Identification of a Heparin-binding Hemagglutinin Present in Mycobacteria

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Summary

Adherence to mammalian host tissues is an important virulence trait in microbial pathogenesis, yet little is known about the adherence mechanisms of mycobacteria. Here, we show that binding of mycobacteria to epithelial cells but not to macrophages can be specifically inhibited by sulfated carbohydrates. Using heparin-Sepharose chromatography, a 28-kD heparin-binding protein was purified from culture supernatants and cell extracts of *Mycobacterium bovis* and *Mycobacterium tuberculosis*. This protein, designated heparin-binding hemagglutinin (HBHA), promotes the agglutination of rabbit erythrocytes, which is specifically inhibited by sulfated carbohydrates. HBHA also induces mycobacterial aggregation, suggesting that it can mediate bacteria-bacteria interactions as well. Hemagglutination, mycobacterial aggregation, as well as attachment to epithelial cells are specifically inhibited in the presence of anti-HBHA antibodies. Immunoelectron microscopy using anti-HBHA monoclonal antibodies revealed that the protein is surface exposed, consistent with a role in adherence. Immunoblot analyses using antigen-specific antibodies indicated that HBHA is different from the fibronectin-binding proteins of the antigen 85 complex and p55, and comparison of the NH_2-terminal amino acid sequence of purified HBHA with the protein sequence data bases did not reveal any significant similarity with other known proteins. Sera from tuberculosis patients but not from healthy individuals were found to recognize HBHA, indicating its immunogenicity in humans during mycobacterial infections. Identification of putative mycobacterial adhesins, such as the one described in this report, may provide the basis for the development of new therapeutic and prophylactic strategies against mycobacterial diseases.

Members of the mycobacterial genus are among the most prominent pathogens causing infectious disease in both humans and animals. Tuberculosis, caused by *Mycobacterium tuberculosis*, results in ~3 million deaths every year (1, 2). Leprosy, a result of infection with *Mycobacterium leprae*, remains a significant unsolved health problem in the developing world (3). Infections by members of the *Mycobacterium avium-intracellulare* complex are among the most frequent opportunistic infections found in patients suffering from acquired immunodeficiency syndrome (4, 5). In addition, the recent dramatic resurgence of tuberculosis in developed countries together with the emergence and spread of drug-resistant *M. tuberculosis* strains (6) highlights the difficulties in controlling mycobacterial diseases.

Despite the importance of mycobacterial infections, little is known about the primary molecular mechanisms involved in the pathogenesis of these diseases (7). One of the initial events in bacterial pathogenesis is the adherence of the microorganism to its target tissues. Although mycobacteria have a tropism for pulmonary macrophages (8), interactions with epithelial cells or with the extracellular matrix (ECM)[1] during initial adherence and subsequent colonization steps of pathogenesis may be critical events (9, 10). There is increasing evidence that interactions with epithelial cells constitute important steps in the pathogenesis of mycobacterial diseases which could result in extrapulmonary dissemination of these microorganisms (11, 12).

Many pathogens, including respiratory pathogens, have recently been shown to produce heparin-binding adhesins that interact with sulfated glycoconjugates that are present on the surface of virtually all epithelial cells and in the

[1] *Abbreviations used in this paper:* CHO, Chinese hamster ovary cells; ECM, extracellular matrix; FHA, filamentous hemagglutinin; GAG, glycosaminoglycans; HBHA, heparin-binding hemagglutinin.

ECM (13). There have been several examples of the use of sulfated glycosaminoglycans (GAG) as ligands for viruses (14–16), parasites (17–19), mycoplasma (20), and bacteria (21–24). The frequency and ubiquitous distribution of sulfated glycoconjugates on cell surfaces and in the ECM make them well-suited for microbial attachment and tissue penetration.

In this study, we have identified and partially characterized a novel 28-kD surface-exposed heparin-binding hemagglutinin (HBHA) produced by *M. bovis* and *M. tuberculosis* and provide evidence that it mediates bacteria-epithelial cell interactions. This protein is also able to induce mycobacterial auto-aggregation which suggests that it could promote mycobacterial colonization at the site of attachment of the pathogen to host tissues.

Materials and Methods

Mycobacterial Adherence Assays. Exponentially growing *M. bovis* BCG (strain 1173P2; World Health Organization, Stockholm, Sweden, passage 3 to 8) were labeled by culturing the mycobacteria for 3 d in Sauton medium containing 5 μCi/ml [6-^{3}H]uracil (24 Ci/mmol; DuPont/New England Nuclear, Boston, MA). The mycobacteria were then harvested by centrifugation (3000 *g* for 5 min), washed twice with Dulbecco's phosphate-buffered saline (DPBS), and resuspended in RPMI 1640 culture medium containing 300 mg/l L-glutamine (GIBCO BRL, Gaithersburg, MD) without added fetal serum (RPMI). The day before the adherence assay, the wells of 24-well tissue culture trays (Nunclon, Nunc, Denmark) were seeded with 10^5 freshly grown Chinese hamster ovary (CHO) cells or J774A.1 macrophage cells (TIB67; American Type Culture Collection, Rockville, MD) resuspended in 2 ml RPMI supplemented with 10% (vol/vol) decomplemented fetal calf serum (RPMI-FCS). The cells were washed three times with 2 ml RPMI, and 1 ml of mycobacterial suspension in RPMI was added to each well to obtain a multiplicity of infection of an estimated 10 bacteria per cell. The adherence assay was performed in the presence of increasing concentrations of D(+)galactose (Sigma Chem. Co., St. Louis, MO) or porcine intestine mucosal heparin (M_r 6 kD; Sigma), or with 20 μg/ml of the indicated carbohydrates (Sigma). After 6 h of incubation at 37°C in 5% CO_2, the cells were washed three times with 2 ml DPBS, and lysed by adding 1 ml distilled water containing 0.1% (wt/vol) sodium deoxycholate. The radioactivity associated with the cellular lysates was determined using a liquid scintillation counter (model LS 6000SC; Beckman Instruments, Carlsbad, CA). Residual adherence is expressed as the percent of radioactivity relative to that obtained in the absence of carbohydrate or antibodies, assayed in quadruplicate. For monoclonal antibody (mAb) inhibition experiments, radiolabeled *M. tuberculosis* H37Ra or BCG were first incubated with a serial dilution of purified mAb 4057D2 (25) or an irrelevant mAb and then assayed for attachment as described above after a 90-min incubation period.

