***Mycobacterium tuberculosis*-infected human macrophages exhibit enhanced cellular adhesion with increased expression of LFA-1 and ICAM-1 and reduced expression and/or function of complement receptors, FcγRII and the mannose receptor**

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The entry of *Mycobacterium tuberculosis* (*Mtb*) into the host macrophage and its survival in this environment are key components of tuberculosis pathogenesis. Following intracellular replication of the bacterium within alveolar macrophages, there is spread of bacilli to regional lymph nodes in the lungs and subsequent presentation of antigens to the host immune system. How this process occurs remains poorly understood, but one mechanism may involve the migration of macrophages containing *Mtb* across the alveoli to lymph nodes, where there is development of a protective host response with formation of granulomas composed in part of aggregated and fused, apoptotic, infected macrophages. Leukocyte integrins, including lymphocyte function-associated antigen-1 (LFA-1) and complement receptors CR3 and CR4, and their counter receptors play a major role in macrophage adhesion processes and phagocytosis. In this study, the appearance of *Mtb*-infected macrophages over time was examined, using inverted-phase microscopy and an *in vitro* culture model of human monocyte-derived macrophages (MDMs). Prior to and immediately following infection of the MDMs with *Mtb*, the macrophages appeared as individual cells in monolayer culture; however, within 24 h of infection with *Mtb*, the MDMs began to migrate and adhere to each other. The kinetics of this response were dependent on both the m.o.i. and the length of infection. Quantitative transmission electron microscopy studies revealed that macrophage adhesion was accompanied by increases in levels of LFA-1 and its counter receptor (ICAM-1), decreases in surface levels of the phagocytic receptors CR3, CR4 and FcγRII, and an increase in major histocompatibility complex Class II (MHC-II) molecules at 72 h post-infection. Decreases in surface levels of CR3 and CR4 had a functional correlate, with macrophages containing live bacilli showing a diminished phagocytic capacity for complement-opsonized sheep erythrocytes; macrophages containing heat-killed bacilli did not show this diminished capacity. The modulation of macrophage adhesion and phagocytic proteins may influence the trafficking of *Mtb*-infected macrophages within the host, with increases in levels of LFA-1 and ICAM-1 enhancing the adhesive properties of the macrophage and decreases in phagocytic receptors diminishing the phagocytic capacity of an already-infected cell, potentially allowing for maintenance of the intracellular niche of *Mtb*.

Keywords: major histocompatibility complex Class II (MHC-II), tubercle bacillus, phagocytosis, granuloma, integrin

INTRODUCTION

The importance of tuberculosis (TB) as a major cause of worldwide morbidity and mortality is well known (Raviglione *et al.*, 1995; Snider & La Montagne, 1994). It is estimated that over one-third of the World's population harbours *Mycobacterium tuberculosis* (*Mtb*), the bacterium that causes TB. Given the growing number of immunocompromised individuals associated with the AIDS pandemic, the increasing prevalence of drug-resistant *Mtb* and the problems associated with current antimicrobial therapies, a better understanding of the pathogenesis of tuberculosis is needed if new treatments and vaccines are to be developed against this disease.

A key step in TB pathogenesis is the ability of the tubercle bacillus to enter and replicate within the macrophages of its human host (Schlesinger, 1996; Fenton, 1998). During primary infection with the organism, aerosol-droplet nuclei containing small numbers of *Mtb* are deposited in the alveoli of the lung(s) and are subsequently phagocytosed by alveolar macrophages (AMs). *Mtb* enters the macrophages via receptor-mediated phagocytosis, a process involving complement receptors (CR1, CR3 and CR4), the mannose receptor and scavenger receptors (Schlesinger, 1993; Zimmerli *et al.*, 1996; Hirsch *et al.*, 1994; Stokes *et al.*, 1993). After intracellular multiplication of *Mtb*, the bacilli spread to regional lymph nodes within the host. In the majority of cases, the host develops a protective response to *Mtb* infection that involves containment of the tubercle bacilli by the formation of granulomas that contain aggregated and fused (giant cells), apoptotic, infected macrophages (Saunders & Cooper, 2000). Characterization of the bacterial and phagocyte molecular determinants that allow for bacterial dissemination during primary infection with *Mtb* and which mediate cell-cell interactions is just beginning to occur (Pethe *et al.*, 2001).

Integrins are $\alpha\beta$ heterodimeric proteins that are found on most cell types; however, they are particularly abundant on leukocytes (Harris *et al.*, 2000; Plow *et al.*, 2000). Most integrins are constitutively inactive in resting cells, but are rapidly and reversibly activated in response to a variety of agonists, leading to highly regulated homotypic and heterotypic cell adhesion. Integrins are divided into families based upon the nature of the β subunit, with the $\beta 2$ (leukocyte) integrin family found predominantly on leukocytes. Members of the $\beta 2$ integrin family share a common β subunit (CD18) that associates with a unique α subunit to form the mature heterodimer.

