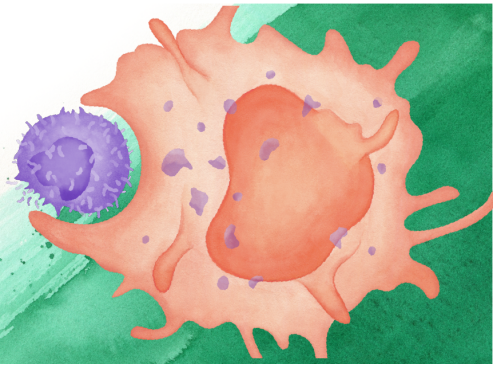




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***Mycobacterium tuberculosis* Resides in Nonacidified Vacuoles in Endocytically Competent Alveolar Macrophages from Patients with Tuberculosis and HIV Infection**¹ ✓

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J Immunol (2004) 172 (7): 4592–4598.

<https://doi.org/10.4049/jimmunol.172.7.4592>

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Mycobacterium tuberculosis Resides in Nonacidified Vacuoles in Endocytically Competent Alveolar Macrophages from Patients with Tuberculosis and HIV Infection¹

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Alveolar macrophages (AM) are the first professional phagocytes encountered by aerosols containing infections in the lungs, and their phagocytic capacity may be affected by these infections or environmental particles. The aim of this study was to evaluate the innate endocytic and phagocytic properties of human AM obtained from patients with pulmonary tuberculosis and to characterize the vacuoles in which *Mycobacterium tuberculosis* bacilli reside *in vivo*. AM were obtained by bronchoalveolar lavage from patients with suspected tuberculosis and from asymptomatic volunteers (controls). Clinical case definitions were based on mycobacterial culture of respiratory specimens and HIV serology. To assess phagocytosis, endocytosis, and acidification of the endosomal system, AM were cultured with IgG-coated polystyrene beads, dextran, and a pH-sensitive reporter (3-(2,4-dinitroanilino)-3-amino-*N*-methylpropylamine) and were evaluated by light and immunoelectron microscopy. Cells from 89 patients and 10 controls were studied. We found no significant difference between the two groups in the ability of AM either to ingest beads and dextran or to deliver them to acidified lysosomes. In AM from patients with tuberculosis, the bacilli were located in vacuoles that failed to accumulate endocytosed material and were not acidified. We concluded that AM from patients with tuberculosis and HIV infections were competent to endocytose and phagocytose material and to deliver the material to functional, acidified lysosomes. *M. tuberculosis* residing in these AM arrests the progression of their phagosomes, which fail to fuse with acidified lysosomes. This confirms, for the first time in humans with tuberculosis and HIV, the conclusions from previous animal and *in vitro* studies. *The Journal of Immunology*, 2004, 172: 4592–4598.

Active tuberculosis (TB)³ continues to cause significant morbidity and mortality worldwide, especially in developing countries (1). This situation is compounded by coinfection with HIV, particularly in sub-Saharan Africa (2, 3).

Much of our understanding of the pathogenesis of TB has come from studies examining the interaction between *Mycobacterium tuberculosis* (MTB), the causative agent of TB, and either murine mononuclear cells or human PBMC (4–7). These studies suggest several mechanisms by which HIV and TB can stimulate a pattern of host cytokine responses capable of exacerbating both infections (8–13). It is, however, unclear whether these mechanisms are accompanied by alterations in the properties and behavior of alveolar macrophages (AM) within the lungs of patients with pulmonary TB.

AM are highly differentiated mononuclear cells found in the walls of alveoli. They are the first professional phagocytes encoun-

tered by MTB during primary and some forms of secondary MTB infections (14). Like all phagocytes, they are capable of internalizing particulate material and delivering it to acidic, degradative lysosomes. The properties of these macrophages fall into two discrete areas: innate responses such as phagocytosis and release of proinflammatory cytokines, and immune responses triggered by the products of activated lymphocytes. Coinfection with MTB and HIV in an individual could affect either arm of macrophage physiology. AM operate at the interface between the host and the environment and can therefore also be influenced by external factors, including carbon from wood fires (15–19). In Malawi there is a very high prevalence of both tuberculosis and HIV infections, and the majority of the population live in simple housing in close proximity to wood fires. In this study our aim was to evaluate the innate physiological properties of AM obtained through bronchoalveolar lavage (BAL) of Malawian adults with and without HIV and/or pulmonary TB.

Previous work conducted on murine bone marrow-derived macrophages and on PBMC in tissue culture has indicated that vacuoles containing pathogenic *Mycobacterium* species fail to acidify and do not fuse with pre-existing lysosomal compartments (4–6). Given the key role played by phagocytosis and endosomal maturation in macrophage function and intracellular survival of MTB, we studied AM from volunteers and patients for their capacity to phagocytose inert particles, to endocytose solutes, and to acidify compartments containing internalized material, including MTB.