Purification of a Heparin-binding Protein from M. bovis BCG. *M. bovis* BCG was grown in static cultures at 37°C using 175 cm^2 Roux flasks (Falcon; Becton-Dickinson, Franklin Lakes, NJ) containing ~150 ml of Sauton medium. At stationary phase, the cultures were centrifuged (10,000 *g* for 20 min), and 500 ml of supernatant was passed through a heparin-Sepharose CL-6B (Pharmacia LKB, Piscataway, NJ) column (1 × 5 cm) equilibrated with DPBS. The column was then washed with 100 ml DPBS, and the bound material was eluted by a 0–500 mM NaCl gradient in 100 ml DPBS. The flow rate during all steps was maintained at 1.5 ml per min, and absorbance at 280 nm was continuously monitored. The eluted 1-ml fractions were analyzed by SDS-PAGE using a 12% gel (26) followed by Coomassie Brilliant Blue R-250 staining.

Mycobacterial Cell Wall Extract Preparations. Mycobacteria were grown in 2 l of Sauton or Long's synthetic medium (Quality Biological Inc., Gaithersburg, MD) until late-log phase. The bacteria were then pelleted by centrifugation, washed once in DPBS containing 0.05% Tween 80 (DPBS/Tw), resuspended in 100 ml of DPBS/Tw, and heated at 80°C for 1 h. The bacteria were centrifuged at 13,000 *g* for 20 min, washed with DPBS/Tw, resuspended in 25 ml of DPBS/Tw containing 5 mM of protease inhibitor 4-(2-aminomethyl)-benzenesulfonyl fluoride hydrochloride, RNaseA and DNaseI. The mixture was sonicated intermittently for 25 min on ice, and then centrifuged at 13,000 *g* for 20 min. The sonication and centrifugation steps were repeated on the cell pellet, and the supernatants were pooled and centrifuged at 34,000 *g* for 3 h. The pellet was discarded, and the final supernatant was diluted 1:2 in DPBS, chromatographed over heparin–Sepharose CL-6B, and eluted as described above.

Hemagglutination Activity. The hemagglutination activity of HBHA purified from culture supernatants was assayed in U-shaped microtiter plates (Falcon; Becton-Dickinson, Franklin Lakes, NJ) in the absence or presence of porcine intestine mucosal heparin, dextran sulfate or dextran at concentrations ranging from 0 to 50 μg/ml. Standard assays contained 70 μl of a 5% fresh rabbit erythrocyte suspension prepared in DPBS and 70 μl of purified HBHA corresponding to 1 μg of protein. Hemagglutination titers were read after 5 h of incubation at room temperature. For mAb inhibition studies, serial dilutions of purified mAb 4057D2 or an irrelevant mAb were pre-incubated with HBHA and hemagglutination was determined as described above.

Auto-aggregation Activity. Bacteria were washed three times using DPBS and centrifuged for 5 min at 200 *g* to remove large aggregates of bacteria. Resuspended bacteria were sonicated for 10 s and passed through a 22-gauge needle five times. Purified HBHA was serially diluted in 100 μl of DPBS in U-bottom microtiter plates (Nunclon, Nunc, Denmark), and 100 μl of an estimated 10^8 bacteria per ml were added to the wells. Plates were agitated and examined macroscopically for aggregation after a 30-min incubation at room temperature.

Electron Microscopy. Mycobacteria were harvested and washed in DPBS as described above and fixed in 2% paraformaldehyde at room temperature overnight. Bacteria were incubated in DPBS containing 0.1 M glycine to remove residual fixative, washed in DPBS containing 0.5% BSA (DPBS/BSA), and cell pellets were incubated with 50 μl of 3921E4 or 4057D2 ascites (25) in 1 ml of DPBS/BSA for 2 h at room temperature. After two washes with DPBS/BSA, the bacteria were incubated for 1 h at room temperature with goat anti–mouse IgG-G10 (Amersham Life Sciences, Arlington Heights, IL) diluted 1:3 in DPBS/BSA. Cells were fixed overnight in 2% glutaraldehyde/2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.3. The fixative was then removed and the samples stored in DPBS containing 4% sucrose at 4°C until they were subsequently dehydrated with graded alcohols and embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate and examined with a EM 912 electron microscope (Carl Zeiss Inc., Thornwood, NY).

Antibody Production. Rat anti-HBHA polyclonal antibodies were prepared as follows: 200 μg of purified BCG HBHA was subjected to preparative electrophoresis on a 15% polyacrylamide gel

in the presence of SDS and then electro-transferred onto a nitrocellulose membrane. After rapid staining with Ponceau Red, the band corresponding to HBHA was carefully excised, cut into small squares, and briefly sonicated in 1.5 ml sterile DPBS. After the addition of 1 ml of a monophosphoryl lipid A solution (MPL + TDM Adjuvant System; Sigma) prepared according to the recommendation of the manufacturer, two Fischer rats were each immunized with 1 ml of the antigen suspension. For each animal, 400 μl were given intraperitoneally, followed by two subcutaneous injections of 300 μl. The animals were boosted the same way with the same amount of antigen one month later, and the serum was collected 3 wk after the boost. The establishment of hybridomas producing the mAbs 4057D2 and 3921E4 has been described elsewhere (25). mAb 4057D2, IgG_3 isotype, was purified by cryoprecipitation using dialysis in 0.01M Tris (pH 8) buffer and mAb 3921E4, IgG_{2a} isotype, was purified by protein A–Sepharose chromatography. Purified mAbs BPG10 and X3E were obtained from the anti-*Bordetella pertussis* antibody collection of CBER, FDA.

Analytical Procedures. Immunoblot analyses were performed using standard procedures as described by Harlow and Lane (27). To identify the presence of anti-HBHA antibodies in human sera, nitrocellulose membranes containing 5 μg of purified HBHA per lane were probed with a 100-fold dilution of human sera and detected as described above (27). Protein concentrations were determined by the method of Bradford (28) using BSA as a standard. NH_2-terminal amino acid sequencing was done as follows: 25 μg of HBHA was subjected to SDS-PAGE using a 15% polyacrylamide gel and after electrophoresis the material was transferred onto a PVDF membrane (ProBlott; ABI, Foster City, CA) by electroblotting. After staining with Coomassie Blue, the band corresponding to HBHA was excised and submitted to automated Edman degradation. A database search in Swiss-PROT for 100% identity was performed.

