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Nonopsonic Binding of *Mycobacterium tuberculosis* to Complement Receptor Type 3 Is Mediated by Capsular Polysaccharides and Is Strain Dependent

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The choice of host cell receptor and the mechanism of binding (opsonic versus nonopsonic) may influence the intracellular fate of *Mycobacterium tuberculosis*. We have identified two substrains of *M. tuberculosis* H37Rv, designated H37Rv-CC and -HH, that differed in their modes of binding to complement receptor type 3 (CR3) expressed in transfected Chinese hamster ovary (CHO-Mac-1) cells: H37Rv-CC bound nonopsonically, whereas H37Rv-HH bound only after opsonization in fresh serum. H37Rv-CC also bound nonopsonically to untransfected CHO cells, whereas H37Rv-HH binding was enhanced by serum and was mediated by the 1D1 antigen, a bacterial adhesin previously identified as a polar phosphatidylinositol mannoside. H37Rv-CC and -HH had identical IS*6110* DNA fingerprint patterns. Of five *M. tuberculosis* clinical isolates examined, four displayed the same binding phenotype as H37Rv-CC, as did the Erdman strain, whereas one isolate, as well as *Mycobacterium smegmatis*, behaved like H37Rv-HH. Nonopsonic binding of H37Rv-CC to CHO cell-expressed CR3 was apparently to the β-glucan lectin site, as it was cation independent and inhibited by laminarin (seaweed β-glucan) and *N*-acetylglucosamine; laminarin also inhibited the binding of H37Rv-CC to monocyte-derived macrophages. Further, binding of H37Rv-CC to CHO-Mac-1 cells was inhibited by prior agitation of bacteria with glass beads (which strips outer capsular polysaccharides) and by preincubation with amyloglucosidase, as well as by the presence of capsular D-glucan and D-mannan from *M. tuberculosis* Erdman, but not by Erdman D-arabino-D-mannan, yeast mannan, or capsular components from H37Rv-HH. Analysis of capsular carbohydrates revealed that H37Rv-CC expressed 5-fold more glucose and 2.5-fold more arabinose and mannose than H37Rv-HH. Flow cytometric detection of surface epitopes indicated that H37Rv-CC displayed twofold less surface-exposed phosphatidylinositol mannoside and bound complement C3 less efficiently than H37Rv-HH; these differences were eliminated after treatment of H37Rv-CC with glass beads. Thus, outer capsular polysaccharides mediate the binding of H37Rv-CC to CR3, likely to the β-glucan site. Moreover, there are strain-dependent differences in the thickness or composition of capsular polysaccharides that determine the mode of binding of *M. tuberculosis* to mammalian cells.

The facultative intracellular pathogen *Mycobacterium tuberculosis* binds to and invades diverse mammalian cells in vitro. Growth and replication within mononuclear phagocytes are well-documented features of the pathogenesis of tuberculosis and are critical to the establishment of an infection (9). In addition, the invasion of nonphagocytic cells by *M. tuberculosis* has been proposed as a means whereby the pathogen gains access to deeper tissues at sites of initial infection and thereby disseminates, although evidence for such a process in vivo is sparse (2, 4, 17).

Regardless of the type of host cell involved, a clear requirement is an invasion strategy that enables efficient intracellular survival and replication. A key question in understanding the molecular basis for intracellular survival is whether survival depends on the initial mode of binding and entry or whether it depends on manipulation of intracellular events subsequent to internalization. Early electron microscopy studies revealed that the capacity for inhibition of phagolysosomal fusion was lost when *M. tuberculosis* was opsonized in immune serum prior to invasion of macrophages in vitro (1), suggesting that entry via specific receptors is required to enable manipulation of the phagosome. Similar conclusions have been drawn from in vitro infection studies with the unrelated pathogens *Toxoplasma gondii* and group B streptococci (13, 33). Moreover, the cytotoxic effect of *M. tuberculosis* for lung epithelial cells was greater after nonopsonic than after opsonic invasion (17). In contrast, several studies have documented the induction of specific *M. tuberculosis* proteins and genes following invasion of macrophages (15, 36), which is consistent with the alternative view that intracellular survival depends on factors that are unrelated to the initial mode of attachment.

We have examined the binding of *M. tuberculosis* to Chinese hamster ovary (CHO) and other nonphagocytic cells (11) and to CHO cells expressing complement receptor type 3 (CR3) (6), with a view to defining these interactions in molecular terms. These studies are part of ongoing efforts to determine whether the mode of binding and invasion plays a role in specifying the subsequent intracellular fate of the pathogen. We have found that binding of some strains of *M. tuberculosis*, as well as of *Mycobacterium smegmatis*, to native CHO cells was mediated in part by surface-exposed phosphatidylinositol mannosides (PIMs) and that this binding was enhanced by opsonization with serum factors that included the mannose binding protein (MBP) (11). In separate studies we have shown that binding of *M. tuberculosis* H37Rv to CR3 expressed by transfected CHO cells was predominantly nonopsonic and was to a site distinct from the C3bi-binding site, which we have specu-

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lated may involve the CR3 β-glucan lectin site (6). In the present study, we have extended our analysis to include five clinical isolates of *M. tuberculosis*, in addition to three laboratory strains of *M. tuberculosis* and also *M. smegmatis*. We found that most of the *M. tuberculosis* strains and isolates bound nonopsonically to CR3 expressed on CHO cells and that this binding was most likely to the CR3 lectin site. Binding was mediated by outer capsular carbohydrates that included the recently identified neutral D-glucan (19, 20). Strikingly, we observed a reciprocal relationship between direct binding to CR3 on the one hand and PIM-mediated binding to native CHO cells on the other: strains that bound nonopsonically to CR3 also bound nonopsonically and PIM independently to CHO cells, and vice versa. These observations can be accounted for by a simple model of strain-dependent variations in the thickness or composition of outer capsular carbohydrates and degree of exposure of PIMs. It remains to be determined whether these strain-dependent modes of binding also correlate with intracellular survival and with virulence.

MATERIALS AND METHODS

Bacteria. Laboratory strains were *M. tuberculosis* Erdman (ATCC 35801), two substrains of H37Rv designated H37Rv-CC and -HH (the origin of these substrains is described below), and *M. smegmatis* ATCC 19420. Clinical isolates of *M. tuberculosis* were GSH-2288 (isolated from a urinary catheter), GSH-2849 (sputum), GSH-3052 (pleural effusion), GSH-3361 (gastric washing), and GSH-3392 (abdominal pus swab). All clinical isolates were provided by the Bacteriology Laboratory, Department of Medical Microbiology, Groote Schuur Hospital, Cape Town, South Africa. Identification of the isolated organisms as *M. tuberculosis* was based on standard microbiological criteria, including growth rate, colony morphology, pigmentation, and positive niacin and nitrate reduction tests (23). All isolates were fully drug sensitive and were at passage three. The distinction between H37Rv-CC and -HH was made because these two substrains displayed distinct and constant phenotypes with regard to their mode of binding to mammalian cells, as reported in Results. These substrains were derived from two separate stocks of strain H37Rv. H37Rv-HH was acquired from the American Type Culture Collection (ATCC 27294) by our laboratory in 1993. H37Rv-CC is the *M. tuberculosis* reference strain of the Bacteriology Laboratory, Groote Schuur Hospital. Although previously assumed to be an American Type Culture Collection strain (6), a detailed investigation revealed that H37Rv-CC was obtained in the early 1950s from the Trudeau Institute. Previous studies conducted in our laboratory were performed either with H37Rv-CC (6) or with H37Rv-HH (11), but the existence of a distinct and constant phenotypic difference between the two substrains became apparent only during the present investigation.

