Diminished Adherence and/or Ingestion of Virulent *Mycobacterium tuberculosis* by Monocyte-Derived Macrophages from Patients with Tuberculosis

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The interaction between the macrophage and *Mycobacterium tuberculosis* is mediated by a variety of macrophage membrane-associated proteins. Complement receptors have been implicated in the adherence of *M. tuberculosis* to macrophages. In the present work, the adherence and/or ingestion of *M. tuberculosis* H37Rv to human monocyte-derived macrophages (MDM) from patients with tuberculosis (TB) and healthy controls was measured by microscopical examination, [3H]uracil incorporation, and CFU. The adherence and/or ingestion was enhanced by fresh serum and inhibited by heat inactivation, EDTA treatment, and anti-CR1 and anti-CR3 antibodies. Comparison of MDM from TB patients and healthy controls showed that the former exhibited a significantly decreased capacity to adhere and/or ingest *M. tuberculosis*, as determined by the number of CFU and [3H]incorporation. The expression of CR1 (CD35) and CR3 (CD11b/CD18) on MDM from TB patients and healthy controls, as determined by flow cytometry, did not show significant differences. These results suggest that the lower ingestion of *M. tuberculosis* by MDM from TB patients is not due to defects in complement receptors, and therefore, there might be other molecules involved in the adherence and/or ingestion process that render MDM from TB patients ingest less mycobacteria than those from healthy controls.

**MATERIALS AND METHODS**

**Subjects studied.** Patients with clinically and bacteriologically diagnosed TB were recruited from Hospital La María, Medellín, Colombia. All patients were under antituberculous treatment for less than 1 month. One patient had meningitis and another had renal TB, both without clinical pulmonary compromise. No patients were receiving an immunosuppressive drug. Blood hemoglobin ranged from 11.8 to 14.6 g/dl. Volunteer healthy donors were also studied as controls for the different experiments. All subjects studied were human immunodeficiency virus negative. Participants were informed of the objectives of the study and voluntarily agreed to participate in it.

**Mycobacteria.** *M. tuberculosis* H37Rv was grown in Proskauer-Boek liquid medium (31), collected, and maintained at −70°C in RPMI 1640 (Gibco BRL, Grand Island, N.Y.) containing 30% glycine and 10% fetal bovine serum (Gibco BRL). The number of bacteria was determined by plating serial dilutions onto petri dishes containing Middlebrook 7H10 solid medium (5) (Becton Dickinson Microbiology Systems, Cockeysville, Md.). For all the experiments described herein, a single batch of mycobacteria was used.

Before each experiment, a vial of *M. tuberculosis* was thawed and incubated in phosphate-buffered saline (0.15 M, pH 7.2) (PBS) containing 50% (vol/vol) fresh pooled human serum (PHS), obtained from seven tuberculin-skin-test-negative healthy subjects, for 20 min at 37°C to opsonize mycobacteria. To disrupt the bacterial clumps, the suspension was passed through a 26-gauge tuberculin syringe at least 20 times without bubble formation as previously described (25). The number of CFU after thawing was 70 to 75% of the original counts.

**Isolation of MNC.** Fifty-milliliter samples of venous blood were poured into Erlenmeyer flasks containing 15 to 20, 2-mm-diameter glass beads. Defibrillation was done by gentle shaking for 10 min until a clot formed. Defibrinated blood was collected and centrifuged at 900 × g for 10 min at room temperature. The buffy coat was recovered and diluted 1:3 with PBS and centrifuged (3:1 [vol/vol]) on Histopaque (Sigma Chemical Co., St. Louis, Mo.). The fraction containing the mononuclear cells (MNC) was recovered and washed twice with PBS. The viability was determined by trypan blue exclusion and was always ≥95%. The percentage of monocytes was determined by Wright’s and nonspecific α-naphthyl-acetate esterase (Sigma) stains of smears obtained by cytocentrifugation of the MNC at 40 × g for 5 min. Monocytes were adjusted to 10⁶/ml in RPMI 1640 (Gibco) (pH 7.2) containing 25 mM HEPES and 1-glutamine without serum and

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antibiotics and cultured under conditions described below for specific experiments.