Results

Inhibition of Mycobacterial Adherence to Epithelial Cells by Sulfated Carbohydrates. To determine if mycobacteria can adhere to eukaryotic cells via sulfated glycoconjugates, we tested whether soluble carbohydrates reduce adherence of *M. bovis* BCG to epithelial cells. As shown in Fig. 1 *A*, as little as 10 μg per ml of heparin significantly inhibited [^{3}H]uracil-labeled BCG adherence to CHO cells, whereas no effect was observed with the addition of up to 100 μg per ml of the non-sulfated sugar D(+)galactose. While heparin-mediated inhibition of adherence was saturable, the inhibition of adherence did not exceed 70%, indicating that other mechanisms of attachment are also involved. Dextran sulfate, fucoidan and chondroitin sulfate, at a sugar concentration of 20 μg per ml, were also able to reduce adherence, but no significant inhibition was observed using the non-sulfated sugars dextran, mannose, or galactose (Fig. 1 *B*). Interestingly, interactions of BCG with J774A.1 macrophage-like cells were not affected by the sulfated carbohydrates, nor by non-sulfated sugars (Fig. 1 *B*), even at concentrations up to 1 mg per ml (data not shown). This is consistent with previous reports implicating complement receptors CR1, CR3, and CR4 as mycobacterial ligands on the surface of blood monocytes and alveolar macrophages (29–31).

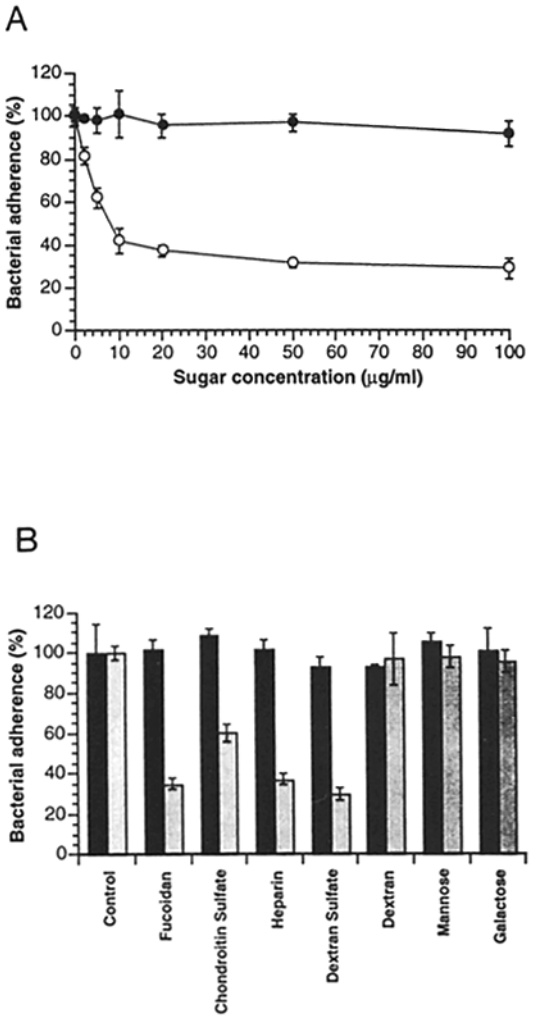

Figure 1. Effect of sulfated and non-sulfated carbohydrates on mycobacterial adherence to CHO cells and macrophages. Exponentially growing *M. bovis* BCG were metabolically labeled and incubated with freshly grown CHO cells (*A* and *B*, *gray bars*) or J774A.1 macrophages (*B*, *black bars*). The adherence assay was performed in the presence of increasing concentrations of D(+)galactose (*A*, *black circles*), porcine intestine mucosal heparin (*A*, *white circles*), or with 20 μg/ml of the indicated carbohydrates (*B*). After 6 h of incubation, the radioactivity associated with the cellular lysates was determined, and residual adherence was expressed as percent of cpm relative to the cpm obtained in the absence of carbohydrate (control). The data represent averages for quadruplicate experiments, and standard deviation bars are shown.

Purification of a Heparin-binding Protein. The inhibition of BCG adherence to epithelial cells by sulfated sugars suggests that mycobacteria express an adhesin which interacts with sulfated glycoconjugates on eukaryotic cell surfaces. As has been shown for other bacteria (32), surface-exposed adhesins may be partially released into the culture medium. Therefore, in order to identify putative adhesins, BCG culture supernatants were fractionated by heparin–Sepharose chromatography. The bound material was eluted with a NaCl gradient, and the eluted fractions were analyzed by SDS-PAGE. As shown in Fig. 2, a 28-kD protein was

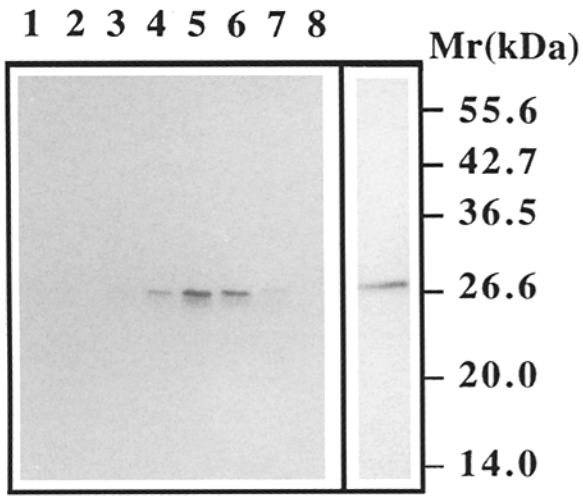

Figure 2. Purification of a heparin-binding protein from *M. bovis* BCG. *M. bovis* BCG was grown to stationary phase, and 500 ml of supernatant were loaded onto a heparin-Sepharose CL-6B column. After washing, the column was eluted with a 0–500 mM NaCl gradient, and eluted fractions were analyzed by SDS-PAGE followed by Coomassie-Blue staining. Fractions eluted in the protein peak at ~350 mM NaCl are shown in lanes *1–8*. The right lane shows the heparin-binding protein purified from a *M. bovis* BCG cell extract preparation. Migration of the molecular mass markers is illustrated in the right margin.