Laboratory strains and clinical isolates were maintained on Lowenstein-Jensen slopes, and stocks for in vitro infection assays were prepared by growth in Kirchner's medium, exactly as described previously (6). In infection assays, bacteria were used live, derived after thawing frozen stocks (6). In some instances, strains H37Rv-CC and -HH were used after heat killing (80°C for 30 min) to facilitate experimental manipulations; in control experiments, heat killing was found not to alter bacterial binding. Bacterial clumping was reduced by successive syringing, and bacterial stocks were quantitated by measurement of optical density or by comparison with a *Candida albicans* reference standard, as described previously (6, 11).

In some experiments, bacteria were treated with glass beads, as follows. Heat-killed *M. tuberculosis* bacteria were pelleted, washed once in phosphate-buffered saline (PBS), resuspended in 5 volumes of PBS, and agitated within an Eppendorf tube by addition of two 4-mm-diameter glass beads and vortex mixing for 5 min. The bacterial suspension was centrifuged, and the supernatant was aspirated and saved; this supernatant was designated the bead-treated supernatant. The remaining bacteria were resuspended in 5 volumes of fresh PBS, quantitated as described above, and used directly in infection assays as bead-treated bacteria.

As an alternative to mechanical treatment with glass beads, bacteria were in some instances treated with hydrolases, as follows. Heat-killed *M. tuberculosis* bacilli were pelleted and resuspended in 1 ml of either α-mannosidase (Boehringer Mannheim, Mannheim, Germany) at 5 U/ml or amyloglucosidase (Sigma Chemical Co., St. Louis, Mo.) at 500 to 800 U/ml. Both enzymes were in 0.05 M sodium acetate–0.1 M NaCl, pH 4.5; as a control, bacteria were resuspended in buffer only. Bacterial suspensions were incubated at 37°C for 18 h and then centrifuged, the supernatants were discarded, and the bacterial pellets were washed once in PBS, resuspended in 1 ml of PBS, and then used directly in in vitro infection assays.

***M. tuberculosis* DNA fingerprinting.** All of the *M. tuberculosis* laboratory strains and clinical isolates described above were subjected to IS*6110* DNA fingerprint analysis. Mycobacterial genomic DNA was extracted, digested with *Pvu*II, and Southern blotted with labelled IS*6110* DNA, exactly as described elsewhere (34, 37).

***M. tuberculosis* capsular carbohydrates.** Analyses of the carbohydrate composition of *M. tuberculosis* H37Rv-CC and -HH bead-treated supernatants were performed as described previously (16, 20). Purified *M. tuberculosis* Erdman outer capsular D-glucan, D-arabino-D-mannan (AM), and D-mannan were prepared as described previously (16, 20).

Mammalian cells. Wild-type CHO (CHO-WT) K1 cells (ATCC CCL61), CHO-Mac-1 cells, and human monocyte-derived macrophages (MDMs) were grown and maintained as described previously (6). CHO-Mac-1 cells are stably transfected with the cDNAs for human CD11b and CD18 (7) and express high levels of the CR3 heterodimer at the cell surface (6).

Mycobacterial binding assays. The binding of the various mycobacterial strains and clinical isolates to CHO-WT cells, CHO-Mac-1 cells, and MDMs was assayed as described previously (6). Briefly, mammalian cells were grown on 12-mm-diameter glass coverslips and then incubated with the bacteria (at multiplicities of infection [MOIs] of 50:1 to 300:1) in the presence or absence of 10% fresh human serum or fetal bovine serum (FBS). Incubations were performed with untreated, bead-treated, or enzyme-treated bacteria, in the presence or absence of various additives, including *M. tuberculosis* bead-treated supernatants, Erdman capsular carbohydrates, laminarin (soluble seaweed β-glucan [Sigma]), *N*-acetyl-D-glucosamine (NADG), animal glycogen (type VII, from the mussel *Mytilus edulis* [Sigma]), EDTA, or monoclonal antibody (MAb) 1D1 (which recognizes mycobacterial, surface-exposed PIMs [11]) (details and concentrations are given in Results). After an 18-h incubation, cells were washed extensively, fixed in glutaraldehyde, stained with acridine orange, quenched in crystal violet, and examined by fluorescence microscopy. Bacterial binding was quantitated as the percentage of cells to which one or more bacteria were bound, by counting 100 to 200 cells per coverslip; each experiment was performed in triplicate. The significance of differences was determined by Student's *t* test.

Flow cytometric analysis of *M. tuberculosis* surface epitopes. Untreated *M. tuberculosis* H37Rv-CC and -HH bacteria and bead-treated H37Rv-CC bacteria were analyzed for C3 opsonization by flow cytometry with a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human C3c antibody, exactly as described previously (6). Bacteria were similarly analyzed for surface expression of the 1D1 epitope, with the following modifications. PBS-washed bacteria were incubated at 4°C with a 1:10 dilution of MAb 1D1 ascites fluid (11) for 45 min, washed in PBS, and then incubated with a 1:20 dilution of FITC-conjugated rabbit anti-mouse immunoglobulin (Boehringer Mannheim) at 4°C for 45 min; the isotypic control consisted of incubation in the secondary antibody only. In all cases, washed and fixed bacteria were briefly vortex mixed and allowed to settle for 5 min, and only the top suspension was applied to the flow cytometer.

RESULTS

IS*6110* DNA fingerprint patterns of *M. tuberculosis* clinical isolates and laboratory strains. The IS*6110* DNA fingerprint patterns of the clinical isolates and laboratory strains shown in Fig. 1 were characteristic of the *M. tuberculosis* species (34, 35). The patterns of the clinical isolates were diverse, as expected for isolates from a large referral center serving numerous, unrelated communities (37); only isolates GSH-2288 and -3361 were clearly related. Of interest is that there was a low degree of similarity between the patterns of the Erdman strain and isolate GSH-3052 (Fig. 1). The IS*6110* DNA fingerprint patterns of H37Rv-CC and -HH were identical to each other, with either the IS*6110* left-arm probe (Fig. 1) or the right-arm probe (35) (data not shown), and these patterns matched those published for the H37Rv strain (28, 29).

