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Nonopsonic Binding of *Mycobacterium tuberculosis* to Human Complement Receptor Type 3 Expressed in Chinese Hamster Ovary Cells

COLETTE CYWES,[1] NICOLE L. GODENIR,[1] HEINRICH C. HOPPE,[1] RENATE R. SCHOLLE,[1] LAFRAS M. STEYN,[2] RALPH E. KIRSCH,[3,4] AND MARIO R. W. EHLERS[1]*

Departments of Medical Biochemistry,[1] Medical Microbiology,[2] and Medicine,[3] and MRC/UCT Liver Research Centre,[4] University of Cape Town Medical School, Observatory 7925, South Africa

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Nonopsonic invasion of mononuclear phagocytes by *Mycobacterium tuberculosis* is likely important in the establishment of a primary infection in the lung. *M. tuberculosis* binds to a variety of phagocyte receptors, of which the mannose receptor and complement receptor type 3 (CR3) may support nonopsonic binding. CR3, a β_2 integrin, is a target for diverse intracellular pathogens, but its role in nonopsonic binding remains uncertain. We have examined the binding of *M. tuberculosis* H37Rv to human CR3 heterologously expressed in Chinese hamster ovary (CHO) cells, thereby circumventing the problems of competing receptors and endogenously synthesized complement, which are inherent in studies with mononuclear phagocytes. The surface expression of CD11b and CD18 was assessed by immunofluorescence, immunobead binding, flow cytometry, and immunoprecipitation with anti-CD11b and anti-CD18 monoclonal antibodies (MAbs). The functional activity of the surface-expressed CD11b/CD18 (CR3) heterodimer was confirmed by rosetting with C3bi-coated microspheres. We found that *M. tuberculosis* bound four- to fivefold more avidly to CR3-expressing CHO cells than to wild-type cells and, importantly, that this binding was at similar levels in the presence of fresh or heat-inactivated human or bovine serum or no serum. In contrast, *Mycobacterium smegmatis* bound poorly to CR3-expressing CHO cells in the absence of serum, but after opsonization in serum, binding was comparable to that of *M. tuberculosis*. The binding of *M. tuberculosis* to the transfected CHO cells was CR3 specific, as it was inhibited by anti-CR3 MAbs, particularly the anti-CD11b MAbs LM2/1 (I domain epitope) and OKM1 (C-terminal epitope), neither of which inhibit C3bi binding. MAb 2LPM19c, which recognizes the C3bi-binding site on CD11b, had little or no effect on *M. tuberculosis* binding. The converse was found for the binding of opsonized *M. smegmatis*, which was strongly inhibited by 2LPM19c but unaffected by LM2/1 or OKM1. CR3-specific binding was also evidenced by the failure of *M. tuberculosis* to bind to CHO cells transfected with an irrelevant surface protein (angiotensin-converting enzyme) in the presence or absence of serum. We conclude that the binding of *M. tuberculosis* H37Rv to CR3 expressed in CHO cells is predominantly nonopsonic and that the organism likely expresses a ligand that binds directly to CR3.

Mycobacterium tuberculosis is a serious public health threat, infecting one-third of the world's population and annually causing 3 million deaths (7). In efforts to develop novel antituberculous strategies, the virulence mechanisms of this organism are under renewed scrutiny. *M. tuberculosis* is a facultative intracellular pathogen that efficiently colonizes mononuclear phagocytes, a process that is critical for the successful establishment of a primary infection (9). In common with other macrophage pathogens, *M. tuberculosis* binds to a restricted set of phagocyte receptors, which include complement receptor types 1, 3, and 4 and the mannose receptor (20, 43, 44, 48). Of these, complement receptor type 3 (CR3) is of particular interest, because it is the target receptor for a diverse group of intracellular pathogens (17, 24) and is unique in promoting phagocytosis without an accompanying respiratory burst (5, 51). It has been speculated that routing of pathogens into macrophages via CR3 specifically enables subsequent intracellular survival and growth (21, 37). At present, the extent to which opsonization with complement component C3 is required for the binding of *M. tuberculosis* to CR3 is unknown. Serum complement enhances the adherence of *M. tuberculosis* to mononuclear phagocytes by approximately threefold (20, 43, 44). However, a component of the binding of *M. tuberculosis* to the mononuclear phagocyte complement receptors, including CR3, is serum and complement independent (20, 43, 44, 48).

The question of nonopsonic binding of *M. tuberculosis* to mononuclear phagocytes is an important one because complement components may be limiting in the alveolar space in the quiescent lung (38), and it is generally agreed that the initial primary infection results from the invasion of alveolar macrophages by bacilli acquired after inhalation of infectious droplets (7). A proportion of nonopsonic binding is mediated by the mannose receptor, although its contribution relative to the complement receptors is uncertain and binding to the mannose receptor is limited to certain strains of the *M. tuberculosis* complex (43). Efforts to determine the extent of nonopsonic binding of *M. tuberculosis* to CR3 and other complement receptors (20, 43, 48) are hindered by the fact that C3 is synthesized endogenously by macrophages (6, 16, 50). In this report, we have reexamined this question by studying the binding of *M. tuberculosis* to human CR3 expressed in Chinese hamster ovary (CHO) cells, thereby avoiding the difficulties of endogenously synthesized complement and the binding of *M. tuberculosis* to competing macrophage receptors. We find that the binding of *M. tuberculosis* H37Rv to CR3 under these conditions is serum and complement independent.

* Corresponding author. Phone: 27 21 406-6335. Fax: 27 21 47 7669. Electronic mail address: mehlers@physio.uct.ac.za.

MATERIALS AND METHODS

Bacteria. *M. tuberculosis* H37Rv (ATCC 27294) and *Mycobacterium smegmatis* (ATCC 19420) were maintained on Lowenstein-Jensen slopes. Bacterial stocks required for the in vitro infection assays were generated as follows. The confluent growth of *M. tuberculosis* on a Lowenstein-Jensen slope was dislodged and suspended in 8 ml of Kirchner's medium, agitated to break up the larger clumps, and allowed to settle for 10 min at room temperature. The top 5 ml was used to inoculate 100 ml of Kirchner's medium and grown with shaking (200 rpm) at 37°C for 2 weeks. Fifty milliliters of the 2-week-old culture was used to inoculate 200 ml of fresh Kirchner's medium and grown as described above for 7 to 10 days. The second culture was allowed to settle, and the top suspension of bacteria, which consisted of single cells and clumps of 3 to 10 organisms, was used directly in the binding assays or was frozen in 0.5-ml aliquots at −20°C. *M. smegmatis* was grown in Kirchner's medium, stationary, at 37°C under 5% CO_2 for 2 days. Bacteria were quantitated from the optical density at 550 nm with reference to a standard curve calibrated by McFarland's standards and serial plating to obtain CFU counts. Prior to quantitation, the bacterial suspension was forced through progressively narrower-gauge syringe needles (ending with an insulin needle, 0.36-mm diameter) to reduce clumping; this procedure was also followed after thawing frozen stocks. The quality of the bacterial stocks was assessed microscopically after Ziehl-Neelsen staining and by regular plating on Kirchner agar.

Human MDMs. Peripheral blood monocytes were obtained from the adherent fraction after Ficoll-Histopaque (Sigma) centrifugation of heparinized blood from healthy volunteers. Monocytes were grown in RPMI 1640 medium supplemented with 20% autologous serum for the first 3 h, after which the conditioned media were saved and the cells were refed with RPMI 1640–10% autologous serum supplemented with 25% conditioned medium. The cells were grown for a minimum of 6 days before use as monocyte-derived macrophages (MDMs).

Transfected cells. CHO-Mac-1 cells, obtained from Timothy A. Springer, Harvard Medical School, are a CHO cell line stably expressing human wild-type CR3 (Mac-1) (12). The cell line was generated by cotransfecting CHO cells (CHO DG 44; containing a deletion of the dihydrofolate reductase gene) with cDNAs for CD11b and CD18 (each in the vector pCDM8) and with the amplifiable vector pCHIP, encoding methotrexate resistance. CHO-Mac-1 cells were maintained in α-modified Eagle medium supplemented with 10% dialyzed, heat-inactivated fetal bovine serum (FBS), 16 μM thymidine, and 0.1 μM methotrexate.

Additional CHO cell lines that transiently express human CR3 were generated as follows. The plasmids πH3M-CD11b (3) and πH3M-CD18 (2), containing the CD11b and CD18 cDNAs, respectively, were obtained from M. Amin Arnaout, Massachusetts General Hospital. The CD11b and CD18 cDNAs were excised and ligated into *Xba*I and *Hin*dIII sites and *Xba*I and *Sma*I sites, respectively, of the eukaryotic expression vector pEE14 (obtained from Christopher R. Bebbington, Celltech Ltd., Slough, England) (see reference 4), to generate pEE-CD11b and pEE-CD18.