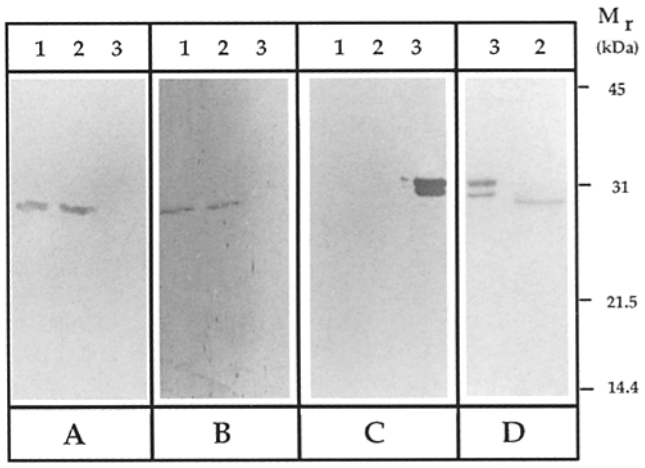

Figure 3. Comparison of the *M. bovis* heparin-binding protein with the 85 antigen complex. The heparin-binding protein purified from *M. bovis* BCG cell extracts (lanes *1*) or culture supernatant (lanes *2*) was compared with the purified antigen 85 complex (lanes *3*) by immunoblot analysis (*A–C*) and Coomassie Blue staining (*D*) after SDS-PAGE. Immunoblot analyses were performed using polyclonal antibodies raised against the purified heparin-binding protein (*A*), the anti-HBHA mAb 4057D2 (*B*), or polyclonal antibodies raised against the antigen 85 complex (*C*). Lanes *1* and *2* of *A–C* contain 2 μg of purified protein, lanes *3* of *A–C* contain 7 μg of purified protein, lanes *2* and *3* of *D* contain 4 and 15 μg of purified protein, respectively. The position of the molecular mass markers is shown in the margin.

eluted at ~350 mM NaCl. The interaction between this protein and heparin appears to be dependent on the sugar sulfation, because it could also be purified using dextran sulfate beads, but not with dextran beads (data not shown).

To determine whether this protein is strictly secreted or also surface associated, cell wall extracts from BCG were prepared and then chromatographed over heparin–Sepharose. As shown in Fig. 2, the 28-kD heparin-binding protein was also present in these preparations, suggesting that it may be surface-associated. A similar size heparin-binding protein was also purified from *M. bovis* Ravenel and *M. tuberculosis* H37Ra cell wall extracts and from tuberculin (purified protein derivative skin test antigen) preparations derived from culture filtrates of *M. tuberculosis* (data not shown).

Cell Surface Localization of the Mycobacterial Heparin-binding Protein. The presence of the 28-kD heparin-binding protein in culture supernatants and cell wall extracts suggest that it may be located at the cell surface and partially released into the culture supernatant. Since the protein does not appear to be species specific, a bank of mAbs directed against *M. avium* antigens (25) was screened to identify antibodies reactive with the heparin-binding protein. Two of the mAbs (4057D2 and 3921E4) recognized the 28-kD protein isolated from BCG (Fig. 3 *B*), from *M. tuberculosis* and from *M. bovis* (data not shown).

These antibodies were then used for the immunoelectron microscopy studies to investigate the localization of this protein on the bacteria. As shown by the gold-labeling in Fig. 4, *A* and *B*, the heparin-binding protein was found to be surface-exposed on *M. tuberculosis* H37Ra, consistent with the fractionation experiments and with a potential role of this surface protein in adherence.

Identification of the Heparin-binding Protein as a Novel Mycobacterial Antigen. Since the size of the 28-kD heparin-

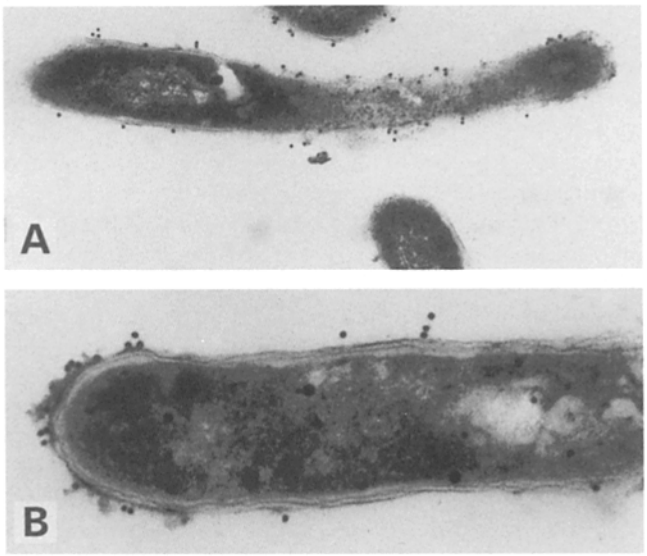

Figure 4. Immunoelectron micrographs of *M. tuberculosis*. *A* and *B* show *M. tuberculosis* H37Ra after incubation with mAbs that specifically recognize HBHA followed by gold-labeled (10 nm) goat anti-mouse immunoglobulin. *A* (×29,500) using mAb 3921E4 and *B* (×61,200) using mAb 4057D2 illustrate that the surface of the bacterium is uniformly labeled.

Table 1. *Inhibition of Hemagglutination, Mycobacterial Adherence, and Mycobacterial Aggregation by Anti-HBHA Monoclonal Antibodies*

	Bioassay*	mAb‡	Concentration of mAb (μg/ml) for maximum inhibition§
I	Agglutination of RBC	4057D2	10
		BPG10	>150
II	Adherence of *M. tb* H37Ra	4057D2	5
	to CHO cells	BPG10	>50
III	Aggregation of *M. tb* H37Ra	4057D2	2.5
		X3E	>100

*Bioassays were performed as described in Materials and Methods using 1 μg/ml of HBHA for the hemagglutination and aggregation assays and radiolabeled-H37Ra bacteria for the adherence assay.