Serum enhancement of binding of *M. tuberculosis* to CHO cells was strain dependent. Binding of H37Rv-HH and *M. smegmatis*, but not of Erdman, to untransfected CHO cells is mediated by surface-expressed PIM and is inhibited by MAb 1D1 (11). Analysis of the binding of the five clinical isolates and of H37Rv-CC to CHO cells revealed that only isolate GSH-2288 behaved similarly to H37Rv-HH, with serum producing a significant (two- to threefold) enhancement in binding (Fig. 2A). In contrast, all other isolates as well as strains H37Rv-CC and Erdman bound in a serum-independent manner. Of these, H37Rv-CC and GSH-3392 and -3361 bound poorly, whereas GSH-2849 and -3052 and Erdman bound as well as or better than H37Rv-HH and GSH-2288, which bound in a serum-dependent manner (Fig. 2A).

MAb 1D1 inhibited the binding of isolate GSH-2288 to

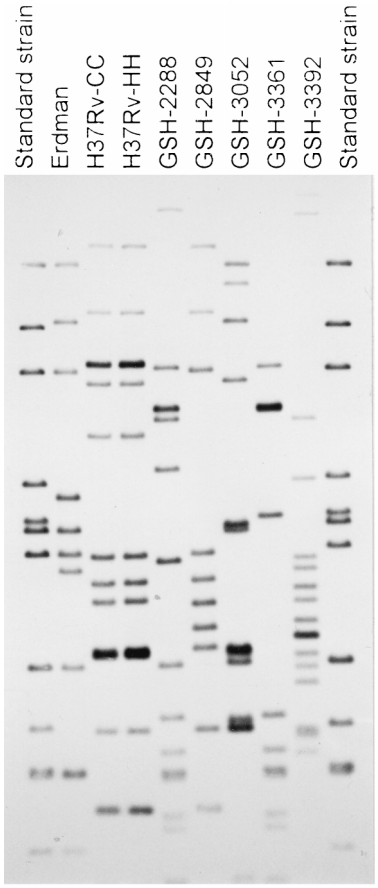

FIG. 1. IS*6110* DNA fingerprint patterns of *M. tuberculosis* laboratory strains and clinical isolates. Fingerprinting was performed by using the IS*6110* left-arm probe, as described previously (34, 37). The standard strain (no. 14323) is a fingerprinting reference strain (35, 37).

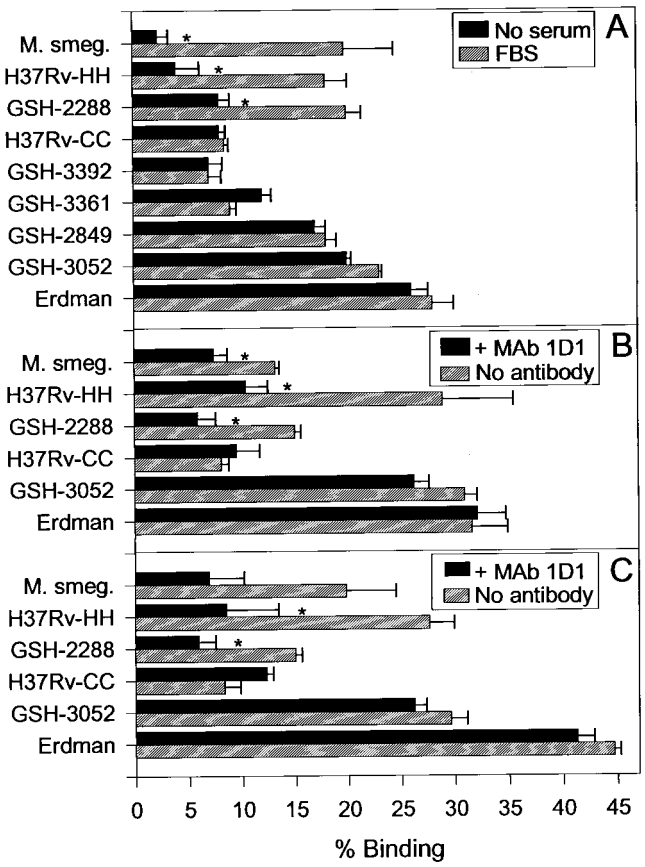

FIG. 2. Effect of serum and MAb 1D1 on binding of *M. tuberculosis* strains and isolates to CHO-WT cells. (A) Cells were incubated with live bacteria for 6 h in the presence (MOI, 100:1) or absence (MOI, 300:1) of 10% FBS, and the percentage of cells that bound one or more bacteria was determined by counting under fluorescence microscopy after staining with acridine orange. (B and C) Cells were infected in the presence or absence of MAb 1D1 (20 μl of ascites fluid per 0.5 ml of medium), either without (B) or with (C) serum. Data are means and standard deviations from four separate experiments. *, $P < 0.01$ compared to the appropriate control (binding in the presence of serum in panel A or in the absence of antibody in panels B and C). M. smeg., *M. smegmatis.*

CHO cells in both the absence (Fig. 2B) and presence (Fig. 2C) of serum (note that binding in the absence of serum [Fig. 2B] was performed at an MOI of 300:1, versus 100:1 in the presence of serum [Fig. 2A and C], in order to achieve comparable levels of binding). Binding was inhibited by approximately 60%, which was similar to the inhibition previously observed for H37Rv-HH and *M. smegmatis* (11) (Fig. 2B and C). In contrast, binding to CHO cells in the presence or absence of serum was not inhibited by MAb 1D1 for strains H37Rv-CC and Erdman and isolate GSH-3052 (Fig. 2B and C). Although the effect of MAb 1D1 on the binding of isolates GSH-3392, -3361, and -2849 was not tested, these results suggested that there was an association between serum enhancement of binding to CHO cells and susceptibility to inhibition by MAb 1D1.

Nonopsonic binding of *M. tuberculosis* to CR3-expressing CHO cells was strain dependent. We have shown previously that *M. tuberculosis* H37Rv bound nonopsonically to CR3 expressed in transfected CHO cells, whereas the binding of *M. smegmatis* to these cells was serum dependent (6). These studies were performed with the substrain that we now designate H37Rv-CC. Here we have examined the binding to CR3-expressing CHO cells (CHO-Mac-1 cells) of the five clinical isolates and strains H37Rv-HH and Erdman. We found that isolates GSH-3392, -3361, -2849, and -3052 and strain Erdman bound in a serum-independent manner (i.e., nonopsonically) to CHO-Mac-1 cells (Fig. 3), in a manner similar to the nonopsonic binding of H37Rv-CC (6). Indeed, binding of some of the strains (GSH-2849, GSH-3052, and Erdman) was moderately inhibited by the presence of serum (Fig. 3). This effect was variable and may have resulted from occlusion of the CR3-binding epitope by serum components. In contrast, the binding of strain H37Rv-HH and isolate GSH-2288 to CHO-Mac-1 cells was serum dependent. In the absence of serum, the binding of H37Rv-HH to CHO-Mac-1 cells was not significantly different from the binding to untransfected CHO cells (compare Fig. 3 and 2B); the binding of GSH-2288 to CHO-Mac-1 cells was reduced in serum-free medium but remained approximately twofold greater than that to CHO cells. The mode of binding displayed by H37Rv-HH resembled that of *M. smegmatis* (6), whereas the GSH-2288 phenotype was intermediate between those of H37Rv-HH and -CC. The addition of MAb 1D1 did not significantly affect the binding of any of the clinical isolates or of the laboratory strains to CHO-Mac-1 cells in the presence or absence of serum (data not shown).