Complement receptor CR3, the major integrin of phagocytic cells (mononuclear phagocytes and polymorphonucleocytes), is also expressed on natural-killer

cells and a subset of lymphocytes. Its major functions include phagocytosis, homotypic aggregation and stable adhesion of leukocytes to endothelial cells, followed by migration into inflamed organs. These functions are mediated through the binding of CR3 to several physiological ligands, including C3bi, ICAM-1 (CD54) and fibrinogen (Ueda *et al.*, 1994; Rieu *et al.*, 1994). Another complement receptor, CR4, shares the functions and ligands of CR3 (Blackford *et al.*, 1996; Ingalls & Golenbock, 1995; Loike *et al.*, 1991) and has been shown to be a particularly important phagocytic receptor for *Mtb* on AMs (Hirsch *et al.*, 1994).

The $\beta 2$ integrin lymphocyte function-associated antigen-1 (LFA-1), although not a phagocytic receptor, plays a major role in cellular adhesion (Stewart *et al.*, 1995). It is expressed on all leukocytes and mediates a broad range of functions, such as T-cell-mediated killing, T-helper-cell and B-cell responses, natural-killer-cell activity, monocyte-mediated antibody-dependent cytotoxicity and leukocyte adhesion to endothelial cells (Stewart *et al.*, 1995). LFA-1 mediates cell interactions through binding to one of its three counter receptors, ICAM-1, ICAM-2 or ICAM-3, which are members of the immunoglobulin superfamily.

Adhesion molecules, such as integrins, play a major role in communication with other cell types involved in the host cellular immune response. Modulation of expression and/or function of $\beta 2$ integrins on *Mtb*-infected cells may serve to regulate cell migration, homotypic cellular adhesion (i.e. granuloma formation) and antigen presentation by influencing cognate interactions with T-cells (Faull *et al.*, 1994; Ochs *et al.*, 1993; Dubey & Croft, 1996). Several other pathogens, including mycobacteria species, have evolved strategies to regulate host-cell-surface molecules (Keller *et al.*, 1995; Tsuyuguchi *et al.*, 1990; Kaye *et al.*, 1986; Mshana *et al.*, 1988; Mohagheghpour *et al.*, 1997). Studies of the effects of *Mtb* on host-cell-surface molecules are limited, but those that have been done include evidence for alterations in major histocompatibility complex Class II (MHC-II) molecules (Gercken *et al.*, 1994; Wade *et al.*, 1995), ICAM-1 (Saha *et al.*, 1994; López Ramírez *et al.*, 1994) and co-stimulatory molecules (Saha *et al.*, 1994).

Using an *in vitro* culture model of human monocyte-derived macrophages (MDMs), we have observed a marked phenotypic change in these cells after their infection with live *Mtb*. Uninfected cultured macrophages appear as a monolayer of largely single cells; however, within 24 h of phagocytosis of *Mtb* by the MDMs (at a ratio of approximately 1 bacillus per macrophage), the macrophages begin to migrate and adhere to each other. As the *Mtb* replicate, the MDMs continue to aggregate; eventually the MDMs detach from the culture plate and are ultimately killed by the

Abbreviations: AM, alveolar macrophage; EC3bi, C3bi-coated erythrocytes; HK, heat-killed; HRP, horseradish peroxidase; ICAM-1, counter receptor of LFA-1; LFA-1, lymphocyte function-associated antigen-1; MDM, monocyte-derived macrophage; MHC-II, major histocompatibility complex Class II; *Mtb*, *Mycobacterium tuberculosis*; TEM, transmission electron microscopy.

replicating bacteria. To begin to determine the mechanism behind these phenotypic changes in the MDMs, we investigated whether there is modulation of several major macrophage surface proteins during intracellular growth of *Mtb*.

METHODS

Isolation and cultivation of MDMs. Human macrophages were obtained from peripheral blood mononuclear cells (PBMCs) as described previously (Schlesinger, 1993). Briefly, heparinized blood was obtained by venapuncture from purified-protein-derivative-negative donors (approved by the institutional IRB). Mononuclear cells were separated on a Ficoll (Amersham Biosciences) cushion and the PBMCs were cultured for 5 days in Teflon wells (Savillex) containing RH (RPMI medium, 20 mM HEPES, 20 mM L-glutamine) plus 20% autologous serum. The resulting MDMs were adhered to glass or plastic (see electron-microscopy section; Waco Pure Chemical Industries) coverslips, or they were adhered directly to wells of a 24-well (Becton Dickinson Labware) or a 6-well (Corning) tissue-culture plate for 2 h. Non-adherent cells were removed from the plates by washing, and the MDMs were cultured for an additional 7 days in RH plus 20% autologous serum, prior to the addition of *Mtb* (Olakanmi *et al.*, 2000). Each experimental condition was performed with ≥ 2 donors.