‡4057D2 is purified IgG mAb directed against HBHA of mycobacteria; BPG10 is purified IgG mAb directed against lipooligosaccharide of *B. Pertussis;* X3E is purified IgG mAb directed against the *B. pertussis* adhesin filamentous hemagglutinin.

§Maximum inhibition is the concentration of mAb that gives a complete inhibition of hemagglutination and aggregation, or inhibits attachment of mycobacteria to CHO cells by 50%.

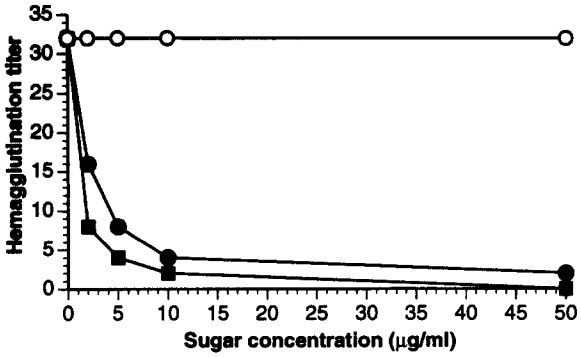

Figure 5. Effect of sulfated and non-sulfated carbohydrates on HBHA-mediated hemagglutination. The hemagglutination activity of the BCG HBHA purified from culture supernatants was assayed in the presence of porcine intestine mucosal heparin (*black squares*), dextran sulfate (*black circles*) or dextran (*white circles*) at concentrations ranging from 0 to 50 μg/ml. Hemagglutination titers were read after 5 h of incubation at room temperature. The data represent averages for quadruplicate experiments, and standard deviation bars are shown.

binding protein is similar to that of the fibronectin-binding antigen 85 complex proteins (33), immunoblot analysis was used to determine whether they are related. Fig. 3 shows that polyclonal and mAbs directed against the 28-kD protein did not recognize the purified antigen 85 complex proteins. Conversely, polyclonal as well as mAbs (data not shown) raised against the BCG antigen 85 complex failed to recognize the 28-kD protein, implying that they are distinct proteins. This finding is also supported by the different migration patterns of these proteins during SDS-PAGE (Fig. 3 *D*). Similar experiments using antibodies directed against the p55 *Mycobacterium vaccae* FAP protein (34) indicate no antigenic cross-reactivity between this fibronectin-binding protein and the 28-kD heparin-binding protein (data not shown).

To further characterize the 28-kD protein, it was subjected to NH_2-terminal amino acid sequencing after purification from BCG. The first 16 amino acids were Ala-Glu-Asn-Ser-Asn-Ile-Asp-Asp-Ile-Lys-Ala-Pro-Leu-Leu-Ala-Ala. The NH_2-terminal amino acids of the heparin-binding protein purified from *M. tuberculosis* H37Ra cell wall extracts were also determined and found to be identical to that of the protein purified from BCG. A similarity search in the protein data bases revealed that this amino acid sequence has not been previously identified, which indicates that the 28-kD protein is a novel mycobacterial protein.

Hemagglutination and Bacterial Attachment Activity of the Mycobacterial Heparin-binding Protein. The ability of bacterial adhesins to agglutinate erythrocytes is often used as a model to study lectin-like microbial attachment to eukaryotic cell receptors (32). We therefore tested the purified heparin-binding protein for its ability to agglutinate erythrocytes. Less than 0.1 μg of purified protein was able to induce the hemagglutination of rabbit erythrocytes, but not sheep, goose, chicken, or human erythrocytes (data not shown). This hemagglutination was completely inhibited in the presence of 10 μg per ml of purified mAb 4057D2 (Table 1). For these reasons we will refer to this protein as HBHA. HBHA-induced hemagglutination was also inhibited by heparin or dextran sulfate, but not by dextran (Fig. 5), similar to the results obtained in the CHO cell adherence assay.

To establish whether mycobacterial adherence to epithelial cells was HBHA mediated, CHO cells were incubated with radiolabeled BCG in the presence of increasing amounts of anti-HBHA polyclonal antibodies or non-relevant polyclonal antibodies. Adherence of BCG to CHO cells was inhibited by ~45% in the presence of anti-HBHA antibodies. Similar experiments using *M. tuberculosis* H37Ra also indicated that anti-HBHA mAb inhibit adherence (Table 1). Attachment was inhibited by 50% in the presence of 5 μg per ml of purified mAb 4057D2 but not by >50 μg per ml of the irrelevant IgG mAb BPG10. These observations indicate that adherence of mycobacteria to epithelial cells is mediated at least in part by HBHA.

Mycobacterial Auto-aggregation Induced by HBHA. Some adhesins also induce auto-aggregation of bacteria (35, 36), and many mycobacterial species grown in vitro readily form aggregates. Fig. 6 demonstrates that *M. tuberculosis* can be aggregated in a dose-dependent manner by the addition of HBHA, with maximum aggregation starting at a concentration of 0.5 μg HBHA per ml. In contrast, 20 μg/ml of purified lipoarabinomannan did not induce aggregation of the bacteria in this assay. Bacterial clumping was completely inhibited by 2.5 μg/ml of the 4057D2 mAb (Table 1),

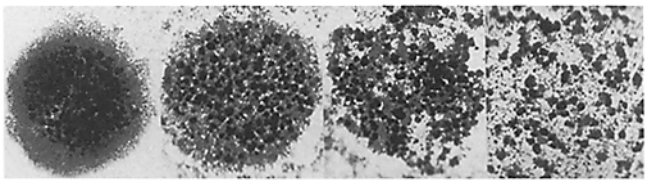

Figure 6. HBHA-mediated aggregation of *M. tuberculosis*. Aggregation of *M. tuberculosis* H37Ra was induced by incubating 10^8 bacteria/ml with 5, 1.25, 0.15, or 0 μg protein/ml final concentration (*left to right*) of HBHA purified from *M. tuberculosis* H37Ra.

suggesting that HBHA is directly involved in auto-agglutination.