Nonopsonic binding of *M. tuberculosis* H37Rv-CC to CR3-expressing cells was inhibited by saccharides and was cation independent. We have shown that binding of *M. tuberculosis*

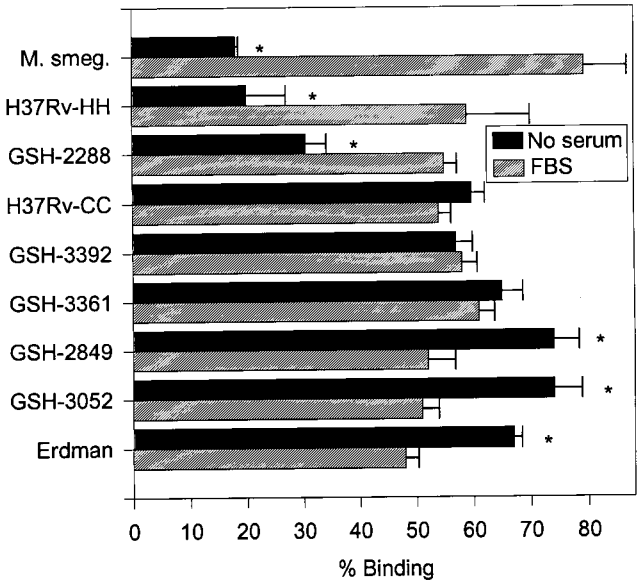

FIG. 3. Serum dependence of binding of *M. tuberculosis* strains and isolates to CHO-Mac-1 cells. Cells were incubated with live bacteria (MOI, 300:1) in the presence or absence of 10% FBS and then analyzed as described in the legend to Fig. 2. Data are means and standard deviations from three separate experiments. *, $P < 0.01$ compared to binding in the presence (*M. smegmatis* [M. smeg.], H37Rv-HH, and GSH-2288) or absence (GSH-2849, GSH-3052, and Erdman) of serum.

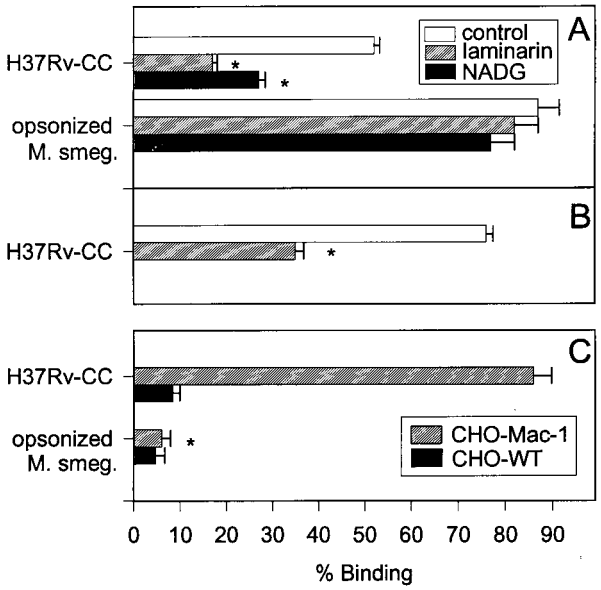

FIG. 4. Effect of saccharides and EDTA on the binding of mycobacteria to CR3-expressing cells. CHO-Mac-1 cells (A) and MDMs (B) were incubated with live bacteria in serum-free medium containing laminarin (5 μg/ml) or NADG (150 mM) at MOIs of 300:1 (CHO-Mac-1 cells) or 50:1 (MDMs). (C) Suspensions of CHO-WT and -Mac-1 cells were incubated with live bacteria (300:1) in serum-free medium containing 5 mM EDTA. In all cases, *M. smegmatis* (M. smeg.) was preopsonized for 1 h in complete, fresh human serum. Bacterial binding was estimated as described in the legend to Fig. 2. Data are means and standard deviations from three separate experiments. *, $P < 0.01$ compared to untreated controls (A and B) or to binding of H37Rv-CC (C).

H37Rv-CC to CHO cell-expressed CR3 was to a site distinct from the C3bi-binding site, as determined by inhibition studies with specific anti-CD11b MAbs (6). In particular, binding was strongly inhibited by MAb OKM1, which binds to the C-terminal region of CD11b that includes the β-glucan lectin site of CR3 (31). To test whether H37Rv-CC binds to the CR3 lectin site, we performed binding studies with CHO-Mac-1 cells in the presence of the saccharides laminarin (a seaweed β-glucan) and NADG, which are known to block the lectin site, and in the presence of EDTA, which does not inhibit the lectin site (31). We found that in the absence of serum, the specific binding of H37Rv-CC to CHO-Mac-1 cells was inhibited by 92 and 66% (calculated after subtraction of background binding, i.e., binding to untransfected CHO cells, estimated at 14% [6] [Fig. 2B]), respectively, by laminarin (5 μg/ml) and NADG (150 mM), whereas these saccharides had no effect on the binding of preopsonized *M. smegmatis* (Fig. 4A). Similarly, the binding of H37Rv-CC to human MDMs in serum-free medium was inhibited by 54% in the presence of 5 μg of laminarin per ml (Fig. 4B). In contrast, binding of H37Rv-CC to CHO-Mac-1 cells in the presence of 5 mM EDTA and the absence of serum was unimpaired, whereas that of preopsonized *M. smegmatis* was completely abolished (Fig. 4C) as would be expected for a complement-coated particle binding to the cation-dependent C3bi-binding site of CR3 (6). These studies were performed with cells in suspension, because the presence of 5 mM EDTA resulted in detachment of the cells; for this reason, the controls consisted of CHO-WT cells also treated with EDTA and not adherent CHO-Mac-1 cells in the absence of EDTA (Fig. 4C). The results strongly suggested that the nonopsonic binding of H37Rv-CC to CR3 expressed in CHO cells was to the cation-independent β-glucan lectin site.