Materials and Methods

Study population

The study was conducted at Queen Elizabeth Central Hospital, Blantyre, a large tertiary and teaching hospital in southern Malawi. All patients were recruited from the TB registry of the hospital where they presented with

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Received for publication October 9, 2003. Accepted for publication January 16, 2004.

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¹ This work forms part of the Malawi-Liverpool-Wellcome Trust Program of Research in Clinical Tropical Medicine. The Wellcome Trust of Great Britain supports H.M., M.N., M.M., S.W., and S.S. D.R. and J.A. are supported by funds from the Public Health Services, National Institutes of Health (Grant HL55936).

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³ Abbreviations used in this paper: TB, tuberculosis; AFB, acid-fast bacilli; AM, alveolar macrophage; BAC, bronchoalveolar cell; BAL, bronchoalveolar lavage; DAMP, 3-(2,4-dinitroanilino)-3-amino-*N*-methylpropylamine; LAMP, lysosome-associated membrane protein; MTB, *Mycobacterium tuberculosis*.

clinical and chest radiographic features suggestive of pulmonary TB, but sputum smear microscopy for acid-fast bacilli (AFB) was negative (defined as smear-negative pulmonary TB). Healthy volunteers were also recruited as controls. The research ethics committees of the College of Medicine, Malawi, Liverpool School of Tropical Medicine, U.K., and Cornell University approved the study protocol, and informed consent was obtained from all study participants.

All patients were investigated as described by Hargreaves and colleagues (20). Sputum samples and BAL fluid were examined for acid-fast bacilli (AFB) using Ziehl-Neelsen staining and were cultured on Löwenstein-Jensen medium. Cultures were examined weekly for a total of 12 wk. All participants were offered HIV counseling and HIV testing was performed using two rapid detection methods (SeroCard, Trinity Biotech, Dublin, Ireland; and HIVSPOT, Genelabs Diagnostics, Singapore). A TB-positive case was defined as a patient from whom MTB was cultured from sputum or BAL fluid. Mycobacterial cultures and smears were negative from all samples in TB-negative cases. The evaluation of the patient population is detailed in the flow diagram (Fig. 1), which indicates the number of patients studied at each stage.

BAL and bronchoalveolar cell preparation

BAL was performed before commencing anti-TB chemotherapy in all patients. Briefly, after obtaining local anesthesia with topical 2 and 4% lignocaine, an fiberoptic video bronchoscope (type BF-1T240; Olympus KeyMed, Southend, U.K.) was introduced by the trans-nasal route and wedged into the abnormal segment of the lung in patients or into a segment of the right middle lobe in healthy volunteers. Sterile, warm 0.9% saline was instilled in aliquots of 50 ml each to a maximum of 200 ml, aiming for a minimum return of 80 ml. The lavage fluid was retrieved by gentle aspiration, pooled, and filtered through a single layer of sterile gauze. The fluid was then centrifuged at $250 \times g$ for 5 min at 4°C, the bronchoalveolar cells (BAC) were resuspended in RPMI 1640 supplemented with 10% heat-inactivated FBS, 1% L-glutamine, 0.2% penicillin, 0.05% gentamicin, and 0.2% amphotericin B (Life Technologies, Paisley, Scotland). A total cell count was performed on a hemocytometer, and cells were adjusted to a concentration of 3×10^5 cells/ml.

AM culture

For light microscopy experiments, BAC were plated onto 13-mm diameter glass coverslips in 24-well plates (Nunc, Hereford, U.K.) at concentration of 3×10^5 cells/ml. For electron and immunoelectron microscopy experiments, 5 ml of the BAC suspension (3×10^5 cells/ml) was placed in T25 tissue culture flasks. The cells were then incubated at 37°C in 5% CO₂ for 24 h. Nonadherent cells were removed by washing twice with warm PBS.

Adherent cells obtained by this method were >98% AM as determined by morphologic examination.

Evaluation of phagocytosis, endocytosis, and acidification of the endosomal system in AM