Immune Reactivity against HBHA in Antisera from Tuberculosis Patients. To determine if HBHA is able to induce an immune response in humans, immunoblot analyses were carried out using purified HBHA and human antisera from tuberculosis patients. Nitrocellulose membranes containing HBHA were probed with 100-fold diluted sera from seven different patients diagnosed with active tuberculosis that had not been vaccinated with BCG. As shown in Fig. 7, all the sera from the tuberculosis patients contained antibodies that recognize the 28-kD HBHA, whereas the serum from a healthy individual did not contain antibodies against HBHA. Four additional sera from healthy individuals showed no reaction in this immunoblot assay. These results indicate that the surface exposed HBHA is immunogenic during the course of tuberculosis disease.

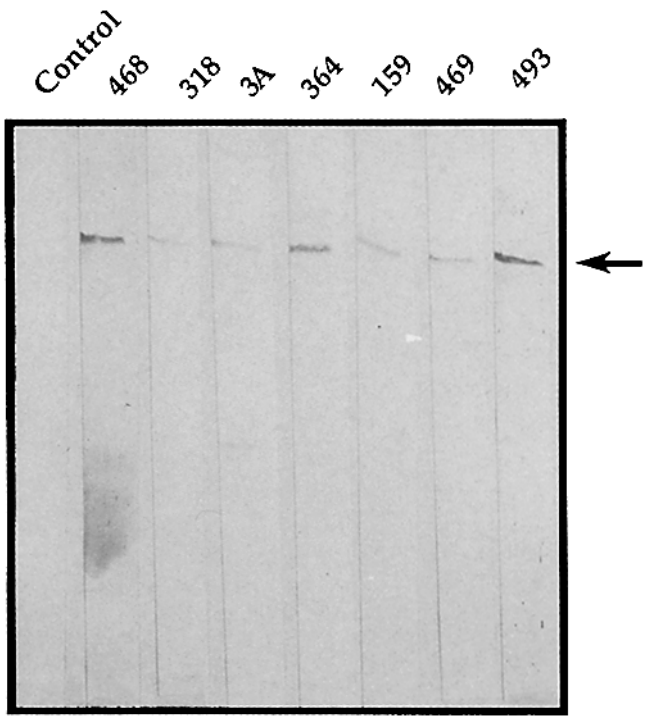

Figure 7. Immunoblot analysis using antisera from tuberculosis patients. Sera from seven patients diagnosed with active tuberculosis (lanes labeled 468 through 493) as well as serum from a healthy subject (control lane) were incubated with nitrocellulose strips containing 5 μg of HBHA and reactivity was visualized as described in Materials and Methods. The arrow denotes the position of purified HBHA.

Discussion

In this study we describe a novel surface-associated heparin-binding adhesin in mycobacteria. As is the case for many bacterial adhesins, this protein expresses hemagglutination activity and is therefore referred to as HBHA. HBHA was purified by heparin–Sepharose chromatography from both culture supernatants and cell wall extracts of BCG and *M. tuberculosis*. This finding and the expression of HBHA on the mycobacterial cell surface, as demonstrated by immunoelectron microscopy, suggests that the protein is appropriately positioned for cell–cell interactions. Similarity searches of the NH_2-terminal amino acid sequence of purified HBHA shows no homology with known proteins in the protein sequence data bases.

A direct role for HBHA in bacterial adherence is strongly suggested by the specific inhibition of HBHA-mediated hemagglutination and attachment of mycobacteria to epithelial cells by mAb directed against HBHA. Since adherence of mycobacteria to epithelial cells is also specifically inhibited by heparin and other sulfated carbohydrates, it is likely that HBHA functions to mediate bacterial adherence via sulfated surface receptors on eukaryotic cells. In addition, similar to the *Bordetella pertussis* filamentous hemagglutinin (FHA) (37) and to the *Yersinia enterocolitica* YadA (36) proteins, the mycobacterial HBHA appears to be a multifunctional adhesin, since it is capable of mediating bacterial auto-aggregation as well as bacteria-eukaryotic cell interactions. The enhancement of inter-mycobacterial contact may also promote virulence through the formation of microcolonies at the initial site of infection, analogous to the role played by the autoagglutination activity of group A streptococci M proteins in virulence (38).

The exploitation of sulfated glycoconjugates as receptors may be particularly advantageous for many infectious agents. Proteoglycans that consist of a protein core with one or more covalently attached GAG chains (13) are expressed on the surface of virtually all animal cells and are also present in the ECM, including the lung mucosa (39). There is evidence that a number of pathogenic microorganisms take advantage of the widespread distribution of sulfated glycoconjugates on cell surfaces to use them as ligands for the attachment phase of infection. The initiation of human cytomegalovirus infection is mediated through an interaction with cell surface heparan sulfate (15). Also, the circumsporozoite protein of *Plasmodium* binds specifically to sulfated macromolecules such as heparin, fucoidan, heparan sulfate, and dextran sulfate (18). In addition, the *B. pertussis* FHA is a multifunctional bacterial adhesin that binds to several ligands including sulfated glycoconjugates (37).

Several observations strongly imply that interactions of pathogenic mycobacteria with sulfated carbohydrates on the surface of cells other than macrophages may contribute

to virulence. Although alveolar macrophages play a well documented role in mycobacterial infection throughout the lungs, dissemination of the tubercule bacilli to other sites via the lymphatic or circulatory system most likely requires attachment to epithelial or endothelial cells. Recent findings suggest that *M. tuberculosis* may indeed gain access to the lymphatic and circulatory systems by direct adherence and penetration of alveolar epithelial cells (11). Infection of pigs with *Mycobacterium avium* also involves essential interactions with epithelial cells for which specific receptors have been postulated but have not yet been characterized (41). Since *M. avium* produces a HBHA-related protein, it is tempting to speculate that its HBHA-like protein is also involved in the recognition of epithelial cells by *M. avium* via interactions with sulfated GAG. In addition, endothelial cells have been demonstrated to contain large amounts of heparan sulfate and other sulfated glycoconjugates on their surface (40). Therefore, the heparin-binding activity of HBHA may be involved in extrapulmonary dissemination of *M. tuberculosis*. This is reminiscent of essential steps in the pathogenesis of infectious diseases caused by other invasive microorganisms such as *Y. enterocolitica* (42).

Furthermore, binding to sulfated sugars may contribute to the spread of *M. tuberculosis* via its attachment to mucus. Interaction of the bacteria with the sulfated polysaccharides contained in mucus may not only increase the likelihood for productive interactions with alveolar macrophages during the initial steps of pathogenesis but may also contribute significantly to the transmission of *M. tuberculosis* when exhaled in the form of droplet nuclei.