CHO-K1 cells (ATCC CCL61) were grown in 50% Dulbecco modified Eagle medium–50% Ham's F-12 medium supplemented with 10% heat-inactivated FBS (complete medium) and cotransfected with 10 μg each of pEE-CD11b and pEE-CD18 by the calcium phosphate precipitate method, essentially as described previously (4). The cells were glycerol shocked after 4 h, washed and incubated overnight in complete medium, grown in glutamine-deficient Glasgow modified Eagle medium (GMEM) supplemented with 10% dialyzed, heat-inactivated FBS, and used in bacterial adherence studies within 72 h after transfection. These transient cell lines were designated CHO-CR3T to distinguish them from the CHO-Mac-1 cells described above.

CHO-ACE cells are a CHO-K1 cell line stably transfected with the plasmid pLEN-ACEVII and express high levels of membrane-bound angiotensin-converting enzyme (ACE), a type I transmembrane protein (14, 15).

The surface areas of CHO-Mac-1 cells and wild-type CHO-K1 (CHO-WT) cells were determined by video-enhanced image analysis of light microscopic sections as follows. Cells were grown in complete medium on 12-mm-diameter glass coverslips to a density of approximately 10^5 cells per coverslip, fixed in 2.5% glutaraldehyde, and maintained in 0.1 M cacodylate buffer (pH 7.2) containing 0.1 M sucrose, 5 mM $CaCl_2$, and 5 mM $MgCl_2$. The cells were examined under light microscopy with a Leica DM-RB microscope, and cell surface areas were measured by analysis with a Leica Q.500-MC image analysis system.

MAbs. The anti-CD11b monoclonal antibodies (MAbs) used were 2LPM19c (immunoglobulin G1k [IgG1k]; Dako, Glostrup, Denmark), LM2/1.6.11 (LM2/1) (IgG1; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City), and OKM1 (IgG2b; American Type Culture Collection, Rockville, Md.). Anti-CD18 MAbs included MHM23 (IgG1k; Dako), TS1/18.1.2.11.4 (TS1/18) (IgG1; Developmental Studies Hybridoma Bank), and IB4 (IgG2a; ATCC). MAbs 2LPM19c and MHM23 were supplied as purified immunoglobulin (approximately 200 μg/ml); the other MAbs were used as hybridoma supernatants. For the immunoprecipitations, MAbs IB4 and OKM1 were purified by protein A affinity chromatography from ascites fluid (stock solutions at 5 to 6 mg/ml). Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human C3c (Dako) is a polyclonal antibody specific for C3, C3b, and the C3c component of C3.

Analysis of CD11b/CD18 expression. The heterologous expression of CD11b and CD18 in transfected CHO cells was analyzed by reverse transcriptase-PCR detection of CD11b and CD18 mRNAs and by immunological detection of the cell surface expression of CD11b/CD18 by immunofluorescence, a cell bead immunoassay, flow cytometry, and immunoprecipitation.

Reverse transcriptase-PCR detection of the CD11b mRNA was performed with oligonucleotide primers specific for the CD11b A (or I) domain, with the sequence 5′-CGGCAGCAGCCCCAGA for the forward primer and 5′-AGCG CTGAAGCCTTCCTG for the reverse primer, which amplify nucleotides 466 to 1146 of the CD11b cDNA (3); for CD18, the primers were 5′-TCAACGAGAT CACCGAGTCC for the forward primer and 5′-GCGTTGTGATCCAGGAA GAC for the reverse primer, which amplify nucleotides 542 to 1190 of the CD18 cDNA (25). Total cellular RNA was extracted from transfected and wild-type CHO cells in Catrimox-14 cationic surfactant (10) and was reverse transcribed and PCR amplified by the protocol of Park (34); the plasmids πH3M-CD11b and -CD18 (see above) were used as positive controls in the PCR. Amplified products were examined by agarose gel electrophoresis.

Cell surface expression of CD11b and CD18 was visualized by indirect and direct immunofluorescence. Transfected and wild-type CHO cells grown on 12-mm-diameter glass coverslips (for details, see "Mycobacterial binding assays" below) were incubated with MAbs 2LPM19c (anti-CD11b) and MHM23 (anti-CD18) for 30 min at 4°C, washed in phosphate-buffered saline (PBS), incubated with FITC-conjugated, rabbit anti-mouse IgG antiserum (Boehringer Mannheim, Randburg, South Africa) for 15 min at 4°C, washed again, fixed in 1% paraformaldehyde, and immediately examined by fluorescence microscopy (with a Zeiss Axioskop with a Pan-Neofluar 100×/1.30 oil immersion objective). Direct immunofluorescence was performed by incubating the cells with phycoerythrin-conjugated MAb 2LPM19c and FITC-conjugated MAb MHM23, giving dual staining of the same field. In addition to immunofluorescence, CD11b/CD18 surface expression was visualized with a cell bead immunoassay (47). Protein A-Sepharose beads (Pierce, Rockford, Ill.) were incubated with MAbs LM2/1 (anti-CD11b) or TS1/18 (anti-CD18) for 1 h at room temperature, coupled in 0.2 M boric acid (pH 9.0) containing 20 mM dimethylpimelimidate (Pierce) for 30 min at room temperature, and then stopped by a 2-h incubation in 0.2 M ethanolamine (pH 8.0) at room temperature. The beads were washed and resuspended in PBS and then incubated with transfected CHO cells for 30 min at room temperature. After washing in PBS, cell surface expression of CD11b and CD18 was estimated by counting, under light microscopy, the number of cells rosetted with beads per 100 cells examined; each cell line was analyzed in triplicate.

Surface expression of CD11b and CD18 was quantitated by dual immunofluorescence flow cytometry. Transfected and wild-type cells (10^5 per determination) were incubated for 15 min at 4°C with phycoerythrin-conjugated 2LPM19c (anti-CD11b) and FITC-conjugated MHM23 (anti-CD18) or with similarly conjugated irrelevant mouse IgG1 as the isotypic (negative) control. After washing in PBS, the cells were detached at 4°C in 5 mM EDTA in PBS, pelleted, and resuspended in 1 ml of 1% paraformaldehyde in PBS. The cell suspensions were analyzed on a Coulter Epics flow cytometer.

The expressed CD11b and CD18 proteins were visualized by immunoprecipitation after surface iodination. Transfected and wild-type cells (4×10^6) were detached by incubation in 5 mM EDTA in PBS at 37°C for 15 min, washed and resuspended in PBS, and surface iodinated with [^{125}I]Na by the lactoperoxidase method, as described previously (42). After washing, the cell pellets were lysed in 280 μl of lysis buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4], 200 mM NaCl, 2 mM $CaCl_2$, 2.5 mM $MgCl_2$, 1% Triton X-100, 0.03 U of aprotinin, 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 12,000 rpm in a microcentrifuge, and then 140 μl of the supernatants was incubated with MAb OKM1 (anti-CD11b) or IB4 (anti-CD18) (6 μg of IgG purified from ascites fluid) at 4°C for 1 h. Protein A-agarose beads were added to the lysates, and the mixture was incubated at 4°C for 1 h, pelleted, washed twice with lysis buffer and once with PBS, and then boiled for 3 min in 20 μl of sodium dodecyl sulfate (SDS) loading buffer. After centrifugation, the supernatants were run on an SDS–7% polyacrylamide gel. The gels were dried, and radiolabelled, immunoprecipitated proteins were visualized by autoradiography.

C3bi-binding activity of CR3 expressed on MDMs and transfected CHO cells. The functional activity of recombinant human CR3 expressed on CHO cells cotransfected with CD11b and CD18 cDNAs was assessed by rosetting with C3bi-coated polystyrene microspheres, prepared as described by Schlesinger and Horwitz (45). Briefly, 1-μm polystyrene microspheres (Sigma) were incubated in a 0.01% suspension of *M. leprae* phenolic glycolipid-1 (PGL-1; obtained from Patrick J. Brennan, Colorado State University, Fort Collins) in carbonate-bicarbonate buffer at 37°C for 24 h. Control microspheres were incubated in buffer without PGL-1; PGL-1 fixes complement component C3, of which a portion is in the form of C3bi (45). The microspheres were washed, incubated in PBS containing 5% bovine serum albumin (BSA), washed, and then incubated in 2.5% fresh human serum in PBS for 30 min at 37°C. The microspheres were washed and resuspended in PBS–0.5% BSA and incubated with CR3-transfected CHO cells and human MDMs seeded on glass coverslips at 37°C, under 5% CO_2, for 24 h. The cells were washed extensively with PBS, fixed in 2.5% glutaraldehyde, briefly stained with trypan blue, and examined under light microscopy. Rosetting was quantitated as described above for the cell bead immunoassay.

Flow cytometric analysis of C3 fixation to opsonized microspheres and bacteria. Opsonized, PGL-1-coated microspheres were prepared as described above. *M. tuberculosis* and *M. smegmatis* (1.2×10^8 bacteria) were incubated in fresh, complete human serum at 37°C for 1 h. Opsonized microspheres and bacteria

were washed in PBS and incubated on ice with a 1:100 dilution of FITC-conjugated rabbit anti-human C3c antibody in PBS–0.5% BSA or with an FITC-conjugated mouse IgG isotypic control. After 1 h, the particles were washed in PBS, fixed in 1% paraformaldehyde, and analyzed on a Coulter Epics flow cytometer.