Bacterial culture and infection of MDMs. *M. tuberculosis* Erdman (ATCC 35801), H37Rv (ATCC 27294^T) and H37Ra (ATCC 25177) were grown for 9–11 days on Middlebrook 7H11 agar. Immediately prior to the infection of the MDMs with *Mtb*, bacteria were scraped from the plates into a 2.0 ml polypropylene tube containing two 3 mm glass beads and 1 ml RPMI. The samples were pulse-vortexed six times (~ 1 s per pulse) and the resulting suspensions were allowed to settle for 30 min as described previously (Schlesinger *et al.*, 1990). This bacterial suspension contained $\sim 1 \times 10^8$ (bacteria ml)⁻¹, with zero to a few bacterial clumps observed by microscopy. The number of bacteria present in the suspension was confirmed by counting in a Petroff–Hauser chamber. Bacteria prepared in this fashion are $\geq 90\%$ viable by c.f.u. assay (Ferguson *et al.*, 2002). Heat-killed (HK) bacteria were prepared as described above, except that the *Mtb* were heated for 1 h at 90 °C.

MDMs were incubated with *Mtb* for 2 h in the presence of 2.5% autologous serum; unattached bacilli were removed from the MDMs by washing. Infected macrophage monolayers and sham-infected (no *Mtb*) control MDMs were cultured for an additional 24–72 h in RH plus 1% autologous serum. The m.o.i. (*Mtb* to MDMs) was $<1:1$ to $10:1$, depending upon the experiment performed. Duplicate or triplicate wells were examined in each test group. Monolayers were examined daily using an inverted-phase microscope (model IMT; Olympus) at 100–400 $\times$ magnification, and representative photomicrographs were taken. Viable MDMs were assessed by trypan-blue exclusion. In this model, at an m.o.i. of $1:1$, the percentage of macrophages that contain intracellular *Mtb* is $32 \pm 3\%$ (mean $\pm$ SEM, $n=9$ experiments) at 2 h, $35 \pm 5\%$ ($n=9$) at 24 h and $42 \pm 6\%$ ($n=5$) at 72 h post-infection, as assessed by fluorescence microscopy (Schlesinger *et al.*, 1990).

Transmission electron microscopy (TEM) quantification of surface expression of macrophage proteins in *Mtb*-infected cells. At 6 min (synchronized phagocytosis; Schlesinger *et al.*, 1990), 24 h or 72 h post-infection with *Mtb*, the macrophage monolayers were fixed with 2% paraformaldehyde, 10 mM

sodium periodate, 75 mM L-lysine HCl and 37 mM phosphate buffer at 4 °C for 4 h. They were then washed three times with PBS (Dulbecco's phosphate-buffered saline without CaCl₂ and MgCl₂; Gibco BRL) plus 0.075% Tween 20. MDMs were blocked with 50% normal goat serum (NGS) in 0.15 M Tris-buffered saline (TBS) with 0.075% Tween 20 (TBST) at room temperature for 1 h with gentle shaking. Primary mAbs to the $\beta 2$ integrins (CD11a, CD11b and CD11c), ICAM-1, Fc γ RII and HLA-DR (Beckman Coulter) were diluted 1:40 and incubated with the MDMs overnight at 4 °C in a solution of TBST with 50% NGS. An IgG-subtypic control was used to detect non-specific staining for each antibody.

After overnight incubation of the MDMs with the primary antibodies, the MDMs were washed and incubated in TBST plus 10% human serum and horseradish peroxidase (HRP)-conjugated goat-anti-mouse IgG secondary antibody for 3 h at room temperature with gentle shaking. The MDMs were then washed six times, and the HRP was developed with 0.05% 3,3'-diaminobenzidine (DAB) tetrahydrochloride and 0.003% H₂O₂ in TBS at room temperature for 30 min. Following development of the HRP, the MDMs were post-fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 15–30 min, rinsed and then immersed in 1% OsO₄ in 0.1 M phosphate buffer for 10 min. Thin-sections of the MDMs were then prepared for TEM.

The intensity of the cross-sectional surface staining of the MDMs was ranked on an ordinal scale from 0 to 3, counting a minimum of 40 macrophage cross-sections per test group examined. Level 0, designated A, indicates background staining; Level 1, designated B, indicates linear staining along the MDM plasma membrane separated by areas greater than the area of staining (i.e. $<50\%$ of the cell's surface stained); Level 2, designated C, indicates linear staining along the MDM plasma membrane separated by areas less than the area of staining (i.e. between 50 and $<100\%$ of the cell's surface stained); Level 3, designated D, indicates linear staining along the entire MDM plasma membrane without breaks (i.e. 100% of the cell's surface stained).

Preparation of C3bi-coated erythrocytes (EC3bi) and zymosan particles, and their uptake by MDMs. Sheep erythrocytes (SRBCs) (BioWhittaker) were washed and suspended in 10 mM HEPES/Hank's balanced salt solution buffer (pH 7.3) (HH buffer). SRBCs (6×10^8) in 10 ml HH buffer were incubated with 100 μ l haemolysin at room temperature for 60 min. The SRBCs were then washed three times, suspended in 1.0 ml HH buffer and 100 μ l C5-deficient human serum and incubated for 60 min at 37 °C, to opsonize the SRBCs with C3bi (EC3bi). EC3bi were washed three times before use.

