of anti-Fn polyclonal antibodies and MABs directed against the CBD, HBD, and GBD of Fn. Anti-Fn polyclonal antibody significantly decreased the Fn-enhanced attachment of *M. tuberculosis* to AMs ($P < 0.05$). Addition of a MAB specific to either CBD or HBD significantly decreased the attachment of *M. tuberculosis* to AMs ($P < 0.05$), whereas MABs specific to GBD showed no effect on attachment of *M. tuberculosis* to AMs. Results are expressed as means \pm SEMs (error bars) of three experiments performed in triplicate.

spectively, whereas MAB specific to GBD showed no significant effect on *M. tuberculosis* attachment to AMs ($40.5\% \pm 1.8\%$ [$P > 0.05$]) (Fig. 3). Thus, these results suggest that Fn-enhanced attachment of *M. tuberculosis* to AMs is mediated, in part, by the HBD of the Fn molecule recognizing *M. tuberculosis* and the CBD of Fn recognizing a surface receptor on AMs (27).

Attachment of *M. tuberculosis* to AMs was significantly decreased in the presence of heparin from $44.2\% \pm 4.2\%$ to $11.8\% \pm 1.1\%$ ($P < 0.05$) and in the presence of RGDS peptide to $15.3\% \pm 1.2\%$ ($P < 0.05$) (Fig. 4). However, the false binding peptide, RGES, had no significant effect ($40.5\% \pm 1.8\%$ [$P > 0.05$]) on *M. tuberculosis* attachment to AMs (Fig. 4). Taken together, these data suggest that Fn may act as a bridge between the microorganisms and the AMs. These data suggest that the HBD of Fn binds to *M. tuberculosis*, whereas the CBD of Fn binds to the AMs.

DISCUSSION

This study suggests that Fn binds to *M. tuberculosis* and that Fn can mediate attachment of *M. tuberculosis* to murine AMs. A MAB specific to the HBD of Fn, but not to the CBD or GBD of Fn, decreased binding of Fn to *M. tuberculosis*, suggesting that the HBD is the site on the Fn molecule responsible for Fn binding to *M. tuberculosis*. Fn-enhanced attachment of *M. tuberculosis* to murine AMs was decreased by the addition of MABs specific to either the HBD or CBD or by RGDS, suggesting that the CBD of Fn likely plays a role in the Fn-mediated attachment of *M. tuberculosis* to AMs. Therefore, Fn may act as a bridge between *M. tuberculosis* and the AM. As Fn is a constitutively expressed protein in the lung (7) and is

increased during inflammation (16), this interaction may represent a normal host response in the initial week of new infection in the alveolar spaces.

Fn is a large adhesive matrix protein with multiple binding domains known to mediate the adherence of many pathogenic microorganisms in a ligand receptor-mediated manner (17). Examples include *E. coli*, *S. pyogenes*, *S. enterica* serovar Dublin, *C. albicans*, *S. aureus*, *T. pallidum*, and *P. carinii* (14, 33, 35, 36, 39, 46, 47). For instance, previous studies from our laboratory have shown that *P. carinii* binds to Fn via the RGDS binding sites of Fn, and Fn appears to mediate the attachment of *P. carinii* to AMs with the RGDS binding site on the other arm of Fn molecule (34). As AMs are important in clearance of this extracellular pathogen from alveolar spaces (26), Fn likely is an important mediator in host defense in *P. carinii* pneumonia.

The present study reveals that Fn binds to *M. tuberculosis* via the HBD of the Fn molecule. Fn binding to *M. tuberculosis* was inhibited by a MAB specific to the HBD of the Fn molecule to levels comparable to that obtained with anti-Fn polyclonal antibodies. This indicates the importance of the HBD of the Fn molecule in Fn-mediated binding to *M. tuberculosis*. In contrast, MABs to CBD and GBD had little effect on Fn binding to *M. tuberculosis*. Further, Fn binding to *M. tuberculosis* could be inhibited by heparin, suggesting the involvement of the HBD of the Fn molecule. A recent study suggests that the *Mycobacterium bovis* BCG interacts with Fn via the C-terminal region adjacent to the heparin binding domain of the Fn molecule (5). Since the type of proteins that bind to the HBD are proteoglycans (30), it is possible that the Fn-binding proteins present on the surface of *M. tuberculosis* are also proteoglycans.

