

# Growth within Macrophages Increases the Efficiency of *Mycobacterium avium* in Invading Other Macrophages by a Complement Receptor-Independent Pathway

LUIZ E. BERMUDEZ,<sup>1\*</sup> AMY PARKER,<sup>1</sup> AND JOSEPH R. GOODMAN<sup>2</sup>

Kuzell Institute of Arthritis and Infectious Diseases, California Pacific Medical Center Research Institute, San Francisco, California 94115,<sup>1</sup> and Department of Pediatric Electron Microscopy, University of California, San Francisco, San Francisco, California 94143<sup>2</sup>

Received 22 April 1996/Returned for modification 20 June 1996/Accepted 27 January 1997

Infections caused by organisms of the *Mycobacterium avium* complex occur in approximately 50 to 60% of patients with AIDS. *M. avium* is an intracellular pathogen that survives and multiplies within mononuclear phagocytes. In this study, we investigated the uptake of *M. avium* grown within macrophages (intracellular growth *M. avium* [IG]) by a second macrophage compared with *M. avium* cultured in broth (extracellular growth *M. avium* [EG]). The results showed that IG was six- to eightfold more efficient than EG in entering macrophages. In addition, while an anti-CR3 antibody was able to inhibit approximately 60% of EG uptake by macrophages, it failed to inhibit the entry of IG. In contrast to EG, IG uptake into macrophages was significantly inhibited in the presence of anti- $\beta$ 1-integrin and anti-transferrin receptor antibodies. Entry into macrophages by alternate receptors was associated with resistance to tumor necrosis factor alpha (TNF- $\alpha$ ) stimulation. While stimulation with TNF- $\alpha$  resulted in inhibition of the growth of EG, it was not associated with inhibition of intracellular growth of IG. Investigation of the reason why *M. avium* is able to sense the changes in the intracellular environment triggering a change to the invasive phenotype suggests a direct relationship with macrophage apoptosis. These results suggest that intracellular growth is associated with novel mechanisms of *M. avium* uptake of macrophages and that those mechanisms appear to offer advantages to the bacteria in escaping the host defense.

Infections caused by organisms of the *Mycobacterium avium* complex occur in approximately 50 to 60% of the patients with AIDS. In patients with AIDS, *M. avium* is associated with bacteremia and disseminated disease (24, 36). *M. avium* infection in these patients is generally acquired by the gastrointestinal tract (25, 30) and is characterized by lack of well-defined granulomas (19). Conversely, *M. avium* infection in patients without AIDS resembles *Mycobacterium tuberculosis* infection. It is usually localized, mainly in the lungs, and the primary route of infection is the respiratory tract (38). In addition, tissue infection results in host immune response, with the formation of granulomas (18).

*M. avium* is an intracellular pathogen that is capable of living and replicating within mononuclear phagocytes (6, 14). It has been shown that the uptake of *M. avium* by macrophages appears to occur after bacterial binding to membrane receptors. Membrane receptors such as complement receptor, mannose receptor, fibronectin receptors (8, 42), and vitronectin receptor (41) have been associated with *M. avium* entry into macrophages. Current evidence suggests that *M. avium* lives inside intracytoplasmic vacuoles that do not acidify (13, 48), although recent work using a live system suggested that acidification of *M. avium* phagosomes may occur (23).

Macrophages are easily infected with *M. avium* in vitro (12). Two of the characteristics of *M. avium*-infected macrophage monolayers are cell detachment and cell death (6, 14). For a given strain, the degree of detachment and death of the macrophages is dependent on both the number of intracellular bacteria and the duration of infection (26).

As with *M. tuberculosis*, early in the infection, a few *M. avium*

bacteria infect a small number of macrophages. Eventually, however, bacteria replicate within macrophages and are released from cells, infecting large numbers of neighboring macrophages (17). This process of intracellular replication, followed by cell death and subsequent infection of other macrophages in the surrounding environment, may represent a manner by which infection occurs in the host. In this study, we investigated whether *M. avium* growing intracellularly can infect macrophages more efficiently than *M. avium* grown in culture medium and whether intracellular growth is associated with alternative ways to enter macrophages.

## MATERIALS AND METHODS

**Mycobacterium.** *M. avium* 101 (serovar 1) was used for the experimental assays described below. It was isolated from the blood of a patient with AIDS, and previous work has determined that this strain is virulent both in macrophages in vitro and in mice (9).