Zymosan A (Zy; Sigma) was suspended in PBS at a concentration of 1 mg ml⁻¹. The suspension was boiled for 30 min and washed twice at room temperature by centrifuging the suspension at 15 800 g for 5 min between washes, to pellet the Zy.

EC3bi (m.o.i. of 33–100 per MDM) or Zy (m.o.i. of 13:1) were incubated with MDM monolayers on glass coverslips for 30 min at 37 °C. To quantify the total number of cell-associated EC3bi per MDM, monolayers of MDMs were washed in PBS and fixed in 2.5% glutaraldehyde for 10 min; the red cells were then enumerated by phase-contrast microscopy. To distinguish internalized EC3bi from attached EC3bi, coverslips were briefly exposed to H₂O prior to fixation of the MDMs to hypotonically lyse the attached EC3bi (Schlesinger & Horwitz, 1991). The number of attached EC3bi were calculated by subtracting the number of internalized EC3bi from the total number of cell-associated EC3bi.

Fig. 1. Macrophage monolayers displayed marked cellular aggregation following infection with *Mtb*. MDM monolayers were incubated with the virulent Erdman strain of *Mtb* for 2 h, washed and then maintained in monolayer culture for 72 h. Monolayers were examined daily by using inverted-phase microscopy. Representative photomicrographs of one experiment ($n > 20$) are shown. (A, B) By 24 h post-infection, there was evidence of macrophage migration with bunching of small groups of cells. Bars, 100 μ m. (C, D) By 72 h post-infection, the degree of migration, cellular aggregation and adhesion had increased. Bars, 60 μ m.

The uptake of Zy by the MDMs was enumerated by phase-contrast microscopy.

Data reported here represent particles associated with 50–300 consecutive MDMs on duplicate or triplicate coverslips per test group. Statistical significance of the data was calculated using the paired *t*-test. The level of phagocytosis by the MDMs varied between individual donors; therefore, certain results are reported as the percentage of particles associated in each treatment group (i.e. live or HK *Mtb*) compared to the number of particles associated with that donor's sham-infected MDMs (normalized to an internal control for each experiment).

RESULTS

Macrophage monolayers displayed marked aggregation and adhesion following infection with *Mtb*

Macrophages in monolayer cultures were infected with the virulent Erdman strain of *Mtb* (m.o.i. 1:1) and examined by inverted-phase microscopy from 2 to 72 h post-infection. Monolayers incubated without *Mtb* served as (uninfected) controls. Our results showed that infection of the macrophages with *Mtb* alters the appearance of the monolayer. MDMs aggregated in individual, small clusters that increased in cell number and size with time. At 24 h post-infection, the differences between the control and infected monolayers were minor; however, there was evidence of macrophage migration with bunching of groups of cells (Fig. 1A, B).

By 72 h post-infection, the degree of migration, cellular aggregation and adhesion increased both in terms of the number of MDMs per cluster and the total degree of aggregation (Fig. 1C, D). Cellular aggregation and adhesion of the MDMs continued to increase over time until the entire monolayer was affected.

Macrophage aggregates ultimately detached from the surface of the tissue-culture well; the majority of the macrophages within these aggregates no longer excluded trypan blue and were presumably dead. However, the MDMs that remained attached to the surface of the culture plate excluded trypan blue and were, thus, viable. The level of aggregation was proportional to the m.o.i. The length of time required to observe marked aggregation was shorter when increased numbers of bacilli were used in the initial infection (m.o.i. 10:1), or extended when decreased numbers of bacilli were used in the initial infection (m.o.i. 0.1:1) (data not shown).

Macrophage surface expression of LFA-1 and ICAM-1 was increased following infection of monolayers with *Mtb* for 72 h

Because of the marked increase in cellular aggregation seen to accompany infection of the macrophages with *Mtb* for 72 h, we examined the level of macrophage surface expression of the $\beta 2$ integrin LFA-1 and its counter receptor ICAM-1, two proteins known to play a major role in cell–cell adhesion. Quantitative TEM was

Fig. 2. Staining of macrophage surface proteins on uninfected and *Mtb*-infected monolayers by development of the HRP. The stained macrophages were examined by using TEM; the amount of macrophage surface staining for the indicated molecules was calculated using the ordinal scale shown. (A) Level A, background staining; (B) Level B, linear staining along the MDM plasma membrane separated by areas greater than the area of staining (i.e. <50% of the cell's surface area stained); (C) Level C, linear staining along the MDM plasma membrane separated by areas less than the area of staining (i.e. between 50 and <100% of the cell's surface area stained); (D) Level D, linear staining along the entire MDM plasma membrane without breaks (i.e. 100% of the cell's surface area stained); (E) subtypic control staining (no staining). Bars, 1 μ m.

used to measure the surface expression of these two proteins on MDM cross-sections of uninfected and *Mtb*-infected cells (Erdman and H37Ra strains). An ordinal scale was developed based on the degree of surface staining of the macrophage proteins as revealed by developing the HRP substrate (Fig. 2A–E). The amount of staining on the surface of ≥ 40 MDM cross-sections per experimental group was calculated using the ordinal scale designated as A–D, where 'A' indicated no antibody staining and 'D' indicated 100% surface staining (see Methods).