Mycobacterial binding assays. CHO cells and MDMs seeded at a density of 10^5 on 12-mm-diameter glass coverslips within 24-well plates were grown for 3 h, washed, and then refed with 0.5 ml of medium containing either (i) 10% fresh human serum from a purified protein derivative-negative, healthy volunteer or 10% recently purchased FBS (complement activity was verified by a complement lysis assay, performed as described previously [29]); (ii) 10% heat-inactivated (56°C for 30 min) human serum or FBS; or (iii) no serum. The bacterial suspension in Kirchner's broth (generally ~50 μl containing 6×10^8 bacilli per ml) was added directly to the medium to give multiplicities of infection (MOIs) of 300:1 and 30:1 (bacteria to cell) for CHO cells and 50:1 for MDMs. In some cases, bacteria were preopsonized by incubation in fresh, complete human serum at 37°C for 1 h before addition to the cells in serum-free medium. After a 24-h incubation (3 h for MDMs), the cells were washed extensively with PBS, fixed in 2.5% glutaraldehyde, stained with acridine orange (0.014%; 1 min at room temperature) followed by quenching in crystal violet (0.5%; 1 min at room temperature), and examined under fluorescence microscopy. Bacterial binding was quantitated as the percentage of cells to which one or more bacteria were bound, and as the mean number of bacteria per cell on each coverslip, by counting 100 to 200 cells per coverslip; bacteria from each cell line were plated on three coverslips per experiment. No attempt was made to distinguish between surface adherence and internalization of the bacteria.

Inhibition of mycobacterial binding to transfected cells. Attempts were made to block the binding of *M. tuberculosis* and *M. smegmatis* to the transfected CR3 by the addition of CR3-specific MAbs. CHO cells were preincubated in serum-free medium containing one or more of the following MAbs: 2LPM19c (1:300 dilution of purified IgG), LM2/1 (undiluted hybridoma supernatant), OKM1 (0.9 μg/ml; protein A column eluate), MHM23 (1:300 dilution; IgG), and TS1/18 (hybridoma supernatant). Preincubations with MAbs were at either 37°C for 30 min or 4°C for 3 h and were followed by the addition of *M. tuberculosis* or preopsonized *M. smegmatis* and incubation at 37°C overnight; in some cases, cells preincubated at 4°C were also incubated with the bacteria at 4°C overnight. Bacterial binding was quantitated as described above, and the significance of differences was determined by Student's *t* test. Effects of the MAbs on cell viability and morphology were assessed by incubating CHO cells with MAb 2LPM19c or LM2/1, as described above, or in serum-free medium only at 4°C for 6 h, before washing in PBS, addition of trypan blue, and examination under light microscopy, and also by image analysis of light micrographs (see above).

RESULTS

Expression of CD11b/CD18 in CHO cells. We examined the heterologous expression of the CD11b and CD18 polypeptides in transfected CHO cells by a number of methods. Reverse transcriptase-PCR revealed the expected 648- and 680-bp products amplified with CD18- and CD11b-specific primers, respectively, in the CHO-Mac-1 cells and in CHO cells transiently transfected with pEE-CD18 and pEE-CD11b (CHO-CR3T cells) (data not shown). By direct and indirect immunofluorescence, ~85% of CHO-Mac-1 cells fluoresced at levels above background when stained with an anti-CD18 MAb, and ~90% fluoresced with an anti-CD11b MAb; of the fluorescent cells, approximately one-third fluoresced brightly and two-thirds fluoresced moderately. Fluorescence was uniform (not punctate) and particularly strong around the cell margins and over pseudopodia (Fig. 1A). A similar percentage of CHO-CR3T cells fluoresced with both MAbs, although at a lower intensity (data not shown). Interestingly, a separate batch of CHO-Mac-1 cells that we obtained from T. A. Springer's laboratory fluoresced weakly with both anti-CD18 and anti-CD11b MAbs, at 6 and 15%, respectively. We designated these CHO-Mac-1LE (for low expressing) and used them as negative controls in some of the binding assays. The immunofluorescence results were reinforced with a cell bead immunoassay using anti-CR3 MAb-coated agarose beads. With anti-CD18-coated beads, rosetting was seen in 90% of CHO-Mac-1 cells, 85% of CHO-CR3T cells, and 10% of CHO-WT cells; with anti-CD11b beads, the results were 92, 90, and 12%, respectively (data not shown).

A more quantitative analysis was obtained by dual immunofluorescence flow cytometry, which revealed that the CHO-Mac-1 cells express 86.4% CD18, 92.9% CD11b, and 86.2% dual CD11b/CD18 (Fig. 1D); these data are in good agreement with those of the immunofluorescence and cell bead assays. Flow cytometry also confirmed the low expression in the CHO-Mac-1LE cells, which express only 2.7% CD18, 4.0% CD11b, and 2.5% dual CD11b/CD18 (Fig. 1E). Final confirmation of the presence of heterologously expressed CD11b/CD18 in the transfected CHO cells was obtained by surface iodination and immunoprecipitation. As shown in Fig. 1F, incubation of cell lysates with MAb IB4 (anti-CD18) or OKM1 (anti-CD11b) resulted in the specific immunoprecipitation of two radiolabelled polypeptides at 170 and 95 kDa, which correspond to CD11b and CD18, respectively, and which constitute the CR3 heterodimer. Taken together, these results indicate that a high percentage of CHO cell transfectants, both the CHO-Mac-1 cells and the CHO-CR3T cells, dually express the CD18 (β_2) and CD11b (α_M) subunits on their cell surfaces.

We determined the surface area of transfected cells to control for the possibility that differences between the adherence of bacteria to transfected cells and that to wild-type cells may result from transfected cells having a larger surface area such that, on average, a greater percentage of cells will bind at least one bacterium. By video-enhanced image analysis of light micrographs of cells grown on coverslips under the conditions used for the binding assays, we found that CHO-Mac-1 cells have an average surface area of 565 ± 174 μm^2 (mean ± standard deviation) compared with 693 ± 142 μm^2 for CHO-WT cells (Table 1). Thus, greater surface area of the transfected cells is not a confounding variable in this study.

C3bi-binding activity of transfected CHO cells. To assess whether the recombinant CD11b and CD18 subunits expressed in transfected CHO cells have assembled into functional $\alpha_M\beta_2$ (CR3) heterodimers, we examined the binding of C3bi-coated microspheres to CD11b/CD18-transfected cells and compared them with untransfected cells (negative control) and MDMs (positive control). An additional negative control was provided by the use of microspheres not coated with C3bi (PGL-1-negative microspheres; see Materials and Methods). C3bi is a well-characterized ligand for CR3, and binding of C3bi-coated particles is an established assay for the functional activity of CR3 (12). Rosetting with C3bi-coated microspheres was observed in 53% of CHO-Mac-1 cells, 76% of CHO-CR3T cells, and 82% of MDMs but in only 12% of CHO-WT (untransfected) cells (Fig. 1B). In contrast, rosetting with control microspheres was observed in 12, 18, 8, and 15% of CHO-Mac-1, CHO-CR3T, MDMs, and CHO-WT cells, respectively (Fig. 1C). These results indicate that a proportion of the surface-expressed CD11b and CD18 subunits have assembled as functional CR3 proteins.

Detection of C3 fixed to opsonized microspheres and bacteria. To confirm that serum opsonization of PGL-1-coated microspheres and of *M. tuberculosis* and *M. smegmatis* bacteria results in the fixation of C3 on the particle surface, we incubated opsonized particles with FITC-conjugated anti-human C3c antibody and analyzed the immunofluorescence by flow cytometry (Fig. 2). Opsonized PGL-1-coated microspheres were 95.6% positive for staining with anti-C3c-FITC compared with 4.9% of unopsonized PGL-1-coated beads (Fig. 2A). Opsonized *M. smegmatis* bacteria were 88.4% positive compared with 11.8% for unopsonized bacteria (Fig. 2B); the fluorescence peak for positively staining bacteria is very broad, unlike that for the opsonized microspheres (compare Fig. 2A and B), presumably because C3 fixation is nonuniform, leading to a heterogeneous population of particles. Results with opsonized