In addition to binding to GAG receptors, which may constitute an initial multivalent low-affinity step in infection with mycobacteria, the pathogen may subsequently associate with greater affinity to other eukaryotic cell receptors via other regions on HBHA or through other bacterial ligands. Additional adhesins for mycobacteria may include proteins of the 85 antigen complex (33) and p55 (34), which both bind to fibronectin. Such a complex dual receptor system involving initial low affinity GAG binding followed by high-affinity binding is required for fibroblast growth factor activity (43). Also, it has been suggested that interaction of herpes simplex virus (44) with heparan sulfate is required to initially concentrate the virus on the cell surface and hence facilitate its binding to other receptors. Indeed, for macrophages, there is evidence for the interaction of complement receptors and mannose receptors with mycobacteria (29–31). An additional role for sulfated receptors is exemplified by the adherence mechanisms used by *Leishmania* which interact with heparan sulfate proteoglycans on epithelial cells (19) and with CR3 and mannose receptors on mononuclear phagocytes (45, 46).

There is an urgent need to prevent pathogenic mycobacterial transmission in endemic areas and in health care settings. The identification of adhesins involved in the initial steps of attachment and colonization may provide new, rational approaches to block infection, either by the development of novel drugs that interrupt early host-pathogen interactions or by new vaccine strategies. Since sulfated polysaccharides are known to have anti-viral activity against a number of enveloped viruses, heparin derivatives, which can be administered to humans without toxic effects (47), are of particular interest for the development of new therapeutics. In addition, since adhesins have proven useful in the control of other infectious diseases, and since there is interest in secreted mycobacterial proteins as vaccine candidates, the mycobacterial heparin-binding protein described in this study deserves some attention as a possible vaccine component. The fact that the antisera from all tuberculosis patients studied in this report recognize HBHA further emphasizes its possible immunological importance and vaccine potential.

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References

1. Smith, P.G., and A.R. Moss. 1994. Epidemiology of tuberculosis. *In* Tuberculosis, Pathogenesis, Protection, and Control. B.R. Bloom, editor. ASM Press, Washington, DC. 47–59.
2. Raviglione, M.C., D.E. Snider, and A. Kochi. 1995. Global epidemiology of tuberculosis. Morbidity and mortality of a worldwide epidemic. *J. Am. Med. Assoc.* 273:220–226.
3. Noordeen, S.K., L.L. Bravo, and T.K. Sundaresan. 1992. Estimated number of leprosy cases in the world. *Bull. WHO.* 70:7–10.
4. Horsburgh, C.R. 1991. *Mycobacterium avium* complex infec-