Our results showed that macrophage surface staining for LFA-1 and ICAM-1 was increased in *Mtb*-infected monolayers relative to uninfected monolayers at 72 h (Fig. 3). This increase in LFA-1 and ICAM-1 levels provides a possible mechanism for the observed increase in homotypic cellular adhesion observed in the *Mtb*-infected monolayers. There was no difference between the observed levels of surface staining on macrophage cross-sections that contained a bacterium and those that did not (data not shown). Results were similar for *Mtb* Erdman and *Mtb* H37Ra.

Macrophage surface expression of CR3, CR4 and Fc γ RII was decreased following infection of the monolayers with *Mtb* for 72 h, whereas the surface expression of MHC-II was increased

In contrast to LFA-1, the $\beta 2$ integrins CR3 and CR4 play a major role in the phagocytosis of *Mtb* by macrophages. To determine whether *Mtb* infection of macrophages leads to altered surface expression of these phagocytic receptors, we quantified the expression of these proteins by using TEM. In contrast to the results obtained for LFA-1 and ICAM-1 expression, macrophage monolayers infected with *Mtb* for 72 h exhibited a marked reduction in their expression of CR3 and CR4 compared to the uninfected control monolayers (Fig. 3). The decrease in expression was greater for CR4 than for CR3.

To determine the specificity of the complement-receptor response upon infection of the MDMs with *Mtb*, we also analysed the surface expression of Fc γ RII. Similar to the results obtained for CR3 and CR4 expression, the expression of Fc γ RII was decreased on monolayers infected with *Mtb* for 72 h (Fig. 3). Thus, in contrast to the macrophage surface proteins LFA-1 and ICAM-1 which play major roles in cell–cell adhesion and whose expression is increased following infection of the monolayer with *Mtb*, the expression of surface proteins that mediate phagocytosis is decreased.

Finally, we assessed whether the expression of the MHC-II molecule HLA-DR, which is involved in antigen presentation, is altered following infection of the macrophages with *Mtb*. Similar to the results obtained for LFA-1 and ICAM-1 expression, the surface expression of MHC-II was increased (Fig. 3). Taken together, our data indicate that macrophages infected with *Mtb* display marked alterations in a variety of surface proteins that are known to play an important role in modulating the host immune response during infection with this bacterium.

Kinetics of changes in macrophage protein surface expression following infection of monolayers with *Mtb*

The studies described above focused on the changes in macrophage protein expression 72 h post-infection with *Mtb*, and were based on the marked degree of cellular aggregation observed in light microscopy studies (Fig. 1). However, changes in the macrophage monolayer began to occur as early as 24 h post-infection with *Mtb*. Also, it was possible that the regulation of specific macrophage surface proteins could begin during the process of *Mtb* phagocytosis. To examine the kinetics of the regulation of macrophage surface proteins following *Mtb* infection, we performed quantitative TEM analyses on a subset of proteins at 6 min post-infection (synchronized phagocytosis) and at 24 h post-infection. As shown in Fig. 3, only small changes in LFA-1 expression were seen at 6 min post-infection; however, there was a marked increase in the expression of this protein by 24 h

Fig. 3. MDM monolayers containing live *Mtb* [Erdman (upper graphs) and H37Ra (lower graphs) strains] demonstrated altered expression of macrophage proteins which varied with time of infection. MDM monolayers containing *Mtb* (incubated with bacteria for 6 min, 24 h or 72 h) or uninfected control monolayers were fixed and prepared for HRP immunoelectron microscopy (see Methods). Surface proteins were stained using a primary mAb against the indicated protein, followed by a secondary HRP-conjugated antibody and HRP development. The vertical axis of each graph shows the percentage of cells within an ordinal rank (A–D; see Fig. 2). The amount of staining on the surface of ≥ 40 MDM cross-sections per experimental group was calculated. Data shown are the mean of two to eight experiments for 6 min, two experiments for 24 h (except the LFA-1 and Fc γ RII groups, $n=1$) and two experiments for 72 h; SEM ranged from 1 to 15%.

post-infection. Fc γ RII expression was decreased at 24 h post-infection. The most striking changes in macrophage protein expression were seen for CR3 and MHC-II. As early as 6 min post-infection, there was a marked reduction in CR3 expression by the macrophages when infected with either Erdman or H37Ra; this reduction in expression persisted at 24 h post-infection. MHC-II expression showed a biphasic response: its expression was decreased at 6 min post-infection, returned towards the level of uninfected cells at 24 h post-infection and then showed increased expression at 72 h post-infection.