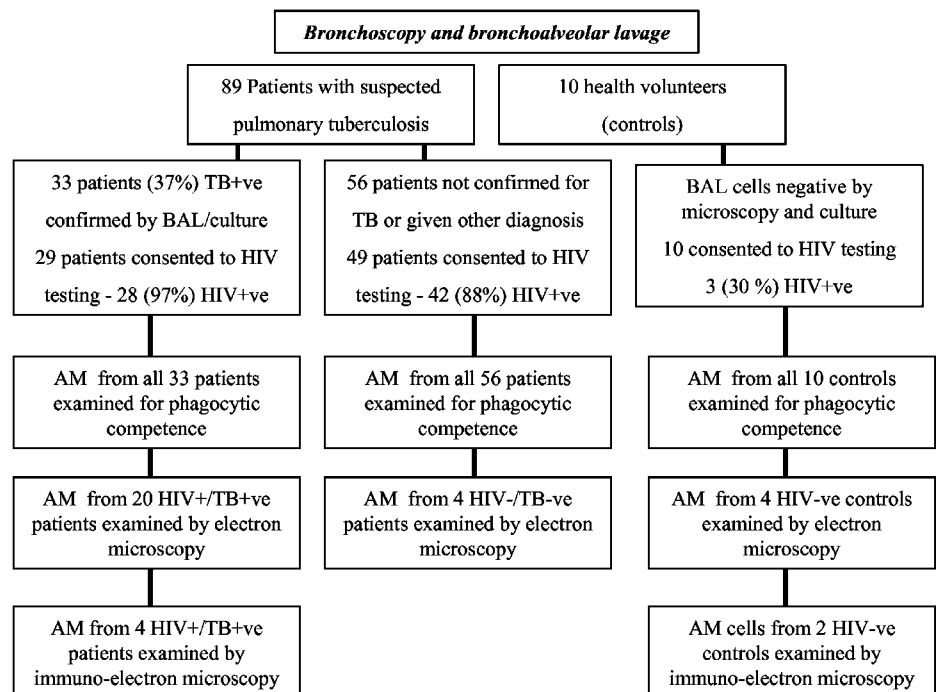
Phagocytosis. To determine the phagocytic capacity of AM by light microscopy, coverslips with adherent cells were incubated with human IgG-coated polystyrene beads (2 μm in diameter) at a bead:cell ratio of 10:1. After a 60-min incubation period (pulse), the extracellular beads were removed by washing twice with warm PBS. The cells were then left in fresh medium for another 60 min (chase), washed twice with warm PBS, and fixed with 2% paraformaldehyde for 30 min before being mounted on glass slides for examination by light microscopy. For each patient or control 100 cells were scored for the number of beads.

Endocytosis. Lysine-fixable dextran conjugated with FITC (FITC-dextran; Molecular Probes, Eugene, OR) was used as a fluid phase marker for late endosomes and lysosomal compartments. Adherent cells in T25 tissue culture flasks were incubated with FITC-dextran (0.5 mg/ml) for 60 min, washed with RPMI 1640, then incubated in fresh medium for an additional 60 min. The cells were then fixed and stored at 4°C for electron microscopy experiments.

Acidification of endosomal and phagosomal compartments. To determine the relative pH of the intracellular compartments, adherent cells in T25 tissue culture flasks were incubated with 20 μM 3-(2,4-dinitroanilino)-3-amino-N-methylpropylamine (DAMP; Oxford Biomedical Research, Oxford, MI), a DNP-derivatized weak base, for 30 min after the addition of either human IgG-coated polystyrene beads or FITC-dextran. DAMP accumulates down pH gradients because, once protonated, it can no longer cross membranes. Therefore, the relative pH can be gauged by the concentration of the label (21). This method was validated previously through more quantitative fluorescence ratio analysis (22). After 30-min incubation, the cells were processed as described above. DAMP was detected using gold-conjugated anti-DNP secondary Ab.

Immunoelectron microscopy. Cells for immunoelectron microscopy were plated in T25 culture flasks as detailed above. After experimentation, cells were fixed in 4% paraformaldehyde in 200 mM PIPES and 0.2 mM MgCl₂ (pH 7.0), scraped, washed, embedded in gelatin, and infiltrated with sucrose 1.86 M and 20% polyvinyl pyrrolidone in PIPES/Mg. The sample was frozen in liquid nitrogen, sectioned, and probed with primary Abs as detailed and with relevant gold-conjugated secondary Abs (Jackson ImmunoResearch Laboratories, West Grove, PA). Finally, the grids were stained with uranyl acetate in methyl cellulose. The distribution of gold label was scored by counting 25 negatives from each sample. No more than three

FIGURE 1. Description of the patient groups. A flow diagram is presented showing the sizes and characteristics of the different patient groups analyzed in the study. The diagram also indicates the numbers of samples assessed for phagocytosis and those examined by electron and immunoelectron microscopy.



vacuoles or lysosomes were scored for an individual negative, and the samples were taken from four different donors. The data from the four individuals were combined and displayed as a histogram (Fig. 6).

Statistical methods

To compare the TB-positive and TB-negative patients with controls, two variables were used: the proportion of cells with no beads, and the mean number of beads per cell. Analysis was performed using Dunnett's multiple comparison procedure with one-way ANOVA tests.