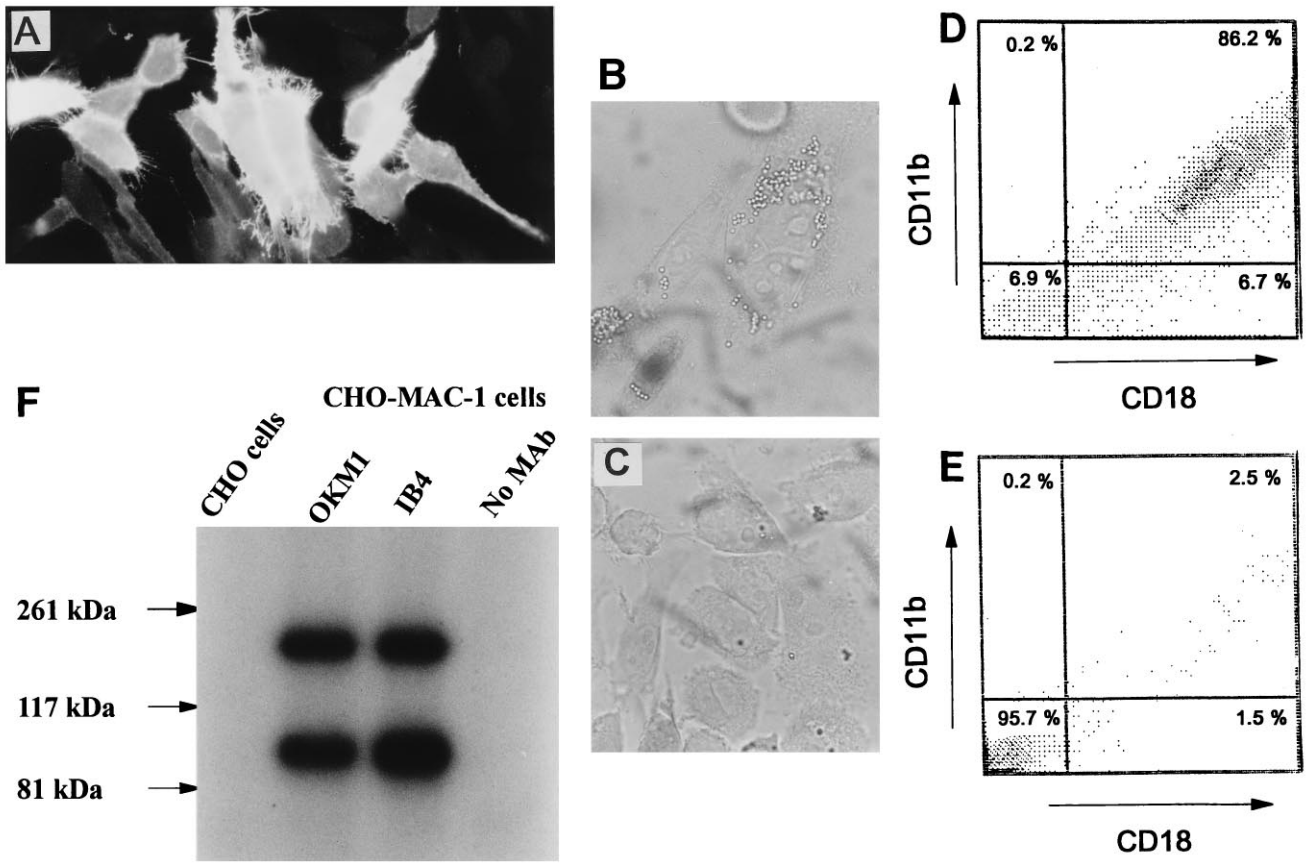


FIG. 1. Cell surface expression and C3bi binding activity of CD11b/CD18 in transfected CHO cells. (A) Direct immunofluorescence of CHO-Mac-1 cells for CD18 with FITC-conjugated MAb MHM23; staining for CD11b with phycoerythrin-conjugated MAb 2LPM19c gave virtually identical fluorescence of the same field (data not shown). (B) Binding of C3bi-coated microspheres (generated by coating microspheres with PGL-1 and then incubating them in fresh human serum [see Fig. 2A]) to CHO-CR3T cells. (C) Incubation of CHO-CR3T cells with control microspheres (not coated with PGL-1). The rod-shaped shadows are artifacts. (D and E) Dual immunofluorescence flow cytometry of CHO-Mac-1 cells (D) and CHO-Mac-1LE cells (E) by using conjugated antibodies as described for panel A above. (F) Surface iodination and immunoprecipitation of wild-type CHO cells (left lane) and CHO-Mac-1 cells (all other lanes). The wild-type CHO cell lysates were immunoprecipitated with a combination of MAbs OKM1 (anti-CD11b) and IB4 (anti-CD18), whereas the CHO-Mac-1 cell lysates were treated with either antibody alone or none; immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

and unopsonized *M. tuberculosis* H37Rv were virtually identical to those obtained with *M. smegmatis* (data not shown).

Binding of mycobacteria to CR3-expressing cells. Incubation of human MDMs with *M. tuberculosis* at an infectivity ratio of 50:1 in the presence of 10% human serum resulted in binding of bacteria to 81% of cells; comparable results have been reported by others (9, 20, 44). In experiments with the CHO cells, an infectivity ratio of 300:1 was found to give useful results, and this was used throughout unless stated otherwise (Fig. 3). Studies on the binding of *M. tuberculosis* to CHO cells led to two striking results: (i) binding of *M. tuberculosis* to CR3-expressing CHO cells was consistently four- to fivefold greater than that to wild-type CHO cells, and (ii) the binding levels of *M. tuberculosis* to CR3-expressing CHO cells were essentially identical in the presence of fresh serum, heat-inactivated serum, or no serum (Table 2 and Fig. 3). Overall, in 10 separate experiments, the percentage of CR3-expressing CHO cells that bound *M. tuberculosis* was 70% ± 11% in the presence or absence of serum, versus 15% ± 3% of wild-type CHO cells. Similarly, on average, 1.36 ± 0.40 bacteria per cell were bound in the presence of serum and 1.66 ± 0.14 bacteria per cell were bound in the absence of serum by CR3-expressing CHO cells, versus a combined average of 0.20 ± 0.03 bacteria per cell by CHO-WT cells in the presence or absence of serum (Table 2). Thus, the mean number of bacteria bound by CR3-expressing cells is seven- to eightfold greater than that bound by wild-type cells.

TABLE 1. Average surface areas of transfected and wild-type CHO cells[a]

Cell line	Section	No. of cells	Area fraction[b]	Surface area (μm^2)	
				Total	Per cell
CHO-WT	1	9	0.2	6,741	749
	2	10	0.16	5,305	531
	3	8	0.19	6,381	798
Mean ± SD		9 ± 1	0.18 ± 0.02	6,143 ± 747	693 ± 142
CHO-Mac-1	1	5	0.11	3,664	733
	2	6	0.09	2,880	480
	3	4	0.08	2,745	686
	4	5	0.05	1,810	362
Mean ± SD		5 ± 0.8	0.08 ± 0.03	2,775 ± 760	565 ± 174

[a] Cells were grown on glass coverslips, fixed in glutaraldehyde, maintained in cacodylate-sucrose buffer, and examined under light microscopy. Surface areas were determined by video-enhanced image analysis.

[b] Fraction of the measured frame, which was 33,354 μm^2 in all sections.