Bead- and glucosidase-treated *M. tuberculosis* H37Rv-CC bound poorly to CHO-Mac-1 cells. It was reported recently that the outermost capsular material of *M. tuberculosis* consists predominantly of polysaccharides and proteins, with the polysaccharides comprising neutral D-glucan, AM, and D-mannan that can be extracted mechanically by agitating the bacilli with glass beads, which does not damage the bacteria (19–21). To test whether these outer capsular polysaccharides mediate nonopsonic binding of H37Rv-CC to CHO-Mac-1 cells, we bead treated H37Rv-CC according to a published procedure (20). Bead treatment reduced the specific binding of H37Rv-CC to CHO-Mac-1 cells in the absence of serum by 92% (calculated after subtraction of background binding to CHO-WT cells) (Fig. 5A and B), whereas the binding of bead-treated H37Rv-HH was unchanged (data not shown). Interestingly, at a lower MOI (50:1), bead treatment virtually abolished the nonopsonic binding of H37Rv-CC to CHO-Mac-1 cells, whereas in the presence of serum, some binding was retained (Fig. 5A); a similar effect was observed for the binding to CHO-WT cells (Fig. 5B). Moreover, there was a modest (32%) inhibition of opsonic binding of bead-treated H37Rv-CC to CHO-WT cells by MAb 1D1, from 25 ± 1% to 17 ± 3% (not shown). These results suggested that bead treatment of H37Rv-CC bacteria resulted in a partial acquisition of the H37Rv-HH binding phenotype.

As an alternative to the mechanical removal of capsular polysaccharides, we treated H37Rv-CC with amyloglucosidase and α-mannosidase, which have been shown to digest the *M. tuberculosis* glucans and AMs, respectively (16). Treatment with amyloglucosidase, but not with α-mannosidase, resulted in an 84% reduction in nonopsonic binding of H37Rv-CC to CHO-Mac-1 cells, whereas binding to CHO-WT cells was unchanged (Fig. 5C). Taken together, the results from the bead and enzyme treatments suggested that outer capsular polysaccharides may mediate the nonopsonic binding of H37Rv-CC to CR3 expressed in CHO cells and that this involved, in part, the

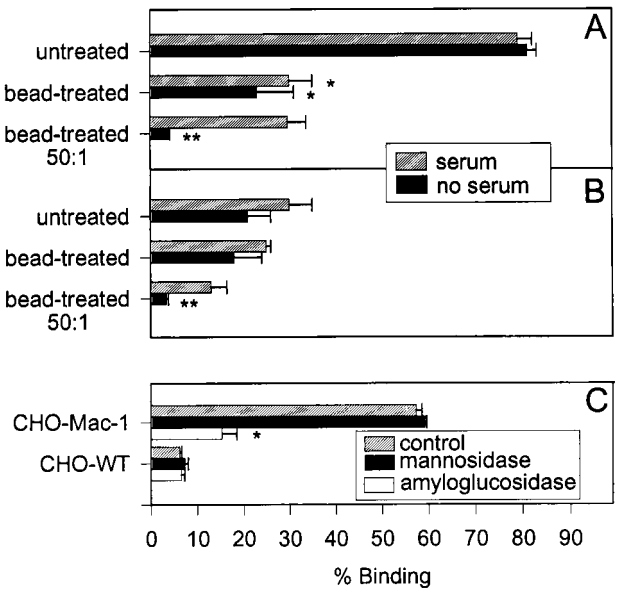

FIG. 5. Binding of bead- and enzyme-treated *M. tuberculosis* H37Rv-CC to CHO-Mac-1 and CHO-WT cells. Heat-killed bacteria were treated mechanically with glass beads and then incubated (MOI of 300:1, except where indicated as 50:1) with CHO-Mac-1 (A) or CHO-WT (B) cells in the presence or absence of 10% fresh human serum. (C) Alternatively, heat-killed bacteria were treated enzymatically with α-mannosidase or amyloglucosidase, or with buffer alone (control), before incubation with cells in serum-free medium. Bacterial binding was estimated as described in the legend to Fig. 2. Data are means and standard deviations from three separate experiments (A and B) or a single experiment (C). *, < 0.01 compared to untreated controls; **, $P < 0.01$ compared to bead-treated bacteria at an MOI of 50:1 in the presence of serum.

capsular D-glucans but apparently not the terminal mannosyl residues of AMs.

***M. tuberculosis* capsular glucans and mannans blocked nonopsonic binding of strain H37Rv-CC to CHO-Mac-1 cells.** To examine further the role of *M. tuberculosis* capsular polysaccharides in the binding to CHO cell-expressed CR3, we incubated H37Rv-CC bacteria with CHO-Mac-1 cells in the presence of purified capsular glucan, mannan, and AM (Erdman strain). As controls, incubations were also performed in the presence of laminarin, yeast mannan, and animal glycogen; all incubations were in the absence of serum. We found that the capsular glucan and mannan both inhibited binding by 78%, whereas capsular AM and yeast mannan inhibited binding poorly (Table 1). Binding was reduced to a similar extent by animal glycogen and by the supernatant of bead-treated H37Rv-CC bacteria (by 78 and 76%, respectively). In contrast, the supernatant of bead-treated H37Rv-HH bacteria had no effect on binding (Table 1). None of these treatments had a significant effect on the nonopsonic binding of H37Rv-HH to CHO-Mac-1 cells (Table 1). Moreover, binding of either strain to CHO-WT cells was not inhibited significantly by any of the treatments (data not shown). These results strengthened the impression that some capsular polysaccharides were involved in the nonopsonic binding of *M. tuberculosis* H37Rv-CC to CR3 expressed in CHO cells. In contrast, substrain H37Rv-HH bound poorly to CHO-Mac-1 cells in the absence of serum, and this binding was not modulated by capsular polysaccharides.

Comparison of outer capsular material from substrains H37Rv-CC and -HH. The supernatants from equivalent numbers of bead-treated H37Rv-CC and -HH bacteria were extracted with organic solvents, acid hydrolyzed, and analyzed for monosaccharides, as described previously (20). For both strains the aqueous phases contained glucose, arabinose, and mannose, whereas the interphases contained only glucose. The total quantities were 11.2 mg of glucose, 0.84 mg of arabinose, and 0.84 mg of mannose per g (wet weight) of H37Rv-CC bacteria and 2.2, 0.34, and 0.32 mg, respectively, per g of H37Rv-HH bacteria. Therefore, H37Rv-CC outer capsular material contained 4- to 5-fold more glucose and 2.5-fold more arabinose and mannose than the outer capsular material from H37Rv-HH. The ratios of glucose to arabinose and mannose were similar to those determined previously for *M. tuberculosis* capsular polysaccharides (19, 20).