Results

Participants

Eighty-nine patients and 10 healthy volunteers (controls) recruited into the study underwent bronchoscopy and BAL, with no complications associated with the procedure. Thirty-three patients (37%) were TB positive. Twenty-nine (88%) of these 33 patients with confirmed TB consented to HIV testing, and 28 (97%) were HIV positive. Of the 56 TB-negative patients 49 (88%) consented to HIV testing, and 42 (86%) were HIV positive. The controls were adults with no clinical or radiographic evidence of respiratory disease. All consented to HIV testing, and three were HIV positive. None of the study participants was an active smoker, and none of the patients was receiving antiretroviral therapy at the time of investigation. A full description of the patient population included in each experimental stage is shown in Fig. 1.

Ultrastructural characterization of AM

Preliminary examination of AM from the study population revealed a high level of carbon loading in cells from 59 patients and eight controls (Fig. 2A). The larger fragments observed inside the macrophages had a charcoal-like appearance (Fig. 2B). In specimens from TB-positive patients, carbon loading was observed in both infected and uninfected AM. The carbon was invariably found in vacuoles discrete from those containing MTB (data not shown). MTB-containing vacuoles in AM were usually "tight" phagosomes (shown in Fig. 2C), although spacious vacuoles containing multiple bacilli were observed infrequently in heavier infections. Intriguingly, we observed structures reminiscent of viral particles budding internally in MTB-infected macrophages from HIV-coinfected patients (Fig. 2C). It has been reported previously that HIV buds internally into endosomal compartments in macrophages (23–25).

Evaluation of the phagocytic capacity of the AM

The distribution of internalized IgG-coated polystyrene beads in the cell population is shown in Fig. 3 (representative experiments from 10 patients and two controls). We compared AM from individual patients and controls. The percentage of cells with no beads and the mean number of beads per cell are summarized in Table I. The proportion of cells with no beads varied between 18 and 61%, with group means varying between 43 and 46%, but no statistically significant difference was found between either patient group and the control group (TB positive, $p = 0.71$; TB negative, $p = 0.62$). Likewise, the mean number of beads varied between 1.4 and 6.2, with group means varying between 3.0 and 3.4, but no statistically significant difference was found between the patient groups and the control group (TB positive, $p = 0.62$; TB negative, $p = 0.41$).

The cells examined showed a range of carbon loading, evaluated empirically and displayed as a range from 0–5+, low to high, on the individual panels of Fig. 3. The AM samples are displayed in groups of HIV⁺/TB⁺ (TB positive), HIV⁺/TB⁻, HIV⁻/TB⁻ (TB negative), and control samples. We did not have sufficient HIV⁻/TB⁺ patients to include this group in this series of experiments. We observed that the distribution of phagocytosed beads was rel-