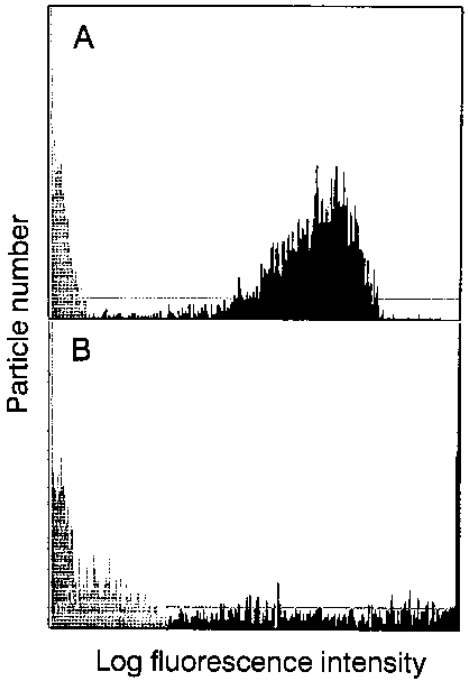


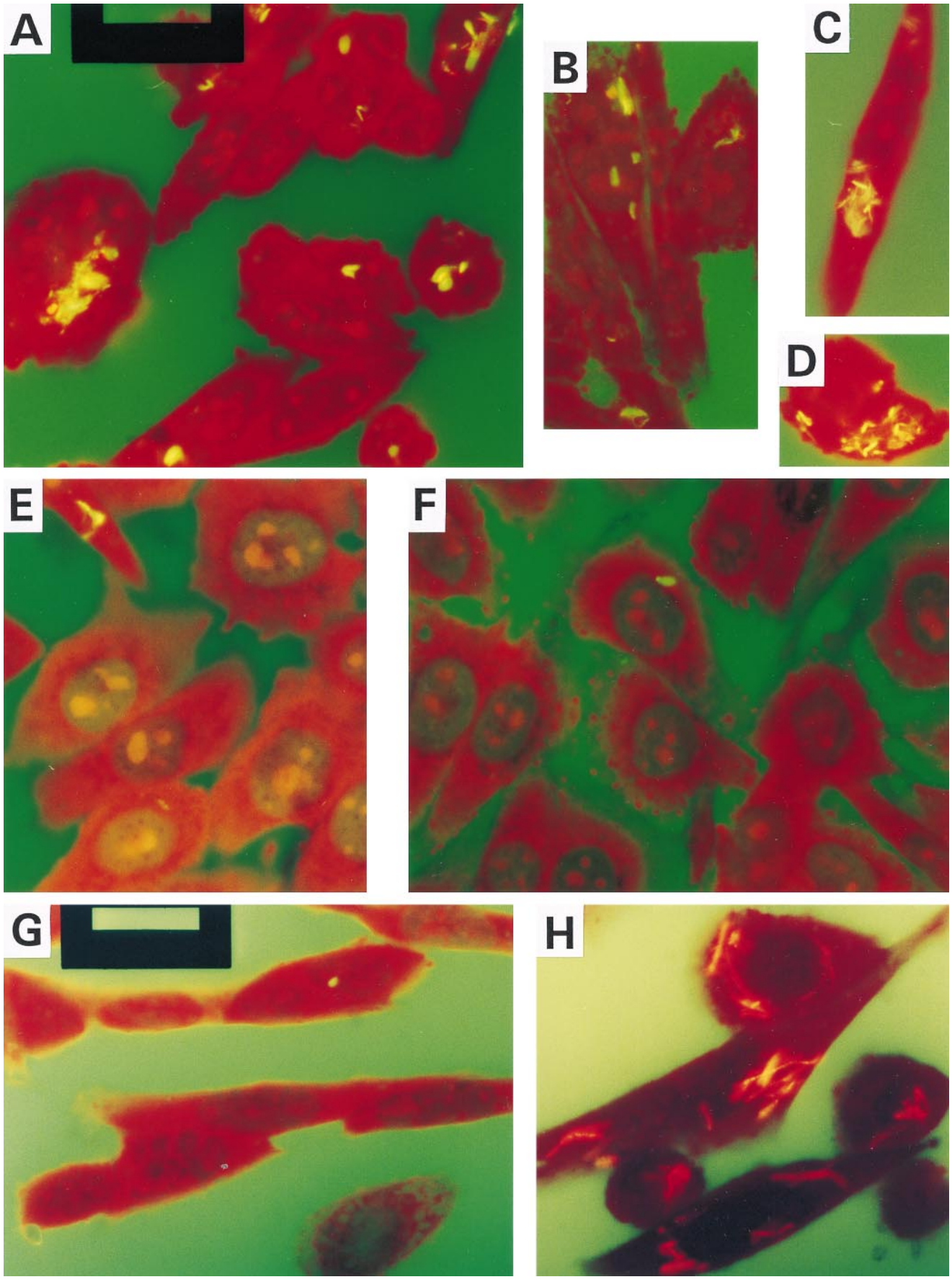
FIG. 2. Immunofluorescence flow cytometry of opsonized particles stained for C3 and C3 breakdown products with FITC-conjugated anti-human C3c antibody. (A) PGL-1-coated microspheres opsonized in fresh human serum (densely shaded, on right; 95.6% positive) compared with control microspheres (not coated with PGL-1) also incubated in serum (lightly shaded, on left); the fluorescence peak for PGL-1-coated, opsonized microspheres stained with an isotypic control antibody was indistinguishable from the peak for the control microspheres. (B) *M. smegmatis* opsonized in fresh human serum (densely shaded broad peak, middle and right; 88.4% positive) compared with unopsonized *M. smegmatis* (lightly shaded, middle and left; 11.8% positive) and opsonized *M. smegmatis* stained with the isotypic control (lightly shaded narrow peak, extreme left).

To exclude the possibility that the CR3-expressing CHO cells are generally "stickier" and will bind opsonized or unopsonized particles more avidly than wild-type cells, we performed two controls. First, we incubated the transfected cells with a control particle that, unless opsonized with a CR3 ligand, does not bind to CR3. In preliminary experiments, we observed that unopsonized *M. smegmatis* bacteria bound poorly to transfected CHO cells and that binding was markedly enhanced after preopsonization in human serum, which leads to the deposition of C3 breakdown products on the bacterial surface (Fig. 2B). We therefore considered *M. smegmatis* a suitable control particle and found that incubation of CHO-Mac-1 cells with unopsonized *M. smegmatis* resulted in only 17% ± 2% of cells bound to bacteria, which is similar to 15% ± 3% binding of CHO-WT cells. In contrast, preopsonized *M. smegmatis* bacteria were bound by 83% ± 6% of CHO-Mac-1 cells versus 21% ± 6% of CHO-WT cells (Table 2). It is interesting that the percentage of cells bound by opsonized *M. smegmatis* is comparable to the results for *M. tuberculosis* but the mean number of bacteria per cell is higher for *M. smegmatis* (Table 2). This may reflect the fact that unlike *M. tuberculosis*, *M. smegmatis* is a fast-growing mycobacterium and has replicated during the course of the incubation (24 h). Second, a nonspecific increased stickiness of transfected cells for *M. tuberculosis* and other particles may be due to altered expression of endogenous surface molecules resulting from high-level expression of recombinant, membrane-bound CR3. To exclude this, we incubated *M. tuberculosis* in the presence and absence of serum with CHO-ACE cells, which express high levels of membrane-bound ACE on the cell surface (14). We found that binding to CHO-ACE cells was low and no different from binding to CHO-WT (Table 3; compare Fig. 3F and E), indicating that expression of recombinant surface proteins in CHO cells does not generally lead to an increased adhesiveness for *M. tuberculosis*. It remains possible that expression of recombinant CR3, unlike ACE, does lead to a nonspecific increased adhesiveness of CHO cells, but this seems unlikely in view of the poor binding to CHO-Mac-1 cells of unopsonized *M. smegmatis*, a mycobacterium that shares with *M. tuberculosis* most of the unique components of the mycobacterial cell wall (8).

Finally, we performed the infections of CHO-Mac-1 cells with 10-fold-fewer *M. tuberculosis* bacteria (30:1 instead of 300:1) to exclude the possibility that no enhancement of binding was seen in the presence of serum, because at high MOIs the system was essentially saturated. However, we found that at an MOI of 30:1, the percentages of CHO-Mac-1 cells bound to *M. tuberculosis* were 47% ± 5% and 52% ± 5% in the presence and absence of serum, respectively, and these were only slightly less than 54% ± 6% and 57% ± 4%, respectively, at an MOI of 300:1 (Table 3). Similarly, although at 30:1 the mean number of bacteria per cell was approximately 31% lower than that at 300:1, there was no increase in the number of bacteria per cell in the presence of serum at either MOI tested (Table 3).

Inhibition of mycobacterial binding to transfected cells. The specificity of the binding of *M. tuberculosis* to CR3 in the transfected CHO cells was confirmed by the use of MAbs to human CR3. Pretreatment of CHO cells with a combination of anti-CD11b and anti-CD18 MAbs reduced binding of *M. tuberculosis* by CR3-expressing CHO cells by an average of 83% and the mean number of bacteria per cell by 88% but had no effect on binding of *M. tuberculosis* to wild-type (CHO-WT) cells (Table 4). Pretreatment with the anti-CD11b MAb 2LPM19c alone resulted in weak inhibition of binding (4 to 27%), whereas MAb LM2/1.6.11 inhibited binding by 68 to 77% and MAb OKM1 inhibited binding by 74% (Tables 4 and 5). It is notable that 2LPM19c binds to the I domain of the CD11b subunit to a site that includes the C3bi-binding site, whereas LM2/1 also binds to the I domain but not to the C3bi-binding site and OKM1 binds to the C-terminal domain of CD11b (12). These data, therefore, suggest that *M. tuberculosis* binds to CR3 at a site that is distinct from the C3bi-binding site. In contrast, the binding of opsonized *M. smegmatis* is inhibited strongly by MAb 2LPM19c (by 90% [Table 5]), as would be expected for a C3bi-coated particle, but not by MAbs LM2/1 and OKM1. Pretreatment with the anti-CD18 MAb MHM23 alone inhibited *M. tuberculosis* binding, although to a lesser and variable extent, at 34 to 65% (Tables 4 and 5). Interestingly, MHM23 had no effect on the binding of opsonized *M. smegmatis*.