TABLE 1. Effect of *M. tuberculosis* capsular polysaccharides and crude capsular fractions on nonopsonic binding to CHO-Mac-1 cells[a]

Polysaccharide or capsular fraction[b]	% Cells bound to bacteria[c]	
	H37Rv-HH	H37Rv-CC
None	16 ± 1	60 ± 2
D-Glucan	13 ± 2	24 ± 2*
D-Mannan	17 ± 1	25 ± 2*
AM	16 ± 2	50 ± 1**
Yeast mannan	17 ± 1	50 ± 4**
Glycogen	16 ± 1	24 ± 2*
Laminarin	16 ± 1	17 ± 2*
CC supernatant	18 ± 1	25 ± 1*
HH supernatant	17 ± 1	59 ± 2

[a] CHO-Mac-1 cells were incubated with heat-killed bacteria (MOI, 300:1) overnight in the presence of the indicated polysaccharides and capsular fractions, and bacterial binding was quantitated as described in the legend to Fig. 2.

[b] All polysaccharides were added at 100 μg/ml except laminarin (5 μg/ml); capsular D-glucan, D-mannan, and AM were from the Erdman strain. Supernatant fractions from bead-treated H37Rv-CC (CC supernatant) and H37Rv-HH (HH supernatant) bacteria were ethanol precipitated (19), lyophilized, and added to the cells at 100 μg of material per ml.

[c] Values are means ± standard deviations from one experiment performed in triplicate. *, $P < 0.0001$; **, $P < 0.02$ (compared to the control [no treatment]).

Flow cytometric analysis of *M. tuberculosis* surface epitopes. In light of the strain-dependent differences in capsular composition and their possible role in the opsonin dependence of binding of *M. tuberculosis* to mammalian cells, we examined the presence of two surface epitopes on untreated H37Rv-HH and -CC bacteria, as well as bead-treated H37Rv-CC bacteria, by flow cytometry. First, we examined the surface expression of the 1D1 antigen (Ag) (a polar PIM), which mediates both direct and opsonin-enhanced binding of H37Rv-HH, but not of H37Rv-CC, to nonphagocytic cells (11). As shown in Fig. 6, 66.5% of H37Rv-HH bacteria expressed surface 1D1 Ag, compared with only 33.8% of H37Rv-CC bacteria. Strikingly, bead treatment of H37Rv-CC increased the percentage of bacteria positive for surface expression of 1D1 Ag to 71.7%. A possible interpretation is that surface expression of 1D1 Ag (i.e., polar PIMs) in the H37Rv-CC substrain is reduced because of the presence of more-abundant capsular polysaccharides; bead treatment partly removes the capsule, resulting in greater 1D1 Ag exposure, similar to that in untreated H37Rv-HH bacteria. Second, we opsonized bacteria in fresh human serum and then analyzed the surface deposition of C3 and C3 breakdown products (Fig. 6). This gave the apparent result that levels of opsonization of untreated H37Rv-HH and -CC were similar, at 58.5 and 54.9% of bacteria, respectively. However, inspection of the recordings revealed that the fluorescence peak for H37Rv-HH was very broad, indicating that surface deposition of C3 was extremely variable, including a significant cell population with very high levels of surface C3 (Fig. 6), similar to that previously found for *M. smegmatis* (6). Bead treatment of H37Rv-CC resulted in a marked increase in the percentage of

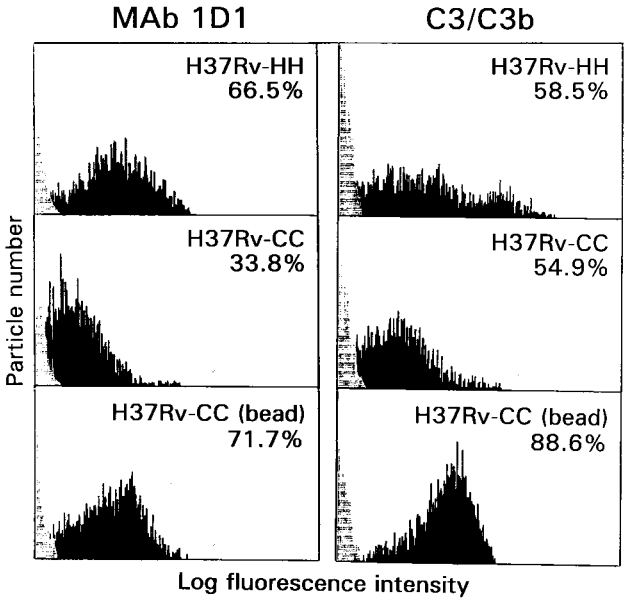

FIG. 6. Flow cytometric detection of *M. tuberculosis* surface epitopes. Untreated H37Rv-HH and -CC bacteria, as well as bead-treated H37Rv-CC bacteria, were reacted first with MAb 1D1 (which is specific for polar PIMs [11]) and then with an FITC-conjugated anti-mouse immunoglobulin antibody (left panels). Alternatively, bacteria were opsonized in fresh human serum and then stained for C3 and C3 breakdown products with FITC-conjugated anti-human C3c antibody (right panels). In each case, the percentage of positively stained bacteria (densely shaded peaks) was determined by comparison to the isotypic control (lightly shaded peaks).

bacteria positive for C3 surface deposition, from 54.7 to 88.6%. This can be interpreted to mean that the more-abundant polysaccharide capsule of H37Rv-CC inhibits C3 fixation. It is notable, however, that even after bead treatment, the H37Rv-CC bacteria lacked a population with high-level C3 deposition, unlike the untreated H37Rv-HH bacteria (Fig. 6).

The data from the carbohydrate analyses of the capsular materials of H37Rv-HH and -CC, and from the flow cytometric analyses of surface epitopes, are consistent with the notion that these two substrains differ in the composition or thickness of the outer capsule, which results in differential exposure of surface epitopes. These differences, in turn, correlate with the opsonic and nonopsonic modes of binding of the H37Rv-HH and -CC substrains to mammalian cells.

DISCUSSION

M. tuberculosis uses diverse ligand-receptor interactions to adhere to host cells, binding by both opsonic and nonopsonic mechanisms to mononuclear phagocytes and to diverse nonphagocytic cells in culture. In many cases the identities of the ligand-receptor pairs are unknown. An important mode of attachment to MDMs and alveolar macrophages is to CR3 and CR4 after opsonization with complement C3 (10, 26, 27). The mechanism of the nonopsonic binding to CR3 has remained unclear, although previous studies established that it is to a site distinct from the C3bi-binding site (6, 30). We have speculated that nonopsonic binding of *M. tuberculosis* to CR3 may involve the β-glucan lectin site on the C-terminal portion of CD11b (6), but this proposal was apparently inconsistent with an earlier report demonstrating that adherence of the pathogen to MDMs was not blocked by the soluble β-glucan laminarin (27). The reported studies (27) were performed with bead-treated bacteria to reduce clumping, which, we reasoned, may have influenced the mode of binding to CR3. In this study we have undertaken an analysis of the role of outer envelope (capsular) components in the binding of several strains and clinical isolates of *M. tuberculosis* to CR3-expressing CHO cells, MDMs, and untransfected CHO cells.