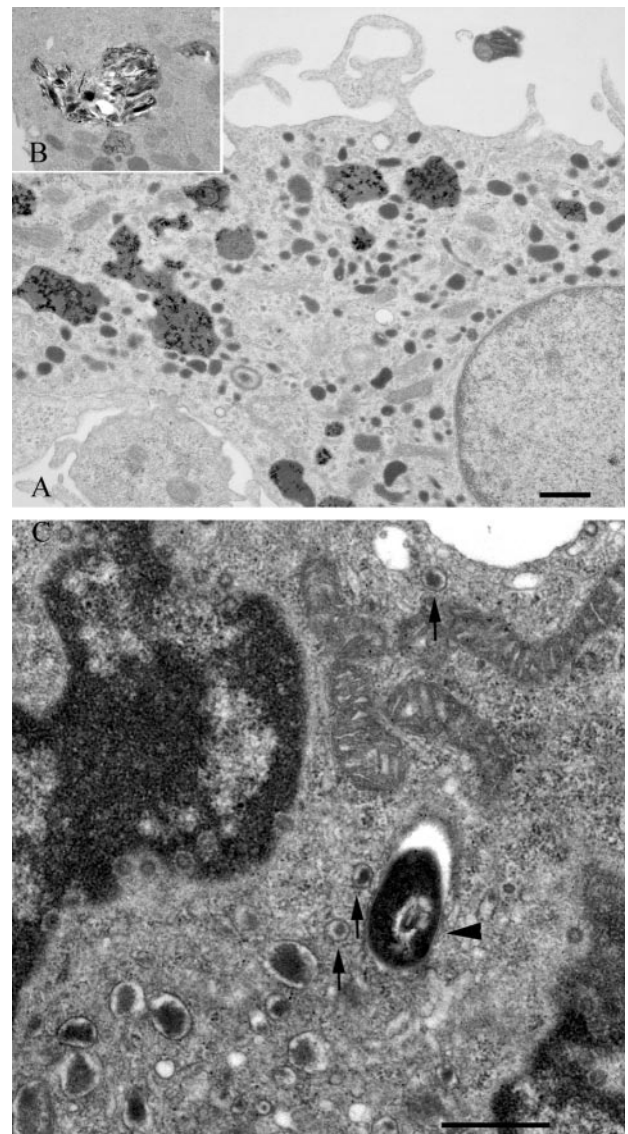


FIGURE 2. Ultrastructural analysis of patients' AM shows evidence of a carbon loading and reveals MTB in tight vacuoles separate from carbon-containing compartments. Electron micrographs are shown of AM recovered from patients (28 samples were processed and examined). The majority of AM from this study population show extensive loading with carbon (A and B). The carbon appears to accumulate in dense lysosomal structures. In many instances the carbon deposits have the appearance of charcoal (B), suggesting that they are derived from wood smoke. C, An AM infected with MTB. Most of the bacteria (arrowhead) in AM are in tight vacuoles with host membrane opposed closely to the bacterial surface. Interestingly, in some AM one can see virus-like particles forming and budding inside the cell (arrows). Although there are no data confirming that these are HIV particles, intracellular budding of HIV has been reported previously in macrophages (23–25). Scale bar = 1 μ m.

atively constant regardless of both the degree of carbon loading and the infection status of the AM donor.

Immunohistological characterization of AM

In initial immunofluorescent analysis of the endosomal system of these AM, cells were incubated with the endocytic tracer FITC-dextran, fixed, and probed with an Ab against the lysosome-associated membrane protein (LAMP-1). Surprisingly, the carbon-containing vesicles in the AM were negative for both markers, suggesting that they were excluded from the endosomal/lysosomal

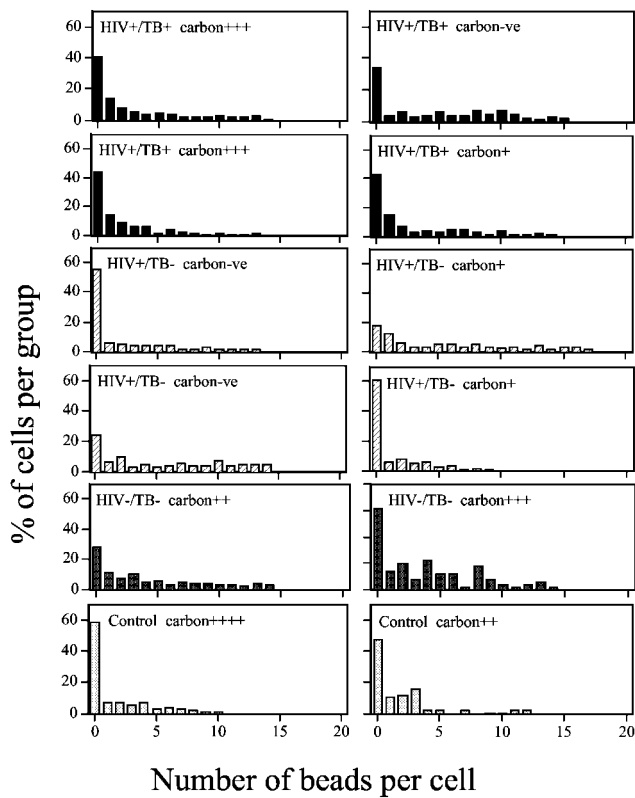


FIGURE 3. The phagocytic capacity of AM appears unaffected by the infection or carbon load status of the phagocytes. Shown are 12 histograms illustrating the uptake of IgG-coated beads by AM from patients or from control, uninfected volunteers. The graphs are clustered according to infection status: HIV⁺/TB⁺ (■), HIV⁺/TB⁻ (◻), HIV⁻/TB⁻ (◻), and controls (□). The degree of carbon loading is indicated by the number of pluses in a range of 0–5. There is no discernible difference between the groups regardless of the infection status of the donor or the level of carbon loading in the AM. Uptake of beads was scored according to the number of beads per cell, and the number of cells in each group is displayed as a percentage of the total.

continuum (data not shown). To confirm this observation we re-examined the samples by electron microscopy and determined that not only were the carbon-loaded vesicles positive for LAMP-1, but they also contained FITC-dextran internalized by the macrophage (Fig. 4A). Comparable results were obtained with samples from control, TB-positive, and TB-negative groups. We postulate that the negative result obtained in the fluorescence studies was a product of localized quenching of fluorescence, because the lack of signal was restricted to the carbon-containing vacuoles, whereas lysosomes elsewhere in the cells labeled strongly.