**Quantification of associated AFB per cell.** The effect of in vitro maturation of monocytes and the optimal dose of infectious inocula was determined by light microscopy and/or electron microscopy. Two hundred microliters of the MNC suspension (approximately \(2 \times 10^5\) monocytes) was dropped into each 15-mm-diameter, round coverslip (Nunc, Inc., Naperville, Ill.) placed in 24-flat-bottom-well plates (Nunc). After 30 to 60 min at 37°C and 5% CO\(_2\), the volume of each well was adjusted to 1 ml with RPMI 1640. After 24 h of culture, PHS inactivated by being heated at 56°C for 30 min (HI-PHS) was added to a final concentration of 5% and the plates were incubated for 1 to 6 days to allow differentiation of monocytes into macrophages (MDM). The day of the experiment, the nonadherent cells were washed by immersing the coverslips seven or eight times in PBS prewarmed at 37°C. Coverslips were placed again in 24-well plates containing 500 \(\mu\)l of RPMI 1640 supplemented with 5% HI-PHS and 100 U of penicillin (Sigma) per ml in each well. Only the confluent monolayers were selected and infected according to the initial number of monocytes. In our hands and by using the method described by Nakagawa and Nathan (14), a maximum of 10% of cells are detached after 1 week in vitro culture. MDM were infected with opsonized or nonopsonized *M. tuberculosis* for 2 h at 37°C at different mycobacterium/MDM ratios (1:1, 5:1, and 10:1). Thereafter, the nonadherent bacteria were washed by immersing the coverslips into PBS at 37°C. Then, the coverslips were immersed for 10 min in 10% formaldehyde, and cells were stained with Kinyoun stain (11). The number of MDM with one or more associated acid-fast bacilli (AFB) and the bacterial load per cell were determined by counting 200 cells at a magnification of \(1000\) with a light microscope. To assess the bacterial load per cell, we used a previously published scoring method (6). We did not differentiate between adhered or ingested *M. tuberculosis* in this study.

**Measurement of \(^{3}H\)Juracil incorporation by MDM-associated *M. tuberculosis* inclusions.** Fifty thousand monocytes were plated in each well of a 96-flat-bottom-well plate (Nunc) and cultured for 7 days in 200 \(\mu\)l of RPMI 1640. Twenty-four hours after plating, HI-PHS was added to a final concentration of 5%. The day of the experiment, the nonadherent cells were removed by washing the wells three times with 37°C-prewarmed PBS. Then, 200 \(\mu\)l of RPMI 1640 and 5% HI-PHS were added. MDM were infected for 2 h at 37°C with preopsonized *M. tuberculosis* H37Rv at a mycobacterium/MDM ratio of 5:1. Nonadhered bacteria were washed with prewarmed PBS. Thereafter, MDM with associated mycobacteria were lysed with 200 \(\mu\)l of RPMI 1640 containing 0.2% saponin (Sigma) and supplement 8X (1.6% L-asparagine, 1.6% sodium glutamate, 0.04% ferric ammonium citrate) to allow mycobacterial extracellular growth (10). After lysis, 0.5 \(\mu\)Ci of \(^{3}H\)Juracil (specific activity, 50 Ci/mmol; Amersham, Little Chalfont, Buckinghamshire, United Kingdom) was added to each well and the plates were incubated for 7 more days at 37°C. Mycobacteria were harvested on glass-fiber filters with a cell harvester (Inotech Biosystems International, Lansung, Mich.), and the \(^{3}H\)Juracil incorporated by *M. tuberculosis* was counted in a \(\beta\)-scintillation counter (model 121; LKB-Wallac, Turku, Finland).

**Determination of macrophage-associated *M. tuberculosis* by CFU.** Three 5-\(\mu\)l droplets were obtained from the lysate before the addition of [\(^{3}H\)Juracil and plated onto petri dishes containing Middlebrook 7H10 agar medium (Becton Dickinson). The petri dishes were incubated for 7 days at 37°C, and the microcolonies were counted microscopically by using a calibrated ocular lens.

**Inhibition of the adherence and/or ingestion of *M. tuberculosis* by MDM.** To determine the type of serum opsonins, *M. tuberculosis* was incubated for 20 min at 37°C with HI-PHS or with serum containing 20 mM EDTA (Sigma) before addition to MDM. In parallel, 7-day-cultured MDM from healthy individuals were incubated for 30 min with monoclonal antibodies against either CR1 (clone E11) (33) or CR3 (clone ICRF444) (20) (both from Pharmingen, San Diego, Calif.) or both or an irrelevant mouse anti-human immunoglobulin G1 (IgG1) (Pharmingen) as the isotype control. MDM were infected for 2 h at a mycobacterium/MDM ratio of 5:1. Thereafter, MDM were lysed and CFU were determined as described above. To establish the role played by the mannose receptor in mediating the adherence of opsonized bacteria to human macrophages, MDM were preincubated for 60 min with different doses (10\(^{-7}\) to 10\(^{-1}\) M) of \(\alpha\)-methyl mannoside (\(\alpha\)-MM) (Sigma) and then infected at a mycobacterium/MDM ratio of 5:1 as described above. The percentage of MDM with associated mycobacteria and the bacterial load per cell were determined by light microscopy.