The antibody inhibition experiments were repeated on nine separate occasions and gave comparable results in every case. MAbs 2LPM19c and LM2/1 did not result in significant cytotoxicity or change in cell morphology or surface area, as judged by trypan blue exclusion (>98%) and examination under light microscopy and image analysis (data not shown). Moreover, pretreatment of the CHO cells with the antibodies, as well as the subsequent incubations with bacteria, was performed at both 37 and 4°C. It is possible that at 37°C the use of intact antibodies, as in this study, can lead to capping and internalization of CR3, and therefore, apparent inhibition of *M. tuberculosis* binding is due to receptor downregulation rather than physical blocking. Additionally, it may be possible that CR3 associates with an unknown, endogenous CHO cell receptor


A
B
C
D
E
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H

FIG. 3. Fluorescence micrographs of transfected and wild-type CHO cells infected with mycobacteria. Cells were incubated with *M. tuberculosis* at 300:1 (A, B, and D to G) or 30:1 (C) or with *M. smegmatis* at 300:1 (H) for 24 h, washed, stained with acridine orange, and quenched in crystal violet. Incubations were in the absence (A and C to G) or presence (B and H) of serum. (A to D and H) CHO-Mac-1 cells, including examples of heavily infected cells at MOIs of 30:1 and 300:1 (C and D, respectively); (E) CHO-WT cells; (F) CHO-ACE cells; (G) CHO-Mac-1 cells preincubated with MAb OKM1. Magnification, ×1,000; bars in panels A and G, 20 μm (inner diameter) and 32 μm (outer diameter).

that is involved in binding *M. tuberculosis* and which is cocapped and internalized after treatment with anti-CR3 antibodies at 37°C. We found that the levels of inhibition of binding of *M. tuberculosis* to CHO-Mac-1 cells by MAb LM2/1 were similar, irrespective of the temperature conditions, which included pretreatment and infection at 37°C (Table 4), pretreatment at 4°C and infection at 37°C, or pretreatment and infection at 4°C (Table 5). Similarly, binding of opsonized *M. smegmatis* was inhibited by MAb 2LPM19c whether infections were performed at 37°C or 4°C (Table 5).

Taken together, the results obtained from the binding and inhibition studies indicate that *M. tuberculosis* binds to functionally active CR3 expressed in CHO cells and that this binding is serum and complement independent. Moreover, *M. tuberculosis* binds to a site that appears to be distinct from the C3bi-binding site. A minor but constant component of binding of *M. tuberculosis* to CHO cells is independent of the heterologous expression of CR3, as is evident from the binding to CHO-WT and CHO-Mac-1LE cells. The mechanism of this binding is unknown.

DISCUSSION

Numerous intracellular pathogens, including the mycobacteria, bind to integrin receptors as a first step in the invasion of mammalian cells (17, 24). This is likely because integrins are cell adhesion proteins that are linked via their cytoplasmic domains to the actin cytoskeleton and proximal components of signal transduction pathways (22). *M. tuberculosis* binds to the β_2 integrins CR3 and CR4 (20, 44, 48), a family of adhesion receptors in lymphoid and myeloid cells (22, 27). CR3 (CD11b/CD18; Mac-1), in addition to its role in cell adhesive interactions, is a primary phagocytic receptor for C3bi-coated particles (27). CR4 (CD11c/CD18; p150,95), although less well understood, also binds to C3bi (12, 27). CR3 appears to be a common mononuclear phagocyte receptor for diverse pathogens, including mycobacteria, *Leishmania* spp., *Legionella pneumophila*, *Histoplasma capsulatum*, and *Bordetella pertussis* (reviewed in reference 17). Phagocytosis via CR3 is usually not accompanied by a toxic respiratory burst (51), unlike, for example, after cross-linking of LFA-1 or CR4 (5), and this may account in part for its preferential use by invading pathogens (21, 37).

It remains to be established whether CR3 is a preferred receptor for *M. tuberculosis*. A difficulty has been to define the role of CR3 in the nonopsonic binding of *M. tuberculosis* to mononuclear phagocytes (43, 48). Complement-independent, nonopsonic binding of *M. tuberculosis* provides a mechanism for the invasion of alveolar macrophages in the complement-poor environment of the lung and is therefore likely to be critical. A component of nonopsonic binding may be mediated by the mannose receptor (43), although this is limited to *M. tuberculosis* strains with mannose-capped lipoarabinomannan moieties (46), and may be restricted to resting macrophages, since activated macrophages downregulate expression of this receptor (19, 30). However, expression of the mannose receptor may be upregulated in macrophages stimulated with surfactant protein A, which results in enhanced phagocytosis of *M. tuberculosis* (18). Moreover, complement-independent binding of *M. tuberculosis* to alveolar macrophages may also be mediated in part by direct opsonization with surfactant protein A (13). Nonopsonic binding to CR3 has been claimed for a number of different pathogens, including *M. tuberculosis*, on the basis of in vitro studies with macrophages in the absence of exogenous complement (reviewed in reference 31), but these data may be spurious because the macrophage synthesizes complement component C3 itself (6, 16, 50). Indeed, a rigorous reanalysis of the binding of *Leishmania* spp. to CR3, previously thought to occur directly in the absence of complement, revealed that complement-independent binding of *Leishmania* spp. to CR3 is very unlikely (31). There are, however, at least two examples of pathogen ligands that bind directly to CR3 in the absence of complement: filamentous hemagglutinin from *B. pertussis* (37) and neutrophil inhibitory factor from the hookworm *Ancylostoma caninum* (32, 39). There is therefore no reason, a priori, why such a ligand should not exist in *M. tuberculosis.*

In an effort to examine, specifically, the interaction between *M. tuberculosis* and CR3 and in particular its dependence on opsonins, we chose a simple model system for our studies, namely a CHO fibroblast cell line heterologously expressing human CD11b/CD18. The use of this cell line avoided the two main difficulties that arise from the use of mononuclear phagocytes: the presence of numerous, competing phagocytic receptors, to which the bacteria may bind both opsonically and nonopsonically, and the de novo synthesis of opsonins, includ-

TABLE 2. Binding of mycobacteria to CR3-expressing CHO cells in the presence and absence of serum[a]

Bacterium and cell line[b]	Fresh serum		No serum	
	% Cells bound to bacteria	No. of bacteria/cell	% Cells bound to bacteria	No. of bacteria/cell
M. tuberculosis[c]				
CHO-WT	14 ± 3	0.18 ± 0.05	14 ± 4	0.18 ± 0.07
CHO-Mac-1LE	16 ± 4		14 ± 3	
CHO-CR3T	76 ± 5	1.25 ± 0.21	76 ± 4	1.59 ± 0.35
CHO-Mac-1	80 ± 8	1.95 ± 0.77	79 ± 4	1.87 ± 0.50
M. tuberculosis[d]				
CHO-WT	15 ± 2	0.20 ± 0.05	19 ± 5	0.25 ± 0.09
CHO-CR3T	82 ± 2	1.19 ± 0.11	81 ± 5	1.56 ± 0.18
CHO-Mac-1	57 ± 6	1.04 ± 0.15	69 ± 7	1.60 ± 0.32
M. smegmatis[e]				
CHO-WT	21 ± 6	0.76 ± 0.04	15 ± 3	0.29 ± 0.01
CHO-Mac-1	83 ± 6	4.34 ± 0.45	17 ± 2	1.04 ± 0.18

[a] CHO cells were incubated with mycobacteria (300:1), washed, stained, and counted under fluorescence microscopy. Data are expressed as the percentage of cells that bound one or more bacteria and as the mean number of bacteria per cell. Values are means ± standard deviations derived from 10 (*M. tuberculosis*) and 2 (*M. smegmatis*) separate experiments, each determination performed in triplicate.

[b] For details on the cell lines used, see the text.

[c] Incubations were performed in the presence or absence of fresh FBS (10%).

[d] Incubations were performed in the presence or absence of fresh human serum (10%). Incubation in heat-inactivated human serum (10%) gave similar results (data not shown).

[e] These bacteria were preopsonized (1 h) in fresh complete human serum (or in PBS as a control) and then incubated with cells (400:1) in serum-free medium.

TABLE 3. Binding of *M. tuberculosis* to transfected CHO cells at different MOIs[a]

MOI and cell line	Fresh serum		No serum	
	% Cells bound to bacteria	No. of bacteria/cell	% Cells bound to bacteria	No. of bacteria/cell
300:1				
CHO-WT	11 ± 3	0.34 ± 0.04	11 ± 2	0.34 ± 0.04
CHO-Mac-1	54 ± 6	1.61 ± 0.04	57 ± 4	1.98 ± 0.23
CHO-ACE	10 ± 3	0.28 ± 0.16	9 ± 1	0.26 ± 0.04
30:1				
CHO-WT	6 ± 1	0.18 ± 0.01	7 ± 4	0.12 ± 0.01
CHO-Mac-1	47 ± 5	1.22 ± 0.20	52 ± 5	1.27 ± 0.11
CHO-ACE	8 ± 2	0.24 ± 0.04	9 ± 3	0.28 ± 0.08

[a] CHO cells were incubated with *M. tuberculosis* at MOIs of 300:1 and 30:1 (bacterium/cell ratio) overnight in the presence or absence of 10% fresh human serum. Bacterial binding was quantitated and expressed as described in Table 2, footnote *a*. Values are means ± standard deviations from one experiment performed in triplicate. CHO-ACE cells are a transfected line expressing an irrelevant recombinant surface protein (see text).

ing complement components. This approach has already proven very useful in clarifying the role of complement in the binding to CR3 by *Rhodococcus equi* and *Leishmania* spp. (21, 31). Moreover, transient and stable expression of integrins, including the β_2 integrins, in CHO cells is a frequently cited experimental tool, and thus this model system is well understood (for examples, see references 12, 23, 35, and 49). Finally, and pertinent to this study, there are a number of reports that clearly establish that CD11b/CD18 expressed in fibroblasts, including CHO cells, is constitutively active (12, 26, 28, 49), as we also demonstrate here by the binding of C3bi-coated beads to CR3-expressing CHO cells (Fig. 1B).