MTB-containing vacuoles do not accumulate either dextran or DAMP

Material endocytosed or phagocytosed by macrophages is delivered down the endosomal continuum to the lysosome, an acidic

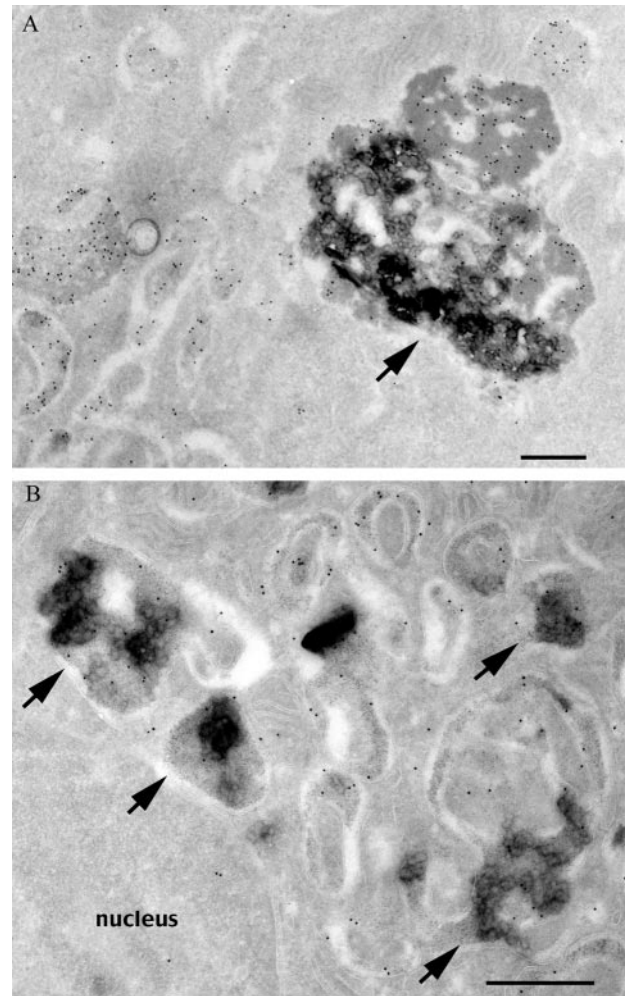


FIGURE 4. AM from patients exhibit normal endosomal trafficking and lysosomal acidification. Immunoelectron micrographs demonstrating that the carbon-containing vacuoles are accessible to FITC-dextran internalized by the cell (A) and capable of acidifying the carbon-loaded lysosomes (B). A, This AM was incubated with FITC-dextran that is visualized with rabbit anti-FITC Ab and 18-nm gold-conjugated goat anti-rabbit IgG. Abundant label is associated with carbon-loaded lysosomes (arrowed). B, This AM was incubated with DAMP, the DNP-derivatized weak base that accumulates down pH gradients in cells. The DAMP was detected by mouse anti-DNP Ab and 12-nm gold-conjugated goat anti-mouse Ab. Label is clearly seen in dense lysosomes containing carbon (arrowed) as well as in lysosomes that do not have carbon. Samples from six individuals were examined by immunoelectron microscopy. Scale bar = 1 μm.

organelle. To determine whether the lysosomes of the patients' cells were acidified, we used the pH indicator DAMP (21) and immunoelectron microscopy. We could clearly detect localization of DAMP within the dense lysosomal compartments, including those that contained carbon deposits (Fig. 4B). The distribution of

Table I. Descriptive statistics for the percentage of cells with no beads and the mean number of beads per cell by group

Variable	Group	n	Mean	SD	Minimum	Maximum
% Cells with no beads	Control	10	45.8	9.6	31	59
	TB ⁺	33	43.8	7.4	25	57
	TB ⁻	56	43.5	10.2	18	61
Mean number of beads	Control	10	3.04	0.94	1.62	4.39
	TB ⁺	33	3.29	0.74	2.1	5.06
	TB ⁻	56	3.38	1.07	1.4	6.18