**Quantification of CR1 and CR3 by flow cytometry.** MDM were obtained as described above. After 7 days of culture, 10\(^6\) MDM were incubated with 20 \(\mu\)g of a monoclonal mouse anti-human CR1 (clone E11) IgG1 (Pharmingen) per ml for 30 min at 4°C. The cells were washed and incubated at 4°C for 30 min with a fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse IgG1 (Becton Dickinson Immunocytometry Systems, San Jose, Calif.). For CR3, the cells were incubated with 10 \(\mu\)l of a phycoerythrin-conjugated monoclonal mouse IgG1 anti-human CR3 (Leu15) (Becton Dickinson) for 20 min. The cells were washed, and the positive fluorescence was determined by flow cytometry (FACSort; Becton Dickinson) by comparison with the respective isotype control antibody (Becton Dickinson). Results are shown as percentage of positive cells, mean of median net fluorescence intensity, and total fluorescence from the product of the two former variables.

**Statistical analyses.** Comparisons between healthy controls and TB patients were done by the unpaired Student t test. One- and two-way analysis of variance (ANOVA) were used to compare the results obtained with different treatments. Correlation analysis was used to compare the results of the different techniques. All statistical analyses were performed with Prism 2 software (GraphPad Software, San Diego, Calif.).

**RESULTS**

To determine the effect of mycobacterial opsonization and time of culture of human MDM on the adherence of mycobacteria, MDM were cultured for different periods of time and incubated for 2 h with opsonized or nonopsonized mycobacteria at all times of MDM culture studied. Seven-day-cultured MDM obtained from six healthy donors were infected for 2 h with *M. tuberculosis* H37Rv opsonized (solid line) or nonopsonized (broken line) with fresh PHS at different mycobacterium/MDM ratios. Nonadhered bacteria were washed, and cells were stained with Kinyoun stain. The results show the means ± standard errors of the means (SEMs) of the percentage of MDM with one or more associated AFB of six different experiments \((P = 0.0002)\) by two-way ANOVA. (A) Effect of the dose of inoculum on the adherence and/or ingestion of *M. tuberculosis* H37Rv by human MDM. MDM cultured for 4 to 7 days were infected for 2 h with *M. tuberculosis* H37Rv preopsonized (solid line) or not (broken line) with fresh PHS at a mycobacterium/MDM ratio of 1:1. Nonadhered bacteria were washed, and coverslips were stained with Kinyoun stain. The results show the means ± standard errors of the means (SEMs) of the percentage of MDM with one or more associated AFB of six different experiments \((P = 0.0001)\) by two-way ANOVA.)
The adherence and/or ingestion of mycobacteria by MDM was dose dependent (Fig. 1B). The percentage of MDM with associated M. tuberculosis under non-opsonizing conditions was lower than under opsonized conditions and was independent of the dose used (P < 0.0001). In the case of opsonized mycobacteria, there was a dose-dependent increase in the percentage of MDM-adhering and/or ingesting bacteria, reaching 71.4% ± 6.2% at a mycobacterium/MDM ratio of 10:1.

In the experiments designed to define the characteristics of the opsonins involved in the adherence and/or ingestion of mycobacteria by MDM (Table 1), it was found that in the absence of serum, there was a reduction of 42 to 75% in the number of CFU/milliliter compared to bacteria opsonized with fresh serum, confirming the microscopic observation described above. Heat inactivation of the serum resulted in a decrease of 35 to 66% in the number of CFU/milliliter recovered from the lysates of MDM from that of mycobacteria opsonized with fresh serum. Similar reductions were observed with EDTA treatment. Incubation with monoclonal anti-CR1 caused a reduction of 45 to 58% in the number of CFU/milliliter. Monoclonal antibody against CR3 caused reductions of 49 and 41% in the number of CFU recovered in two of the subjects studied. When anti-CR1 and anti-CR3 were used together, the inhibitory effect increased to 61% in subject 1 and to 68% in subject 2. The use of an isotype antibody control did not affect the number of CFU/milliliter recovered.