Macrophage monolayers infected with live *Mtb* were reduced in their phagocytic abilities via complement receptors and the mannose receptor

TEM results revealed that macrophage surface expression of the phagocytic receptors CR3 and CR4 decreased following infection of the monolayer with *Mtb*. To determine if the decreased expression of these receptors had a functional correlate (i.e. decreased phagocytosis), we examined the effect of *Mtb* infection on macrophage complement-receptor activity, by measuring the association of EC3bi with *Mtb*-infected monolayers at 72 h post-infection. EC3bi, which bind to CR3 and CR4, were incubated with monolayers for 30 min at 37 °C and the mean number of EC3bi associated with individual

macrophages was enumerated by phase-contrast microscopy. For these experiments, we elected to compare results from monolayers infected with the virulent strains of *Mtb* (Erdman and H37Rv), and the importance of bacterial viability by comparing the effects of live and HK *Mtb* on the monolayers. The results showed that macrophage monolayers infected with live *Mtb* Erdman or *Mtb* H37Rv, but not HK *Mtb*, demonstrated a reduced total cell association of EC3bi via CR3 and CR4 ($P=0.003$) (Fig. 4a and Table 1). Concomitant with the observed reduction in the mean number of EC3bi particles per infected macrophage, there was a significant reduction ($P=0.006$) in the percentage of EC3bi-associated macrophages compared to the uninfected control or HK *Mtb* groups (Fig. 4b). In a similar fashion, infection of the macrophages with live H37Ra, but not HK H37Ra, decreased EC3bi association with the macrophages (live bacteria $60 \pm 13\%$, $n=4$, and HK bacteria $123 \pm 4\%$, $n=3$; results are expressed as a percentage of the control value).

In parallel wells, monolayers of MDMs were subjected to hypotonic lysis to remove extracellular EC3bi from them. The number of attached EC3bi was calculated by subtracting the number of ingested EC3bi from the total number of EC3bi per cell. Our results showed that the decrease in the total number of cell-associated EC3bi

Fig. 4. Effect of live and HK *Mtb* infection of monolayers on macrophage complement receptor activity as assessed by the total number of EC3bi associated with the monolayers. MDM monolayers were incubated with the indicated strain of *Mtb* for 2 h, washed and then maintained in monolayer culture for 72 h. After washing of the monolayers, EC3bi were incubated with monolayers for 30 min at 37 °C. To quantify the total number of cell-associated EC3bi per MDM, monolayers were washed in PBS, fixed in glutaraldehyde and the red cells were enumerated by phase-contrast microscopy. (a) Data are presented as a percentage of the control (uninfected cells) for the number of EC3bi per cell; mean results are shown $\pm$ SEM. (b) Data are presented as the percentage of macrophages with associated EC3bi. The total number of EC3bi associated with uninfected macrophages ranged from 0.59 to 2.23 EC3bi per cell. $n=5$ For Erdman; $n=4$ for H37Rv; $n=3$ for HK Erdman; $n=2$ for HK H37Rv. Asterisk, $P \leq 0.05$, relative to the uninfected group.

reflects reduced numbers of both attached and ingested red cells in live but not in HK *Mtb*-infected monolayers of macrophages, with no consistent difference in results between infection of the macrophages with the Erdman or the H37Rv strain (Table 1).

Another macrophage receptor for *Mtb* phagocytosis is the mannose receptor. To determine whether the functional activity of this receptor was altered in *Mtb*-infected monolayers, we assessed the association of unopsonized zymosan, a particle that is phagocytosed primarily by the mannose receptor on human macrophages (Speert & Silverstein, 1985), with the macrophages. Similar to the results obtained for EC3bi association, there was a reduction in the mean number of zymosan particles per macrophage in *Mtb*-infected monolayers compared to uninfected (control) monolayers ($51 \pm 8\%$ and $55 \pm 17\%$ of control, respectively, for live Erdman-infected and live H37Rv-infected cells, $n=2$). Thus, together with the TEM data, our results

Table 1. Effect of live and HK *Mtb* infection of monolayers on macrophage complement-receptor activity, as assessed by the attachment and ingestion of EC3bi

See Methods for details of MDM infection and enumeration of the EC3bi. The number of attached EC3bi was calculated by subtracting the number of ingested EC3bi from the total number of EC3bi per cell. The results shown are given with the mean $\pm$ SD of triplicate coverslips in each test group for a representative experiment ($n=2$ to 5; see also Fig. 4).

Monolayer infected with	No. of EC3bi		
	Total	Ingested	Attached
Nothing	1.45 ± 0.58	0.72 ± 0.10	0.73
Erdman	0.45 ± 0.27	0.15 ± 0.07	0.30
HK Erdman	1.47 ± 0.52	0.88 ± 0.25	0.59
H37Rv	0.74 ± 0.61	0.45 ± 0.57	0.29
HK H37Rv	1.97 ± 0.81	1.35 ± 0.56	0.62

indicate that macrophage monolayers infected with live *Mtb* have reductions in expression of the major classes of receptors for phagocytosis on these cells.