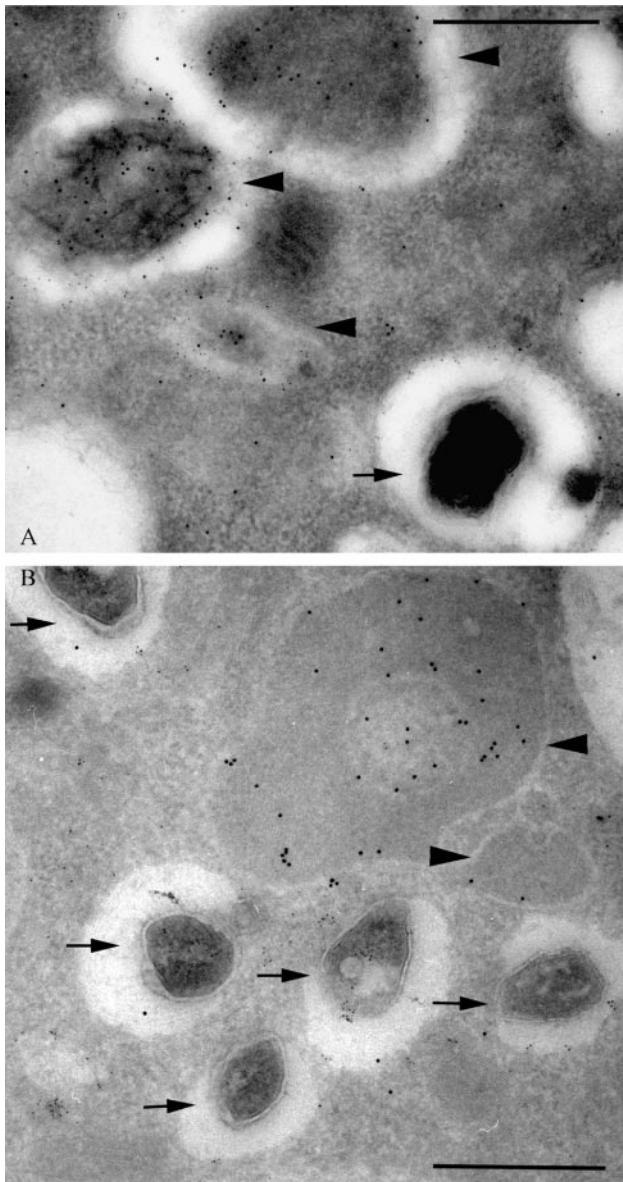


FIGURE 5. MTB-containing vacuoles in patients' AM show minimal accumulation of lysosomal tracer and are at a higher pH than neighboring lysosomes. Immunoelectron micrographs demonstrating that the vacuoles containing MTB are not readily accessible to FITC-dextran internalized by the cell (A) and avoid acidification (B). A, This AM was incubated with FITC-dextran that is visualized with rabbit anti-FITC Ab and 12-nm gold-conjugated goat anti-rabbit IgG. Abundant label is associated with dense lysosomes (arrowhead), but little label is observed in bacteria-containing compartments (arrow). The sections were colabeled with rat anti-LAMP-1/6-nm gold-conjugated goat anti-rat IgG. LAMP1 is an endosomal protein that is enriched in lysosomes. B, This AM was incubated with DAMP, the DNP-derivatized weak base that accumulates down pH gradients in cells. The DAMP was detected by mouse anti-DNP Ab and 18-nm gold-conjugated goat anti-mouse Ab. Label is clearly seen in dense lysosomes (arrowhead), but not in vacuoles containing bacteria (arrows). The sections were colabeled with rabbit anti-lipoarabinomannan/6-nm gold-conjugated goat anti-rabbit IgG. The label is associated primarily with the bacteria, but can also be observed in vesicles discrete from those containing bacilli. Samples from six individuals were examined by immunoelectron microscopy. Scale bar = 1 μ m.

DAMP within the lysosomal compartments of AM from patients and controls was similar.

We analyzed the distribution of both FITC-dextran (Fig. 5A) and

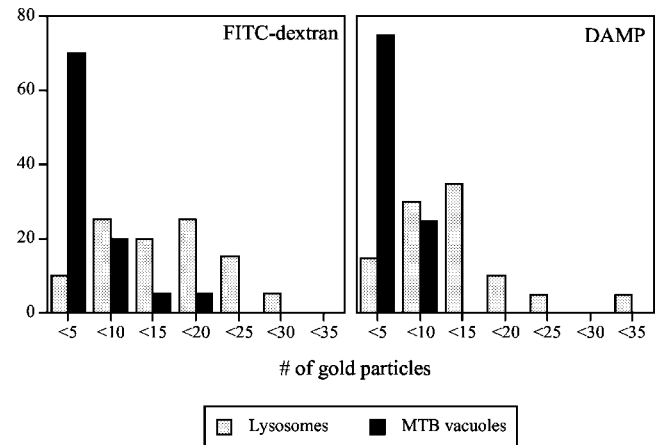


FIGURE 6. Quantitation of lysosomal tracer and DAMP labeling in MTB-containing vacuoles in patients' AM reveals minimal accumulation of lysosomal tracer and a high relative pH. Histograms of the relative labeling intensity observed in lysosomes and MTB-containing vacuoles are presented. Micrographs of infected AM incubated with FITC-dextran or DAMP were scored for the number of gold particles associated with lysosomes or mycobacteria-containing vacuoles. In both instances the number of gold particles associated with lysosomes was much greater, demonstrating that the mycobacteria-containing vacuoles had a higher pH and did not fuse with FITC-dextran-containing lysosomes. Samples from four individuals were scored and combined to generate these distribution plots.

DAMP (Fig. 5B) in MTB-infected AM and found that the vacuoles containing bacteria had significantly lower levels of label intensity for both FITC-dextran and DAMP than those observed in neighboring lysosomes within the same cell. The number of gold particles was scored for four patients' samples; the values were combined and are displayed in the histogram in Fig. 6. The low levels of label indicate that the compartments in which the bacilli reside are not acidified strongly and failed to accumulate endocytosed tracers that have been shown previously to concentrate in lysosomal compartments (26). These observations are consistent with the findings of studies performed on macrophages infected in vitro that show access to early endosomes, but fail to concentrate tracers delivered to lysosomes (26, 27).