Since the adherence of mycobacteria to MDM could also be mediated by other membrane molecules, including mannose receptors (20), we used different concentrations of α-MM trying to block these receptors and therefore the adherence of mycobacteria. At the doses used, α-MM had no significant effect on the adherence of opsonized M. tuberculosis to MDM, as detected either by the percentage of cells with adhered mycobacteria or by the score of the bacterial load per cell (Fig. 2).

Analysis of the adherence and/or ingestion of M. tuberculosis to MDM from both TB patients and healthy controls by counting the CFU (Fig. 3A) showed that MDM from TB patients had significantly fewer associated M. tuberculosis than MDM from healthy controls, with values of (1.1 ± 0.1) × 10^3 and 2.0 ± 0.1/ml × 10^5, respectively (P < 0.00001). The difference between controls and TB patients was also demonstrated by [3H]uracil incorporation (Fig. 3B). Cultures from controls exhibited a mean of 7,526 ± 1,030 cpm, while the incorporation in cultures from TB patients was 1,590 ± 272 cpm (P < 0.0001). The determination of the adherence and/or ingestion of M. tuberculosis by counting the CFU and counts per minute showed a significant correlation (r = 0.81, P = 0.0004).

Since the differences observed between healthy controls and TB patients could be due to differences in the number of CR1 or CR3 expressed on the membranes of MDM, we determined the expression of these molecules by flow cytometry. As shown in Table 2, we did not find significant differences in the percentage of CR1- or CR3-positive cells when we compared MDM from TB patients and healthy controls. It is also shown in Table 2 that the mean of median fluorescence intensity for CR1 in the group of TB patients was 333.7 ± 26, while in healthy controls, it was 366.5 ± 46, showing no significant difference. In the case of CR3 expression, the mean of median fluorescence intensity was 632.9 ± 40 and 695 ± 22.15 for TB patients and healthy controls, respectively, with no significant differences between the values for the two groups. Moreover, when we compared the total expression of these molecules on the membranes of MDM from TB patients and healthy controls, we did not find any significant differences (Table 2).

Thus, there was no significant differences in the two groups in the expression of CR1 and CR3 present on the membranes of MDM.

**DISCUSSION**

The initial contact between intracellular microorganisms and phagocytes can be mediated by opsonic (4, 10) and non-opsonic interactions (16). The former are mediated by either immunoglobulins or C3b/C4b complement fractions that interact with Fc receptors and CR, respectively (4, 10). In our experiments, the role played by serum immunoglobulins was ruled out by opsonizing mycobacteria with pooled serum from healthy, tuberculin-skin-test-negative subjects with no clinical history of tuberculosis. Previous experiments in our laboratory showed that sera of skin-test-negative individuals are negative for antibodies against M. tuberculosis.
H37Rv organisms were pulsed with 0.5 pl and plating the lysate onto Middlebrook 7H10 agar. (B) MDM were lysed, and bacteria were washed, and MDM were lysed. (A) CFU were determined by liquid scintillation. Each dot represents the mean for three samples from each subject.

Seven days later, cultures were collected and the counts per minute were counted for 20 min with fresh PHS at a 5:1 mycobacterium/MDM ratio. Nonadhered controls and TB patients were infected for 2 h with M. tuberculosis H37Ra produced by an enzyme-linked immunosorbent assay for antimonycobacterial IgG and IgM antibodies (13a). The findings that opsonin activity was heat labile and EDTA sensitive and that monoclonal antibodies against CR1 and CR3 significantly reduced the number of CFU recovered from infected MDM, as previously found by microscopical observations (7, 20, 22, 32), are evidence that complement plays an important role in the adherence and/or ingestion of M. tuberculosis. However, total inhibition was not obtained with any of the treatments described, suggesting that other mechanisms of adherence are involved.

Mannose receptors are one of the major molecules that mediate nonopsonic interaction of mycobacteria through LAM (20, 24, 28, 32). Although it has been reported (3) that α-MM inhibited the adherence of nonopsonized M. avium to MDM, our finding that α-MM did not affect the adherence and/or ingestion of opsonized M. tuberculosis suggests that in our system, the main adherence was mediated by receptors other than the mannose receptor. Another interesting possibility is CD14, a glycosphatidylinositol-anchored membrane molecule that can serve as LAM receptor on macrophage membranes (17, 19). It is noteworthy that in body compartments with low levels of opsonins, LAM can interact with CR3 through the mannose residues at a different site than the iC3b-binding site (25). A recent report suggests that class A scavenger receptor can also play an important role in nonopsonic binding of M. tuberculosis to macrophages (32).