The results obtained with our model system establish that under these experimental conditions, (i) CR3 is a receptor for *M. tuberculosis* and (ii) binding of *M. tuberculosis* to CR3 can be complement and serum independent (i.e., nonopsonic). Our experiments were performed on 16 separate occasions by two different individuals, with comparable results in every case. To rule out the possibility of artifact, we performed a variety of control experiments. First, we showed that the CR3-transfected CHO cells do not have a larger surface area than that of the wild-type cells (Table 1), and therefore, the higher percentage of transfected cells bound to *M. tuberculosis* is not simply a reflection of greater average cell size. Second, we included transfected CHO cells expressing high levels of an irrelevant surface protein (CHO-ACE cells) and found that binding of *M. tuberculosis* to these cells did not differ from binding to wild-type cells (Table 3). This reduces the likelihood that high-level expression of a recombinant surface protein per se leads to a generalized increase in stickiness of CHO cells for *M. tuberculosis*. Third, we used opsonized and unopsonized *M. smegmatis* bacteria as control particles to establish the specificity of the binding of unopsonized *M. tuberculosis* to CHO-CR3 cells. We found that CHO-Mac-1 cells bound unopsonized *M. smegmatis* poorly and to the same extent as CHO-WT cells, whereas binding was enhanced fourfold to opsonized *M. smegmatis* (Table 2). These controls suggest that the binding of unopsonized *M. tuberculosis* to transfected, CR3-expressing CHO cells is due to a direct interaction with the recombinant CR3 and is not an artifact resulting from a generalized, nonspecific enhancement of the adhesiveness of the transfected cells for *M. tuberculosis.*

Further support for the specificity of the association of *M. tuberculosis* with the CR3-expressing cells derives from the inhibition of binding by anti-CR3 MAbs. Anti-CD11b and anti-CD18 antibodies combined inhibited binding by up to 83%, and individual anti-CD11b or anti-CD18 antibodies inhibited binding by up to 77% (Tables 4 and 5). This inhibition was not the result of MAb-induced cytotoxicity or changes in cell morphology. The inhibition was apparently also not the result of capping and internalization of CR3 or cocapping of an associated, unknown endogenous CHO cell surface protein that may bind *M. tuberculosis*, since binding to CHO-CR3 cells was inhibited by anti-CR3 MAbs at 4°C as well as at 37°C (Table 5). Of considerable interest is our finding that of three anti-CD11b MAbs tested, LM2/1 and OKM1 were strongly inhibitory but 2LPM19c was not. MAb 2LPM19c recognizes an I-domain epitope that includes the C3bi-binding site, whereas LM2/1 also binds to the I domain but does not inhibit C3bi binding (12). OKM1 binds to the C-terminal domain of CD11b and inhibits C3bi binding weakly (12, 40, 49). These results are in agreement with those reported by Stokes et al. (48), who found that the nonopsonic binding of *M. tuberculosis* to murine macrophages was inhibited strongly by anti-CR3 MAb 5C6 (which

TABLE 4. Binding of *M. tuberculosis* to CR3-expressing CHO cells is inhibited by anti-CR3 MAbs[a]

MAb[b]	Target antigen	CHO-CR3T cells		CHO-Mac-1 cells		% Inhibition[c] of:	
		% Cells bound to bacteria	No. of bacteria/cell	% Cells bound to bacteria	No. of bacteria/cell	% Cells bound to bacteria	No. of bacteria/cell
None		78 ± 7	1.33 ± 0.37	67 ± 7	1.15 ± 0.19		
Mouse IgG		67 ± 9	1.04 ± 0.02				
2LPM	I dom., C3bi[d]	79 ± 5	1.15 ± 0.11	61 ± 4	0.94 ± 0.08	4	18
LM2	I dom., non-C3bi[e]	31 ± 6*	0.57 ± 0.08*	27 ± 7*	0.44 ± 0.09*	77	64
MHM23	CD18	55 ± 2*	0.82 ± 0.10*	52 ± 14*	0.78 ± 0.21*	34	42
2LPM, LM2, MHM, and TS1	CD11b/CD18	25 ± 9*	0.32 ± 0.13*	26 ± 13*	0.31 ± 0.14*	83	88

[a] CHO cells were preincubated (30 min) with the indicated MAbs before incubation with *M. tuberculosis* (300:1) overnight, all at 37°C. The cells were processed and bacterial binding was quantitated as described in Table 2, footnote *a*. Data are expressed as means ± standard deviations and are drawn from seven separate experiments, each determination performed in duplicate or triplicate. An asterisk indicates that the value is significantly different ($P < 0.01$) from the value of the control (no antibody).

[b] Abbreviations: 2LPM, 2LPM19c; LM2, LM2/1.6.11; MHM, MHM23; TS1, TS1/18.1.2.11.4.

[c] Calculated for combined CHO-CR3T and CHO-Mac-1 data and based on decrease from control (no antibody) after subtraction of background binding (i.e., binding to CHO-WT cells). Binding to CHO-WT cells (data not shown) did not vary with any of the treatments and gave a combined average of 16% ± 3% cells bound to one or more bacteria and a mean of 0.19 ± 0.03 bacteria per cell.

[d] CD11b I domain including C3bi-binding epitope.

[e] CD11b I domain excluding C3bi-binding epitope.

TABLE 5. Inhibition of binding of mycobacteria to CHO-Mac-1 cells by anti-CR3 MAbs at 4 and 37°C

Temp[a] and MAb	*M. tuberculosis*		*M. smegmatis*	
	% Cells bound to bacteria[b]	% Inhibition of binding[c]	% Cells bound to bacteria[b]	% Inhibition of binding[c]
4°C				
None	55 ± 1		92 ± 3	
2LPM19c	40 ± 1*	27	5 ± 1*	95
LM2/1	13 ± 1*	76	71 ± 3*	23
37°C				
None	74 ± 14		90 ± 3	
2LPM19c	64 ± 1*	14	26 ± 4*	71
LM2/1	24 ± 2*	68	91 ± 1	−1
OKM1	19 ± 5*	74	88 ± 1	2
MHM23	26 ± 4*	65	88 ± 2	2

[a] CHO-Mac-1 cells were preincubated in all cases with the indicated MAb at 4°C for 3 h, before incubation with mycobacteria (300:1) overnight at 4 or at 37°C.

[b] Bacterial binding was quantitated as described in Table 2, footnote *a*, and data are means ± standard deviations of the percentage of cells that bound one or more bacteria (from two separate experiments, each coverslip in duplicate or triplicate). An asterisk indicates that the value is significantly different ($P < 0.01$) from that of the control (no antibody).

[c] Calculated as the percent inhibition of binding with respect to that of the control (no antibody); no correction was made for background binding (i.e., binding to CHO-WT cells).

binds an epitope distinct from the C3bi site) but not by M1.70 (which recognizes the C3bi-binding site). In contrast to our results with *M. tuberculosis*, binding of opsonized *M. smegmatis*, which has C3 breakdown products on its surface (Fig. 2), is blocked by MAb 2LPM19c by 71 to 95% (Table 5), as would be expected for a C3bi-coated particle.

On the basis of these data, we propose that *M. tuberculosis* binds, at least in part, to CR3 nonopsonically, to a site distinct from the C3bi-binding site. Because of the strong inhibition of binding to CHO-Mac-1 cells by the C-terminal CD11b MAb OKM1, it is tempting to speculate that *M. tuberculosis* binds to, or close to, the CR3 lectin site (40, 49). Although originally controversial, there is increasing and strong evidence that CR3 expresses a lectin site, which likely constitutes the monocyte/macrophage β-glucan receptor (26, 40, 41, 49). This has been demonstrated most convincingly in heterologous systems, in which CR3 expressed in fibroblasts (including CHO cells) avidly bound unopsonized yeast (26) and zymosan particles (yeast cell walls) (49). A soluble zymosan polysaccharide extract (SZP) that bound strongly to CR3 was found to consist of 95% mannose, indicating that the CR3 lectin site has a broad sugar specificity, including mannose-containing polysaccharides, *N*-acetyl-D-glucosamine, and β-glucan (49). A detailed analysis of SZP binding to a range of CR3/CR4 chimeras expressed in CHO cells and inhibition by a panel of 21 anti-CR3 MAbs indicated that the CR3 lectin site is situated in the C-terminal domain of CD11b, distinct from the C3bi binding site located in the I domain (49). SZP or zymosan binding was not inhibited by MAb Leu-15, which like 2LPM19c selectively inhibits the C3bi binding site of CR3, but was strongly inhibited by the C-terminal MAb OKM1 (40, 49). These results may have direct relevance to the observation that OKM1 was shown to inhibit the binding of opsonized *M. tuberculosis* to MDMs to a greater extent than that shown by Leu-15 (44 to 50% versus 34 to 39%, respectively) (44), which strongly suggests that a significant component of the binding of *M. tuberculosis* to CR3 is opsonin independent, irrespective of the presence or absence of C3 on the bacterial surface. This interpretation parallels the conclusions reached for the opsonin independence of the binding of zymosan particles to neutrophil CR3 (40) or group B streptococci to CR3 on neutrophils and monocytes (1), where in both cases binding was not inhibited by a large excess of Fab anti-C3.