Previous studies suggest that the BCG interacts with Fn via the C-terminal region adjacent to the HBD of the Fn molecule (5). α antigen, also known as 85B secreted protein, of myco-

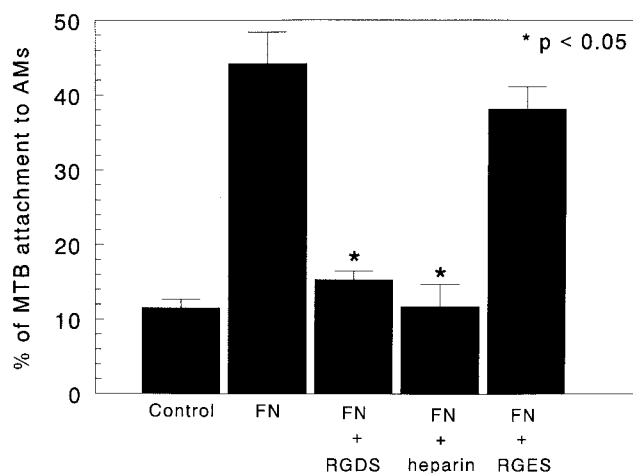


FIG. 4. Effects of RGDS, RGES, and heparin on in vitro attachment of *M. tuberculosis* to AMs. *M. tuberculosis* organisms were incubated with murine AMs at 4°C for 4 h in the presence or absence of tetrapeptide RGDS, a control tetrapeptide (RGES), and heparin. Addition of RGDS or heparin significantly decreased the attachment of *M. tuberculosis* to AMs ($P < 0.05$). However, RGES did not have a significant effect on the attachment. Results are expressed as means \pm SEMs (error bars) of three experiments performed in triplicate.

bacteria interacts with either the C-terminal HBD or the more central CBD (29). In contrast, other studies have shown that gelatin but not heparin inhibited the binding of Fn to 85B protein (32). The gelatin binding site is located on the collagen binding domain of Fn. These studies suggest that the 85B complex protein possesses multiple binding affinities for Fn.

Attachment of *M. tuberculosis* to murine AMs was enhanced in the presence of exogenous Fn. This enhanced attachment was competitively inhibited by the addition of the synthetic peptide RGDS, the tetrapeptide sequence for the CBD of the Fn molecule. Further, addition of MAbs directed against the CBD and HBD decreased Fn-enhanced attachment, indicating that the CBD and HBD of the Fn molecule are involved in the *M. tuberculosis* attachment to AMs. As expected, attachment of *M. tuberculosis* to AMs was Ca²⁺ dependent, a property required for the functioning of both the HBD and CBD of the Fn molecule. Others have suggested that Fn may mediate mycobacterial attachment. For instance, mycobacteria have been shown to attach via Fn to epithelial and Schwann cells (44). Further, in vivo studies have indicated that Fn may facilitate the uptake of BCG by bladder epithelium (20). Fn-mediated interactions may involve different sites on the Fn molecule. Our study shows Fn can increase attachment of *M. tuberculosis* to AMs; however, increased attachment does not necessarily mean there is an increase in phagocytosis by AMs (14).

In all likelihood *M. tuberculosis* attachment and phagocytosis are multifactorial, involving multiple mechanisms and receptors (12, 40). *M. tuberculosis* organisms have developed a number of mechanisms to gain entry into the macrophages using specific cell surface receptors (12). Like other microorganisms, *M. tuberculosis* binds to CR1, CR3, and CR4, which in turn results in the phagocytosis of the bacilli and entry into the phagosomes (42). *M. tuberculosis* can enter macrophages through the mannose receptors (41). Surfactant apoproteins such as surfactant protein A (SP-A) or SP-D may also facilitate attachment and/or phagocytosis of *M. tuberculosis* to macrophages (13). Our study clearly demonstrates that Fn enhances attachment of *M. tuberculosis* to AMs. The lung is a rich source of Fn, and Fn production increases in response to infection or inflammation (7). Multiple studies suggest that Fn plays a role in recognition of various species of mycobacteria. No studies have yet delineated which of these several mechanisms predominate in vivo. Because mycobacteria are intracellular pathogens, some mechanisms of attachment may enhance infection, whereas others may facilitate clearance of mycobacteria (4, 8, 31).

In summary, these Fn binding studies suggest that Fn binds to the *M. tuberculosis* organisms, likely through its HBD, whereas Fn-mediated binding of *M. tuberculosis* to the AM likely occurs through the CBD of the Fn molecule. Thus, Fn may promote the attachment of *M. tuberculosis* to AMs by acting as a bridge between the organism and the host cell. Further insights into the mechanisms of *M. tuberculosis* attachment to AMs may provide important information in the pathogenesis of this disease and may permit the development of therapeutic strategies to modulate this infectious process.

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