MDM from TB patients had a lower capacity to adhere and/or ingest M. tuberculosis compared to those from healthy controls. This difference was observed by CFU results as well as by [3H]uracil incorporation; it must be noted that a high correlation between the two techniques was detected, as previously published (2). There are several possible explanations of the differences between healthy controls and TB patients. Since all patients were receiving antituberculous treatment, it was possible that the drug persisted inside the endocytic vacuoles of MDM, affecting the phagocytosed mycobacteria (5) and reducing the number of live and culturable bacilli. However, it is unlikely that after 7 days in culture there were enough active antimycobacterial compounds within MDM; this scenario is even more unlikely when the interaction of mycobacteria with the MDM was only 2 h. A deactivated state of MDM from TB patients might also explain the differences observed with MDM from healthy controls. M. tuberculosis-infected macrophages or macrophages exposed to LAM produce interleukin 10 (1), which inhibits the production of macrophage-activating cytokines (13, 29). It has been previously reported (12) that monocytes infected with M. tuberculosis H37Ra produced transforming growth factor β1 (TGF-β1) and that treatment of monocytes with TGF-β1 decreased the uptake of M. tuberculosis. Moreover, the spontaneous release of TGF-β1 was found to be higher in monocyte supernatants from TB patients than in those of healthy controls (27). Thus, it is possible that in our experiments, MDM from TB patients had an increased production of interleukin 10 or TGF-β and consequently reduced monocyte activity. Since MDM were not exposed to lymphocyte-derived cytokines, the diminished capacity of MDM from TB patients to adhere and/or ingest M. tuberculosis may be due

TABLE 2. Expression of CR1 and CR3 on MDM from TB patients and healthy controls*  

| CR   | Subject               | % of positive cells | MFI# | Total expression |  
|------|-----------------------|---------------------|------|------------------|  
|      |                       |                     |      |                  |  
| CR1  | TB patients (n = 6)   | 70 ± 10.0d          | 333.7 ± 26d | 22,840 ± 2,849d |  
|      | Healthy controls (n = 6) | 59 ± 7.0q          | 366.5 ± 46 | 21,780 ± 4,213 |  
| CR3  | TB patients (n = 10)  | 47 ± 8.0d          | 632.9 ± 40d | 31,040 ± 6,075d |  
|      | Healthy controls (n = 16) | 53 ± 6.0d         | 695.3 ± 22 | 38,370 ± 5,100 |  

* Peripheral blood mononuclear cells were cultured for 7 days as described in Materials and Methods. Nonadherent cells were washed, and adherent cells (MDM) were incubated at 4°C for 30 min and recovered by washing with cold PBS. Once recovered, the MDM were counted and incubated with either a monoclonal mouse anti-human CR1 at 4°C for 30 min and then with an FITC-conjugated rat anti-mouse IgG1 or with monoclonal mouse phycoerythrin-conjugated anti-human CR3 IgG1. Cells were incubated for 20 min at room temperature, and the percentage of positive cells and the fluorescence intensity were determined by flow cytometry. Means ± standard errors of the means are shown.

# MFI, mean of median fluorescence intensity.

d Not statistically significant compared with the value for healthy controls.
to either an acquired defect secondary to the infectious process or a constitutional characteristic of MDM from TB patients.

One interesting possibility is that the differences observed in TB patients and healthy controls in the adherence of opsonized M. tuberculosis were due to variations in the number of CR1 or CR3 molecules expressed on the surfaces of their macrophages. The number of CR1 molecules expressed on the cell surface varies in different cell types (9), and it seems to be genetically regulated by a CR1-linked gene (30). Using flow cytometry, we were unable to demonstrate these differences, which suggests that the deficient adherence and/or ingestion of M. tuberculosis observed in MDM of TB patients is due to abnormalities in receptors other than CR1 or CR3. The fact that none of the patients had advanced anemia suggests that their general clinical status was not responsible for the defective macrophage phagocytic capacity. It remains to be determined whether these abnormalities can be reversed after the antituberculous treatment and whether patients with other diseases, in similar clinical conditions, exhibit similar phagocytic defects. It must be remembered that clinical TB is the final result of many host and mycobacterial factors interacting within particular epidemiological and environmental conditions. The identification of such variables will eventually lead to a better understanding of the disease and to develop more-effective methods to prevent and treat it.

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