Despite these conclusions, our result that serum opsonization was completely ineffective in enhancing the binding of *M. tuberculosis* to CHO-CR3 cells was unexpected. A careful analysis of the literature reveals that it has been clearly established that serum, and specifically C3, opsonization enhances the binding of *M. tuberculosis* to mononuclear phagocytes and that serum-enhanced binding is mediated by complement receptors, including CR3 (20, 43, 44). However, as noted before, in some studies it was also shown that *M. tuberculosis* can bind nonopsonically to monocyte/macrophage complement receptors, predominantly CR3 (43, 48), although no attempt was made to control for macrophage-derived C3. It is evident from these studies that the extent of nonopsonic binding to CR3 specifically can be difficult to dissect in experiments with mononuclear phagocytes, because C3 deposition leads to enhanced binding to multiple complement receptors and addition of anti-CR3 antibodies blocks both opsonic and nonopsonic binding to CR3. Nevertheless, antibodies that are specific for the CD11b C3bi-binding site, such as Leu-15, moderately block the binding of opsonized *M. tuberculosis* to MDMs (by 34 to 39%) (44), and therefore it is reasonable to conclude that a component of the binding of *M. tuberculosis* to MDM CR3 is opsonic. We can offer two explanations for the apparent discrepancy between this conclusion and our result that binding of *M. tuberculosis* to CHO cell-expressed CR3 is completely serum independent.

First, the opsonin dependence of binding to CR3 may be strain dependent. Stokes et al. (48) observed considerable variations among different *M. tuberculosis* complex strains and other mycobacterial species in the extent of nonopsonic binding to murine macrophages. Similarly, Antal et al. (1) noted that the C3-independent binding of another gram-positive bacterium, group B streptococcus, to CR3 is likely strain dependent. The reported studies on *M. tuberculosis* binding to monocyte/macrophage complement receptors were performed predominantly with the Erdman strain (43, 44) or the H37Ra strain (20) grown in rich medium (7H9 broth). Our studies were performed throughout with the H37Rv strain grown in a minimal medium (Kirchner's broth). We have shown here that unlike *M. tuberculosis* H37Rv, the fast-growing mycobacterium *M. smegmatis* is completely dependent on serum opsonization for binding to CHO-CR3 cells. In preliminary experiments, we have observed differences in the extent of opsonin independence of binding to CHO-CR3 cells among various *M. tuberculosis* laboratory strains and clinical isolates (9a), and this phenomenon clearly merits a thorough investigation. Taken together, we propose that in our studies, *M. tuberculosis* H37Rv expresses an endogenous CR3 ligand, which binds CR3 with similar or greater affinity than C3bi, since binding is not augmented by opsonization in serum, and hence binding to CR3-expressing CHO cells is predominantly nonopsonic.

Second, it is possible that the heterologous expression of CD11b/CD18 in CHO cells confers cell-specific activation signals on CR3 that enable the receptor to bind *M. tuberculosis* nonopsonically to an extent not typical in its native environment, the mononuclear phagocyte. This is a theoretical consideration that arises from the observation that the integrin heterodimers are complex molecules whose functional activity is determined by cell-specific signals that dictate both their affinity state and adhesion-modulating postreceptor events (33, 35). The quantitative significance of this possibility can only be

formally evaluated once the putative *M. tuberculosis* CR3-binding ligand has been isolated in a homogeneous form and its ability to bind macrophage-expressed CR3 has been determined. It should be noted, though, that the CHO-Mac-1 cells used here have been studied extensively with respect to the binding of four distinct CR3 ligands, and no anomalies were detected (12). Moreover, these and related CHO cell transfectants were used to study the nonopsonic binding of zymosan to CR3, and again, no significant differences were observed in comparison with leukocyte-expressed CR3 (49), making this possibility unlikely. Nevertheless, in a study with murine macrophages, the nonopsonic binding of *M. tuberculosis* was strongly dependent on macrophage phenotype, even though in all cases binding was mediated predominantly by CR3 and levels of CR3 expression were comparable in each of the macrophage populations studied (48). These results led the authors to conclude that the CR3 epitope that binds *M. tuberculosis* nonopsonically exists in a phenotype-dependent active or inactive state (48). It is therefore possible, similarly, that in transfected CHO cells, CR3 is expressed with the nonopsonic, *M. tuberculosis*-binding epitope in a constitutively active state. A detailed survey of human macrophages of various phenotypes has not been undertaken in regard to phenotype dependence of nonopsonic binding to *M. tuberculosis.*

An additional consideration relevant to the question of the functional equivalence of recombinant CR3 expressed in CHO cells compared with macrophage CR3 arises from the recent observation that CR3 associates with glycosylphosphatidylinositol-linked proteins, such as CD14 and CD16b in neutrophils and urokinase plasminogen activator receptor in monocytes (reviewed in reference 36). This association involves the CR3 β-glucan lectin site (26, 36), and it is therefore possible that if the nonopsonic binding of *M. tuberculosis* to CR3 involves this or a related site, then a subset of CR3 on monocyte/macrophages is unavailable for nonopsonic binding, unlike in transfected CHO cells in which CR3 likely does not form such multiprotein complexes. Interestingly, the CR3-CD14 complex in neutrophils dissociates upon cell spreading and adherence (36), which, it can be speculated, may provide an additional explanation for the phenotype dependence of the nonopsonic binding of *M. tuberculosis* to murine macrophages (48).

Unidentified at present is the presumed endogenous *M. tuberculosis* ligand that binds directly to CR3. Although we have raised the possibility that the binding of *M. tuberculosis* to CR3 may involve the CR3 lectin site, this is at present speculation. Schlesinger et al. (44) have shown that the attachment and ingestion of the Erdman strain of *M. tuberculosis* by human monocytes were not inhibited by laminarin, a soluble β-glucan. It is possible that if other strains of *M. tuberculosis* are examined, or if a wider range of soluble sugar antagonists of the CR3 lectin site (49) are tested, an inhibitory effect would be observed. The sugar specificity of the CR3 lectin site is broader than originally suspected and includes mannose-containing polysaccharides (49), which are prominent in the mycobacterial cell wall, and thus this possibility warrants further investigation. It has been shown recently that CR3 also binds to heparin and heparan sulfate glycans. The lectin site mediating this binding is situated in the I domain and therefore appears to be distinct from the β-glucan site (11). It is doubtful that *M. tuberculosis* binds to the heparin site, since this site is inhibited strongly by MAb 2LPM19 and weakly by LM2/1 and OKM1 (11), which is the reverse of what we have found for inhibition of the binding of *M. tuberculosis* to CHO-CR3 cells. Nevertheless, the *M. tuberculosis* cell wall is rich in diverse saccharides, including glycolipids, peptidoglycolipids, glycoproteins, and acyl trehalose 2′-sulfates (sulfolipids) (8), and it is conceivable that one or more of these can function as a ligand for one or the other CR3 lectin site. Notwithstanding these considerations, we cannot exclude the possibility that *M. tuberculosis* expresses a protein that functions as a ligand for CR3.

In conclusion, we have shown that *M. tuberculosis* can bind to CR3 nonopsonically, to a site other than the C3bi-binding site, and that the binding of this pathogen to CR3 heterologously expressed in CHO cells is not augmented by opsonization in serum. It remains to be established what proportion of the binding of *M. tuberculosis* to CR3 expressed by mononuclear phagocytes is nonopsonic in vivo. We speculate that this may be dependent on the strain of *M. tuberculosis* and on the monocyte/macrophage phenotype. Also unknown at present is the potential importance of nonopsonic binding versus opsonic binding to CR3. In addition to providing an alternative mode of binding to this receptor in a complement-poor environment, the nonopsonic interaction may give an advantage to the pathogen. For instance, binding of C3bi-coated particles to CR3 on unactivated neutrophils fails to stimulate phagocytosis, whereas binding of unopsonized zymosan does (40, 41). We believe that the CR3-expressing CHO cells provide a useful model system to examine these and related questions, and these cells provide a tool for attempts at isolating the putative CR3-binding ligand expressed by *M. tuberculosis*. If identified, such a ligand could be a useful target for the design of novel preventive and therapeutic strategies in tuberculosis.

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