We have been working with two independently acquired stocks of *M. tuberculosis* H37Rv, which we have designated H37Rv-CC and -HH. Our original studies on the binding of strain H37Rv to CHO-CR3 cells (6) were all performed with H37Rv-CC. To our surprise, during the course of the present study, we found that, unlike H37Rv-CC, H37Rv-HH did not bind nonopsonically to CHO-CR3 cells. Instead, H37Rv-HH behaved like *M. smegmatis*, in that binding was dependent on opsonization in fresh serum, suggesting that these strains lacked a ligand mediating direct binding to CR3. Four of five clinical isolates and Erdman bound nonopsonically to CHO-Mac-1 cells in the same manner as H37Rv-CC, whereas one of the clinical isolates, GSH-2288, behaved like H37Rv-HH and *M. smegmatis*, requiring the presence of fresh serum to bind efficiently to the CHO-Mac-1 cells. Thus, strong nonopsonic binding to CHO cell-expressed CR3 is not a property peculiar to laboratory strains but may in fact represent a common characteristic of clinical isolates, although a larger number and diversity of isolates must be examined to reach a firm conclusion.

Our previous studies on the nonopsonic binding of H37Rv (substrain CC) to CHO cell-expressed CR3 indicated that binding was not to the C3bi-binding site, since it was unaffected by MAb 2LPM19c, which blocks this site (6). Instead, binding was significantly inhibited by MAb OKM1, which has been shown to inhibit the cation-independent β-glucan lectin site situated C terminal to the I domain on the CD11b subunit of CR3 (31). Indeed, the nonopsonic binding of H37Rv-CC to CHO-CR3 cells was not affected by 5 mM EDTA, unlike the serum-dependent binding of *M. smegmatis*, whereas the binding of H37Rv-CC was significantly inhibited by laminarin and NADG, which bind to the CR3 lectin site, but not by yeast mannan, which does not block the lectin site (31). Importantly, nonopsonic binding of H37Rv-CC to human MDMs was also inhibited by laminarin, unlike what was previously reported (27); however, the earlier study was performed with bead-treated bacteria, which lose the capacity for nonopsonic binding to CR3. Our results strongly suggested that H37Rv-CC expressed a glycoconjugate that served as a ligand for the CR3 lectin site. Presumably that was also true for isolates GSH-2849, -3052, -3361, and -3392 and the Erdman strain, although we did not test the mechanism of nonopsonic binding to CHO-Mac-1 cells for these strains.

Our results are of considerable interest in light of the recent demonstration that the outermost layer of the cell envelope, or capsule, of *M. tuberculosis* consists of a carbohydrate-protein matrix containing surprisingly small quantities of lipids (19–21). This matrix is presumably external to the outer leaflet of the postulated pseudo-outer lipid bilayer (to which the mycolic acids contribute the inner leaflet [3]), although constituents of the outer leaflet, including PIMs, are embedded in the matrix in small amounts (21). The dominant carbohydrates in this capsule are a glycogen-like D-glucan polysaccharide (70% of the total), AM, and D-mannan (the latter two comprise 30% of the total) (19, 20). These polysaccharides are easily stripped from the bacterial surface by gentle agitation with glass beads, which does not damage the bacteria (20, 21).

Our studies revealed that following mechanical agitation with glass beads, nonopsonic binding of H37Rv-CC bacteria to CHO-Mac-1 cells was significantly inhibited. Moreover, non-

opsonic binding of untreated H37Rv-CC to CHO-Mac-1 cells was inhibited by incubation in the presence of purified capsular D-glucan and D-mannan from the Erdman strain (19) and in the presence of supernatants from bead-treated bacteria, which contained significant quantities of carbohydrates. These results strongly suggested that outer capsular polysaccharides from H37Rv-CC mediate the nonopsonic binding to CR3 expressed in CHO cells. However, we are presently unable to determine with certainty which of the capsular polysaccharides is primarily responsible for binding to CR3. The D-glucan is clearly involved, based on the inhibition of H37Rv-CC binding to CHO-Mac-1 cells by both Erdman D-glucan and glycogen and by pretreatment of bacteria with amyloglucosidase (which efficiently hydrolyzes the *M. tuberculosis* D-glucan [16]). The roles of AM and D-mannan are uncertain, however, because although the addition of D-mannan effectively inhibited binding of H37Rv-CC to CHO-Mac-1 cells, neither purified AM nor pretreatment with α-mannosidase had an effect. One interpretation is that D-mannan but not AM binds to CR3. The failure of α-mannosidase pretreatment to inhibit binding of H37Rv-CC to CHO-Mac-1 cells may result from the relative resistance of D-mannan to this enzyme. Whereas the terminal (1→2)-linked mannosyl residues of AM are susceptible to cleavage by α-mannosidase (12, 16), the structure and enzyme susceptibility of the D-mannan have not been studied. An alternative possibility is that neither AM nor D-mannan binds to CR3 and that the apparent effect of D-mannan on binding is due to the presence of an unidentified contaminant. These questions can be resolved by the use of highly purified capsular carbohydrates and of soluble CR3 in in vitro binding assays. It is worth noting that the CR3 lectin site, originally identified as a β-glucan-binding site, appears to have a broad specificity that may include mannose-containing saccharides (31). Our results suggest that the binding range of the CR3 lectin site can be extended to include a glycogen-like α-1,4-glucan, and possibly a D-mannan, from the *M. tuberculosis* capsule.

In contrast to the case for the H37Rv-CC bacteria, bead treatment of H37Rv-HH bacteria or incubation in Erdman capsular polysaccharides did not alter the extent of their binding to CHO-Mac-1 cells in the presence or absence of serum. Moreover, chemical analysis revealed that the H37Rv-CC capsule contained 5-fold more glucose and 2.5-fold more arabinose and mannose than the H37Rv-HH capsule. These data suggested that the molecular basis for the difference between strains H37Rv-CC and -HH, in terms of the ability to bind nonopsonically to CHO cell-expressed CR3, was in the thickness or composition of the outer polysaccharide envelope or capsule.

The role of strain-dependent capsular differences in adherence to mammalian cells was studied further with wild-type (untransfected) CHO cells. We have shown that H37Rv (substrain HH) binds to CHO-WT cells and to primary endothelial cells via surface-exposed PIMs and that the binding is enhanced by serum components that include MBP, whereas binding of Erdman to CHO-WT cells is serum independent and not mediated by PIMs (11). We now find that H37Rv-CC and four of five clinical isolates bound in a serum- and PIM-independent manner, whereas isolate GSH-2288 bound in a serum- and PIM-dependent manner to CHO-WT cells. It is striking that the strains that bound nonopsonically to CHO-Mac-1 cells also bound nonopsonically to CHO-WT cells, whereas the strains that required serum to bind to CHO-Mac-1 cells also bound in a serum- and PIM-dependent manner to CHO-WT cells. The mechanism of nonopsonic binding of H37Rv-CC, and related "nonopsonic" strains, to CHO-WT cells remains unclear, however, as capsular polysaccharides appeared not to be involved.