tion in the acquired immunodeficiency syndrome. *N. Engl. J. Med.* 324:1332–1338.
5. Barnes, P.F., A.B. Bloch, P.T. Davidson, and D.E. Snider. 1991. Tuberculosis in patients with human immuno-deficiency virus infection. *N. Engl. J. Med.* 324:1644–1650.
6. Bloch, A.B., G.M. Cauthen, G.M. Onorato, K.G. Dansbury, G.D. Kelly, C.R. Driver, and D.E. Snider. 1994. Nationwide survey of drug-resistant tuberculosis in the United States. *J. Am. Med. Assoc.* 271:665–671.
7. Bloom, B.R., and C.L. Murray. 1992. Tuberculosis: commentary on a reemergent killer. *Science (Wash. DC).* 257: 1055–1064.
8. Riley, L.W. 1995. Determinants of cell entry and intracellular survival of *Mycobacterium tuberculosis*. *Trends Microbiol.* 3:27–31.
9. Shepard, C.C. 1957. Growth characteristics of tubercule bacilli and certain other mycobacteria in HeLa cells. *J. Exp. Med.* 105:39–48.
10. Mapothar, M.E., and J.G. Sanger. 1984. In vitro interaction of *Mycobacterium avium* with intestinal epithelial cells. *Infect. Immun.* 45:67–73.
11. McDonough, K.A., and Y. Kress. 1995. Cytotoxicity of lung epithelial cells is a virulence-associated phenotype of *Mycobacterium tuberculosis*. *Infect. Immun.* 63:4802–4811.
12. Bermudez, L.E., and J. Goodman. 1996. *Mycobacterium tuberculosis* invades and replicates within type II alveolar cells. *Infect. Immun.* 64:1400–1406.
13. Yanagishita, M., and V.C. Hascall. 1992. Cell surface heparan sulfate proteoglycans. *J. Biol. Chem.* 267:9451–9454.
14. Nahmias, A.J., and S. Kibrick. 1964. Inhibitory effect of heparin on herpes simplex virus. *J. Bacteriol.* 87:1060–1066.
15. Compton, T., D.M. Nowlin, and N.R. Cooper. 1993. Initiation of human cytomegalovirus infection requires initial interaction with cell surface heparan sulfate. *Virology.* 193:834–841.
16. Shieh, M.-T., and P.G. Spear. 1994. Herpesvirus-induced cell fusion that is dependent on cell surface heparan sulfate or soluble heparin. *J. Virol.* 68:1224–1228.
17. Ortega-Barria, E., and E.A. Pereira. 1991. A novel *T. cruzi* heparin-binding protein promotes fibroblast adhesion and penetration of engineered bacteria and trypanosomes into mammalian cells. *Cell.* 67:411–421.
18. Frevert, U., P. Sinnis, C. Cerami, W. Shreffler, B. Takacs, and V. Nussenzweig. 1993. Malaria circumsporozoite protein binds to heparan sulfate proteoglycans associated with the surface membrane of hepatocytes. *J. Exp. Med.* 177:1287–1298.
19. Love, D.C., J.D. Esko, and D.M. Mosser. 1993. A heparin-binding activity on *Leishmania* amastigotes which mediates adhesion to cellular proteoglycans. *J. Cell Biol.* 123:759–766.
20. Krivan, H.C., L.D. Olson, M.F. Barile, V. Ginsburg, and D. Roberts. 1989. Adhesion of *Mycoplasma pneumoniae* to sulfated glycolipids and inhibition by dextran sulfate. *J. Biol. Chem.* 264:9283–9288.
21. Menozzi, F.D., R. Mutombo, G. Renauld, C. Gantiez, J.H. Hannah, E. Leininger, M.J. Brennan, and C. Locht. 1994. Heparin-inhibitable lectin activity of the filamentous hemagglutinin adhesin of *Bordetella pertussis*. *Infect. Immun.* 62: 769–778.
22. Hannah, J.H., F.D. Menozzi, G. Renauld, C. Locht, and M.J. Brennan. 1994. Sulfated glycoconjugate receptors for the *Bordetella pertussis* adhesin filamentous hemagglutinin (FHA) and mapping of the heparin-binding domain on FHA. *Infect. Immun.* 62:5010–5019.
23. Isaacs, R.D. 1994. *Borrelia burgdorferi* bind to epithelial cell proteoglycans. *J. Clin. Invest.* 93:809–819.
24. Van Putten, J.P.M., and S.M. Paul. 1995. Binding of syndecan-like cell surface proteoglycan receptors is required for *Neisseria gonorrhoeae* entry into human mucosal cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:2144–2154.
25. Rouse, D.A., S.L. Morris, A.B. Karpas, J.C. Mackall, P.G. Probst, and S.D. Chaparas. 1991. Immunological characterization of recombinant antigens isolated from a *Mycobacterium avium* λgt11 expression library by using monoclonal antibody probes. *Infect. Immun.* 59:2595–2600.
26. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
27. Harlow, E., and D. Lane. 1988. Antibodies, A Laboratory Manual. Cold Spring Harbor Laboratory. Cold Spring Harbor, NY. 726 pp.
28. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. *Anal. Biochem.* 72:248–254.
29. Schlesinger, L.S., C.G. Bellinger-Kawahara, N.R. Payne, and M.A. Horwitz. 1990. Phagocytosis of *Mycobacterium tuberculosis* is mediated by human monocyte complement receptors and complement component C3. *J. Immunol.* 144:2771–2780.
30. Schlesinger, L.S., and M.A. Horwitz. 1990. Phagocytosis of leprosy bacilli is mediated by complement receptors CR1 and CR3 on human monocytes and complement component C3 in serum. *J. Clin. Invest.* 85:1304–1314.
31. Hirsch, C.S., J.J. Ellner, D.G. Russell, and E.A. Rich. 1994. Complement receptor-mediated uptake and tumor necrosis factor-mediated growth inhibition of *Mycobacterium tuberculosis* by human alveolar macrophages. *J. Immunol.* 152:743–753.
32. Menozzi, F.D., C. Gantiez, and C. Locht. 1991. Interaction of the *Bordetella pertussis* filamentous hemagglutinin with heparin. *FEMS Microbiol. Lett.* 78:59–64.
33. Wiker, H.G., and M. Harboe. 1992. The antigen 85 complex: a major secretion product of *Mycobacterium tuberculosis*. *Microbiol. Rev.* 56:648–661.
34. Ratliff, T.L., R. McCarthy, W.B. Telle, and E.J. Brown. 1993. Purification of a mycobacterial adhesin for fibronectin. *Infect. Immun.* 61:1889–1894.
35. Menozzi, F.D., P.E. Boucher, G. Riveau, C. Gantiez, and C. Locht. 1994. Surface-associated filamentous hemagglutinin induces autoagglutination of *Bordetella pertussis*. *Infect. Immun.* 62:4261–4269.
36. Balligaund, G., Y. Laroch, and G. Cornelis. 1985. Genetic analysis of virulence plasmid from a serogroup 9 *Yersinia enterocolitica* strain: role of outer membrane protein P1 in resistance to human serum and autoagglutination. *Infect. Immun.* 48:782–786.
37. Locht, C., P., Bertin, F.D. Menozzi, and G. Renauld. 1993. The filamentous hemagglutinin, a multifaceted adhesin produced by virulent *Bordetella spp. Mol. Microbiol.* 9:653–660.
38. Tyewska, S.K., V.A. Fischetti, and R.J. Gibbons. 1988. Binding selectivity of *Streptococcus pyogenes* and M-protein to epithelial cells differs from that of lipoteichoic acid. *Curr. Microbiol.* 16:209–216.
39. Ruoslahti, E. 1989. Proteoglycans in cell regulation. *J. Biol. Chem.* 264:13369–13372.
40. Imai, Y., L.A. Lasky, and S.D. Rosen. 1993. Sulfation requirement for GlyCAM-1, an endothelial ligand for L-selectin. *Nature (Lond.).* 361:555–557.
41. Bermudez, L.E., and L.S. Young. 1994. Factors affecting in-

vasion of HT-29 and HEp-2 epithelial cells by organisms of the *Mycobacterium avium* complex. *Infect. Immun.* 62:2021–2026.

42. Flügel, A., H. Schulze-Koops, J. Heesemann, K. Kühn, L. Sorokin, H. Burkhardt, K. von der Mark, and F. Emmrich. 1994. Interaction of enteropathogenic *Yersinia enterocolitica* with complex basement membranes and the extracellular matrix proteins collagen type IV, laminin-1 and -2, and nidogen/entactin. *J. Biol. Chem.* 269:29732–29738.
43. Yayon, A., M. Klagsbrun, J.D. Esko, P. Leder, and D.M. Ornitz. 1991. Cell surface heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell.* 64:841–848.
44. Trybala, E., B. Svennerholm, T. Bergström, S. Olofsson, S. Jeansson, and J.L. Goodman. 1993. Herpes simplex virus type 1-induced hemagglutination: glycoprotein C mediates virus binding to erythrocyte surface heparan sulfate. *J. Virol.* 67: 1278–1285.
45. Mosser, D.M., and P.J. Edelson. 1985. The mouse macrophage receptor for C3bi (CR3) is a major mechanism in the phagocytosis of *Leishmania* promastigotes. *J. Immunol.* 135: 2785–2789.
46. Wilson, M.E., and R.D. Pearson. 1988. Roles of CR3 and mannose receptors in the attachment and ingestion of *Leishmania donovani* by human mononuclear phagocytes. *Infect. Immun.* 56:363–369.
47. Leizorovicz, A., M.C. Haugh, F.-R. Chapuis, M.M. Samama, and J.-P. Boissel. 1992. Low molecular weight heparin in the prevention of perioperative thrombosis. *Brit. Med. J.* 305:913–920.