DISCUSSION

Tissue macrophages, including those containing intracellular mycobacteria, are long-lived cells that are in constant communication with matrices and other cell types involved in the host cellular immune response. Alterations in the cell-surface expression of adhesion molecules, antigen-presenting molecules and co-stimulatory molecules on *Mtb*-infected macrophages will likely play an important role in the innate immune response to infection with *Mtb* and in shaping the subsequent specific cellular immune response of the host to infection with this bacterium, including the formation of granulomas. Here, we report that MDMs infected with live *Mtb* at a low m.o.i. are markedly altered in their biology, showing enhanced homotypic cellular adhesion and increased surface expression of LFA-1 and ICAM-1. These infected MDMs also have a diminished phagocytic ability and display decreased surface expression and/or activity of the major classes of phagocytic receptors on these cells, i.e. complement receptors, Fc γ Rs and the mannose receptor. The viability of the infecting bacteria was found to be important for the alteration in the phagocytic potential of the macrophages, since infection of the macrophages with HK bacteria abolished the diminished effect.

During primary infection of the host with *Mtb*, inhaled *Mtb* encounters AMs. AMs are highly effective at clearing foreign particulate matter from the lungs, including microbes deposited on the epithelial surface of the lungs, and at the same time serve to downregulate the activation of the immune system present in lung tissue and in draining lymph nodes (Thepen *et al.*, 1994;

Herscowitz, 1985; Schauble *et al.*, 1993). The alveoli also contain type I and type II alveolar epithelial cells which express ICAM-1 (the counter receptor for $\beta 2$ integrins) and, thus, may mediate adhesion and migration of professional phagocytes within the lungs (Simon & Paine, 1995).

After the intracellular multiplication of *Mtb* within AMs, there is spread of the bacilli to regional lymph nodes. The mechanism by which the bacilli traverse the alveoli remains poorly understood. One mechanism may involve the migration of AMs containing *Mtb* across the alveoli to the nodes of the lung. Evidence for this comes, in part, from studies in dogs, in which red and green fluorescently labelled microsphaeres instilled separately into the trachea of these animals were found together within single macrophages in the draining lymph nodes, and from a more-recent study that followed the movement of inflammatory macrophages in a murine model (Harmsen *et al.*, 1985; Bellingan *et al.*, 1996). Our data support the idea that increases in the expression of LFA-1, ICAM-1 and potentially other integrins by *Mtb*-infected macrophages may enhance the cellular migration of these macrophages in the lungs. Alternatively, it has also been proposed that mycobacteria may directly penetrate damaged alveolar epithelial tissue, may move intercellularly to gain access to lymph nodes and the circulatory system or may transit to the lymph nodes by dendritic cells (Lambrecht *et al.*, 2001; Demangel *et al.*, 1999; McDonough & Kress, 1995; Zhang *et al.*, 1997).

Human AMs and MDMs express CR3, CR4 and the mannose receptor, receptors known to mediate the phagocytosis of opsonic and non-opsonic *Mtb* and other mycobacteria in the naïve host (Schlesinger, 1996). Along with the production of specific antibodies by the host during infection with *Mtb*, Fc γ Rs may also play an important role in mycobacterial entry and the host response (Teitelbaum *et al.*, 1998). Our data provide evidence that there is downregulation of the expression of these phagocytic receptors during the intracellular growth of *Mtb* in macrophages. Whether these changes in receptor expression are to the benefit of the microbe or the host is not known. We speculate that decreases in the expression of these receptors act to reduce the phagocytic capacity of an already-infected cell, potentially allowing for maintenance of the intracellular niche of *Mtb* for a longer period of time. With further intracellular growth of the bacterium, *Mtb*-infected macrophages undergo apoptosis and, ultimately, cell lysis (Fratazzi *et al.*, 1999; Placido *et al.*, 1997; Keane *et al.*, 1997). Consistent with our results, treatment of human monocytes with *Mycobacterium avium* complex (MAC) or MAC-derived lipid resulted in significant decreases in the expression of CR3 (Tsuyuguchi *et al.*, 1990; Pourshafie *et al.*, 1993). Furthermore, in earlier studies, *Mtb*-infected mononuclear phagocytes and *Mycobacterium bovis* BCG-infected mouse peritoneal macrophages were found to have altered phagocytic receptor functions (Mariano *et al.*, 1977; Ezekowitz *et al.*, 1981).

A significant component of the granulomas found in hosts infected with *Mtb* is composed of aggregated and fused macrophages. The cellular adhesion that we observed with MDM monolayers infected with live *Mtb* could represent a stage in the formation of granulomas. The mechanism(s) underlying the changes in macrophage phenotype and integrin expression upon infection with *Mtb* is the focus of current studies. These studies include evaluating whether the changes in cellular adhesion are due to autocrine and/or paracrine effects – our data are suggestive of a paracrine effect. We observed global changes in the macrophage monolayer despite only a proportion of the cells containing *Mtb*, as assessed by fluorescence microscopy. Also, as assessed by TEM, there was no observed difference in the levels of surface-protein-staining on macrophage cross-sections that contained a bacterium when compared with those that did not. However, TEM is limited by it being a cross-sectional analysis of cells.