A flow cytometric analysis of bacterial surface epitopes revealed that surface exposure of the 1D1 Ag was twofold greater on H37Rv-HH than on H37Rv-CC; this difference was eliminated after bead treatment of H37Rv-CC. The 1D1 Ag has been identified as a polar PIM (11), and the data are in agreement with recent reports indicating that although the major constituent of the *M. tuberculosis* capsule is a carbohydrate-protein matrix, PIMs comprise a minor but detectable surface-exposed glycolipid moiety (21). Our results are the first direct demonstration that there are strain-dependent differences in surface exposure of PIMs, and potentially other lipids, in *M. tuberculosis*. Based on the effect of bead treatment and on the quantitative analysis of capsular carbohydrates, these strain-dependent differences in PIM exposure appear to result from variations in capsule thickness. This may similarly be the explanation for the recent observation, also based on flow cytometric analyses, that the more virulent of the *M. tuberculosis* strains in the pairs H37Rv-H37Ra and RIRv-RIRa display smaller amounts of surface-exposed mannoconjugates (22). Although it has been shown that variations in the amounts of envelope AMs and other mannoconjugates do not correlate with the known virulence of established *M. tuberculosis* strains (19), the correlation of quantitative or qualitative differences in D-glucan and other capsular polysaccharides with virulence remains to be determined.

Both H37Rv-HH and H37Rv-CC were opsonized with C3, but only H37Rv-HH included a subpopulation with high levels of surface deposition of C3. Although opsonization of H37Rv-CC was significantly enhanced after bead treatment, these bacteria still did not include a high-fluorescence subpopulation. This may explain why bead treatment produced only a modest serum enhancement of binding of H37Rv-CC bacteria to CHO-Mac-1 cells; presumably only particles with very high levels of surface deposition of C3 bind effectively to the C3bi site on CHO cell-expressed CR3. Of considerable interest, however, is the inference that the efficiency of C3 opsonization is reduced in the presence of abundant capsular polysaccharides. This is a well-known phenomenon that has been documented for a number of gram-positive and gram-negative bacterial pathogens, in which sialic acid-rich capsules inhibit C3 deposition and activation (25). Sialic acid is not a constituent of *M. tuberculosis* capsular polysaccharides, and therefore the mechanism of C3 inhibition is unclear. However, the observation that H37Rv-CC displays lower levels of surface-exposed PIMs may contribute to reduced C3 activation. We have shown that PIMs bind MBP (11), which in turn promotes the so-called lectin pathway of complement activation and C3 deposition (32). Unlike H37Rv-HH, H37Rv-CC does not bind wild-type CHO cells in a serum opsonin- and 1D1 Ag-dependent manner (this study and reference 11). Although we have not shown this directly, we would predict that H37Rv-CC is opsonized inefficiently with serum MBP, which would be in agreement with studies showing that the capsules of bacterial and fungal pathogens inhibit MBP binding (5, 32).

In conclusion, the various lines of evidence presented in this study indicate that outer capsular materials from *M. tuberculosis*, most likely in the form of neutral polysaccharides, modulate the binding of this pathogen to mammalian cells. Clinical isolates and laboratory strains can be divided into two groups, displaying either a nonopsonic or an opsonic binding phenotype. The nonopsonic group, exemplified by H37Rv-CC, binds efficiently in the absence of serum to the phagocytic receptor CR3; based on studies with H37Rv-CC, binding is to the β-glucan lectin site and appears to be mediated by capsular

D-glucan and perhaps D-mannan. This group of strains also binds nonopsonically to wild-type CHO cells, but the ligands and receptors involved are unknown. In contrast, the opsonic group, of which H37Rv-HH is the type strain, requires opsonization in fresh serum (presumably with complement component C3) to adhere to CR3. Serum also enhances the binding of the opsonic group to CHO-WT cells, in part via opsonization with MBP binding to surface-exposed PIMs (11); the CHO cell receptor involved has yet to be identified. Compared with H37Rv-HH, H37Rv-CC expresses significantly greater quantities of capsular carbohydrates, displays less surface-exposed PIM, and is opsonized less effectively with C3. In light of these functional data and the structural data recently reported by Ortalo-Magné et al. (19–21), we propose the following model to account for the two, strain-dependent binding phenotypes. The nonopsonic strains express abundant capsular polysaccharides that can act as ligands for direct binding to CR3 and possibly other receptors. The opsonic strains express less capsular polysaccharide and in addition may be deficient in a specific saccharide required for direct binding to CR3. Moreover, the thinner capsule of the opsonic strains results in greater surface exposure of outer capsular PIMs, which are hence available for binding to nonphagocytic cells, in part following opsonization with MBP. The molecular basis for the strain-dependent differences in expression of capsular components remains to be determined.

What might be the advantages to *M. tuberculosis* of opsonic versus nonopsonic modes of binding to host cell receptors? We have previously discussed the proposal that nonopsonic binding by *M. tuberculosis* to an important phagocytic receptor such as CR3 may confer an advantage in settings in which complement is sparse, as may be the case in the quiescent lung (6). Additional, more subtle benefits to the pathogen can be considered. First, nonopsonic invasion of host cells may result in increased intracellular survival or in a higher degree of cytotoxicity compared to opsonic invasion, as has been shown for both *M. tuberculosis* and group B streptococci (17, 33). Second, even if complement were abundant in the quiescent lung, complement coating alone might not be sufficient to promote efficient invasion. It has been shown that C3bi-coated erythrocytes are not ingested by freshly explanted alveolar macrophages or monocytes, whereas unopsonized zymosan and *Saccharomyces cerevisae* and *Histoplasma capsulatum* yeasts are rapidly phagocytosed by these cells (18, 24, 31); unopsonized yeasts and zymosan bind to the CR3 lectin site (31). This has led to the suggestion that on CR3 the lectin and C3bi sites are fundamentally different, in that binding to the lectin site, but not the C3bi site, leads to receptor activation and stimulation of phagocytosis (18, 31). As has been proposed for the fungi *H. capsulatum* and *Blastomyces dermatitidis* (8, 14, 18) and for various bacterial pathogens (25), expression of a capsule by *M. tuberculosis* may modulate its adherence to host cells and may be altered to suit its requirements, depending on the stage of the disease process. Strains producing abundant capsular polysaccharides may be better able to rapidly invade resting alveolar macrophages during the initial infection by binding to the CR3 lectin site. However, these strains are poorly opsonized by complement or MBP and thus during periods when opsonins are abundant (e.g., during hematogenous dissemination) may be less efficient in invading either phagocytic or nonphagocytic cells than strains with low levels of outer polysaccharides (acapsular variants). It remains to be established whether *M. tuberculosis* can switch spontaneously between states of high or low capsular polysaccharide expression, depending on disease progression, or whether the strains we have identified are stable variants. Thus far we have not observed spontaneous conversion of one type to the other on continuous subculturing in the laboratory.

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