In Fig. 5B, the sections were colabeled with Ab against the mycobacterial cell wall component lipoarabinomannan to confirm the identity of the bacilli. The label associated strongly with the bacteria, but could also be observed in vesicles discrete from those containing bacteria. This release and trafficking of cell wall constituents are consistent with previous observations from tissue culture infections (26, 28).

Discussion

The synergy between MTB and HIV coinfections is well documented, and it is a commonly held belief that both infections modify the host's immune environment to enhance both viral and bacterial replication and spread (8–13). The emphasis of these studies has been on the lymphocytes and cytokines present in or produced by the BAL cells. Few studies have examined the physiology of the AM from patients with TB. Given the central role played by AM, at least in the initial stages of pulmonary TB, it was important to evaluate the physiology of these cells with respect to phagocytosis and the functionality of their endosomal/lysosomal network.

We found that the phagocytic index of the AM was constant and could discern no variation in uptake of IgG-coated particles that

correlated with either infection status or the degree of carbon-loading observed in the AM. These data are contrary to the findings of Bonecini-Almeida and colleagues (10), who reported a 2-fold decrease in the phagocytic index of AM from HIV-infected patients, but are consistent with the data presented by Gordon and co-workers (22), who failed to demonstrate any change in the AM phagocytic index in HIV-infected patients who had recovered from an episode of invasive pneumococcal disease in Malawi. Although the current study does have the caveat that we have no information on the duration or status of either HIV or MTB infection, our data do indicate that the phagocytic capacity of the AM is not affected grossly by HIV, MTB, or carbon loading.

The lysosomal system is an extremely effective barrier against infection by many microbes, and MTB depends on its ability to halt the progression of its phagosome through this system to enable it to survive inside the phagocyte. We examined both the delivery of endocytosed marker (FITC-dextran) and the acidification of the lysosome (DAMP) by immunoelectron microscopy and concluded that the endosomal/lysosomal continuum was functionally intact in all AM regardless of the TB and HIV status of the host or the degree of carbon loading. These results indicate that the AM from TB- and HIV-infected patients retain the capacity to acidify compartments containing endocytosed material.

Examination of MTB-containing vacuoles in infected AM demonstrated that whereas lysosomes in these same cells were acidified and accessible to dextran, the MTB-containing vacuoles showed minimal label with both markers. The paucity of label for both endocytosed markers and vacuolar acidification indicates that MTB-containing vacuoles have a high pH and do not fuse with lysosomes. We had established previously through fluorescence ratio analysis that the pH of *Mycobacterium*-containing vacuoles in murine bone marrow-derived macrophages was pH 6.1–3, which is consistent with the low level of labeling observed in these preparations (22). This is, however, the first report demonstrating the nonacidification of MTB-containing phagosomes in human TB infections. This is encouraging for the tissue culture models that form the basis of our current understanding of the parasitization of macrophages by MTB.

An additional complication that has also been observed previously is the potential impact of environmental factors, such as carbon from wood smoke or diesel fumes. Carbon-loaded macrophages are reported to have a diminished capacity to produce reactive oxygen intermediates (15), possess a reduced amount of lysosomal enzymes (16), and show impaired phagocytosis (17, 18). Our current study, in common with previous analyses of AM from TB patients in Africa, noted that many of the patients had AM that were extensively loaded with carbon, probably from wood fires.

However, the carbon-containing vesicles in these macrophages were acidified and were accessible to endocytosed dextran, indicating that, at least with the assays used, the endosomal/lysosomal continuum was functionally intact.

In conclusion, we have demonstrated, for the first time in human TB infections, that MTB-containing phagosomes fail to acidify and fuse with lysosomes. Our data also demonstrate that AM in TB- and HIV-infected individuals are competent to endocytose and phagocytose material and to deliver that material to functional lysosomes. This trafficking pathway does not appear to be disturbed by the carbon deposits that have accumulated within the lysosomes of many AM. Furthermore, MTB arrests the progression of its phagosomes in AM in a manner indistinguishable from that reported in *in vitro* studies. Because of the known association between macrophage activation and acidification of the mycobacterium-containing phagosome, these data suggest that the milieu of

the lung in patients with pulmonary TB and HIV coinfections may be low in IFN- γ (29, 30). We are in the process of assaying the BAL fluid for cytokines and the BAL lymphocytes for their cytokine profiles to test this hypothesis. The value of the current study is that it establishes a baseline for ongoing analysis of the lymphocytes and cytokine environment within the lungs and of the ability of AM to respond effectively in this environment to limit mycobacterial and viral replication.

Acknowledgments

We thank Prof. Ed Zijlstra, Rose Malamba, and Anthony Chipolopolo for their invaluable assistance during recruitment and bronchoscopy of study participants.

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