Cognate interactions between antigen-presenting cells and T-cells involve both T-cell-receptor–MHC–antigen binding and binding between several accessory molecules, such as LFA-1 and ICAM-1, as well as costimulatory molecules. The upregulation of the expression of LFA-1, ICAM-1 and HLA-DR observed in our study suggests enhanced T-cell interactions; however, other accessory molecules that have not yet been studied may be downregulated, so it is difficult to predict the ultimate result of this upregulation. Our results with regard to the regulation of MHC-II expression on *Mtb*-infected human macrophages deserve comment. We observed an early decrease in MHC-II expression followed by an increase in MHC-II expression at 72 h post-infection with *Mtb*. Several studies support the idea that mycobacteria-infected cells have a diminished capacity for MHC-II-restricted antigen presentation (Gercken *et al.*, 1994; Pancholi *et al.*, 1993; Hmama *et al.*, 1998). However, the degree to which this relates to MHC-II expression remains uncertain. Different studies have shown decreased expression of MHC-II, no change in its expression or an increase in its expression (Noss *et al.*, 2000; Pancholi *et al.*, 1993; Gercken *et al.*, 1994; Ezekowitz *et al.*, 1981; VanHeyningen *et al.*, 1997; Tsuyuguchi *et al.*, 1990).

The requirement for viable bacteria to infect macrophages to generate the MHC-II response varies in different studies (Hmama *et al.*, 1998; Gercken *et al.*, 1994; Noss *et al.*, 2000). *Mtb* cell-wall components alone can effect the expression of MHC-II (Wadee *et al.*, 1995; Noss *et al.*, 2001). In some cases, IFN- γ -induced MHC-II expression is decreased rather than the constitutive expression of MHC-II after infection with *Mtb* (Wojciechowski *et al.*, 1999; Hmama *et al.*, 1998) as shown in this study. Changes in the expression of MHC-II also relate to the initial m.o.i. used (Gercken *et al.*, 1994; Hmama *et al.*, 1998). Whereas other studies have required a high m.o.i. to observe an effect on MHC-II expression, our studies revealed a marked effect on the expression of MHC-II at a low m.o.i. (1:1). The type and differentiation state of the macrophage cells used, as

well as their time in culture, may be important factors for determination of the MHC-II response (Pancholi *et al.*, 1993; Hmama *et al.*, 1998; Hamerman & Aderem, 2001). Studies have been conducted with murine macrophages, the myeloid-cell-line THP-1 and human monocytes (Pancholi *et al.*, 1993; Gercken *et al.*, 1994; Noss *et al.*, 2000). Monocytes in short-term culture may be particularly sensitive to MHC-II regulation (Hmama *et al.*, 1998; Gercken *et al.*, 1994). Our studies were conducted with MDMs. The biphasic response we observed in this study most closely resembles that seen with *M. bovis* BCG and mouse peritoneal macrophages (Hamerman & Aderem, 2001; Ezekowitz *et al.*, 1981). Thus, the mechanism(s) underlying the regulation of MHC-II expression/function is complex. The kinetic analysis done in our study indicates that changes in protein expression in *Mtb*-infected human MDMs vary markedly with time in culture. Early changes in protein expression may relate, in part, to protein sorting during phagocytosis and later changes may depend more on intracellular growth. These possibilities are being explored.

Apart from MHC-II molecules, *Mtb*-containing macrophages from a susceptible mouse strain manifest reduced expression of the co-stimulatory molecule B7 (CD80) and increased expression of ICAM-1, resulting in T-cell unresponsiveness that appeared to be linked to prostaglandin synthesis (Saha *et al.*, 1994). Incubation with *Mtb* or lipoarabinomannan enhanced the expression of ICAM-1 by THP-1 cells, in part by secretion of TNF- α (López Ramírez *et al.*, 1994).

The alterations in $\beta 2$ integrin protein expression (and in expression of other surface molecules) that we have observed in the MDMs provide evidence that the phenotype and function of *Mtb*-infected human macrophages will be markedly altered compared to uninfected macrophages. Several published studies on mycobacterial-infected cells, including those using arrays, support this view (Stenger *et al.*, 1998; Ehrt *et al.*, 2001; Wang *et al.*, 2000; Ragno *et al.*, 1997). Given that the expression and/or function of CR3, CR4, FC γ Rs and the mannose receptor are decreased and the expression and/or function of LFA-1, ICAM-1 and HLA-DR are increased, we hypothesize that there is a shift from a cell potent in phagocytosis to a cell with enhanced adhesive capabilities that influence cell-cell interactions (e.g. attachment to and migration across epithelial tissue and cognate interactions with T-cells), in a fashion resembling the maturation of dendritic cells. In support of this hypothesis, a functional transitional state of macrophages was recently found in murine peritoneal macrophages infected with *M. bovis* BCG (Hamerman & Aderem, 2001).

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