For the assays, *M. avium* was grown in one of two manners. To prepare extracellular growth *M. avium* (EG), *M. avium* was cultured in Middlebrook 7H10 agar for 10 days, and isolated transparent colonies were washed and resuspended in Middlebrook 7H9 broth for 5 days (logarithmic phase). Prior to the assays, bacteria were washed in Hanks' buffered salt solution (HBSS) and passed throughout an 18-gauge needle 10 times, and the suspension was added to a 15-ml plastic tube. It was then vortex agitated for 2 min. The suspension was then placed to rest at room temperature; after 5 min, the top 1 ml of the suspension was removed and used as the source of bacteria. To prepare intracellular growth *M. avium* (IG), *M. avium* cultured in vitro as described above was treated as follows. First, a large-scale uptake assay was carried out at 37°C in a six-well tissue culture plate (Falcon) containing  $5 \times 10^5$  macrophages per well. The infection period was 2 h, and the bacterium/cell ratio used for infection was 100:1. After phagocytosis, monolayers were washed with HBSS three times to remove extracellular bacteria and then incubated at 37°C for 1, 2, 3, 5, 7, and 10 days. The percentages of viable cells both adherent to the plastic and detached at each time point were determined by trypan blue exclusion assay and acridine orange staining and compared to those of uninfected controls. The resulting culture at each time point was harvested, and the cells attached to the plastic were detached by adding sterile water for 20 min at 4°C. Detached cells were recovered by centrifugation at  $300 \times g$  for 10 min at 4°C and resuspended in

\* Corresponding author. Mailing address: Kuzell Institute, 2200 Webster St., Room 305, San Francisco, CA 94115.

sterile water. The combined cell lysate and supernatant (after cell lysis by freeze-thaw) were then centrifuged for 10 min at  $2,000 \times g$  to pellet the bacteria and macrophage lysate. The pellet was then resuspended in 1 ml of HBSS and centrifuged for 3 min at  $150 \times g$  at  $4^\circ\text{C}$ . The supernatant containing bacteria was found to be free of intact macrophages by light microscopy. Bacteria were then pelleted at  $5,000 \times g$  for 20 min at  $4^\circ\text{C}$  and resuspended in HBSS. The bacterial suspension was vortex agitated for 2 min and allowed to sediment as described above. Approximately  $10^6$  to  $10^7$  bacteria were recovered. Examination by light microscopy demonstrated good dispersion of the inoculum. Different numbers of monolayers were necessary to obtain bacteria for each time point. Bacteria were pooled, divided into samples containing approximately  $5 \times 10^6$  organisms, and frozen. On the day of the assays, bacteria (EG and IG) were thawed, and the suspension was plated onto 7H11 agar for quantitation of the viable bacteria.

The viability of inocula were also determined by the LIVE-DEAD assay (Molecular Probes, Portland, Oreg.). Viability of EG ranged from 61 to 75%, and viability of IG ranged from 68 to 82%.

*M. avium* 104 (serovar 1) expressing green fluorescence protein (GFP) was cultured as described above for EG and IG. The *gfp* gene in this strain is integrated in the bacterial chromosome as previously described (37). The GFP-expressing *M. avium* was used in the TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) assay as described below.

**Other bacteria.** *Bacillus subtilis* and *Listeria monocytogenes* were acquired from the American Type Culture Collection, cultured in blood agar, and stored in tryptic soy agar (TSA; Difco) as previously reported (2). For the experiments, bacteria were cultured to late exponential growth, collected by centrifugation, washed, and resuspended in HBSS to give an inoculum of  $10^9$  CFU/ml. For the experiments, *B. subtilis* was opsonized with 10% fetal bovine serum for 1 h at  $37^\circ\text{C}$ .

**Monocyte-derived macrophages.** Monocyte-derived macrophages were obtained from healthy donors and purified as previously described (7). Monolayers were seeded with  $5 \times 10^5$  cells. For the assays, monolayers were cultured in RPMI 1640 supplemented with 5% serum-inactivated fetal bovine serum (Sigma Chemical Co., St. Louis, Mo.) and 2 mM L-glutamine.

**Uptake and intracellular killing assays.** Uptake assays were carried out as follows. *M. avium*, *B. subtilis*, or *L. monocytogenes* ( $10^6$  organisms) was added to macrophage monolayers ( $10^5$  cells) in the absence of serum for 1 h at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ . The monolayers were then washed three times with HBSS. To lyse macrophages, the monolayers were incubated with 0.5 ml of water for 10 min. Then 0.5 ml of another lysing solution made of 1.1 ml of 7H9 medium and 0.4 ml of 0.25% sodium dodecyl sulfate (SDS) in phosphate buffer was added to each well for 10 more min. The wells were vigorously scrapped with a rubber policeman, and the macrophage lysates were resuspended in 0.5 ml of 20% bovine albumin in sterile water to neutralize the SDS effect. The suspension was then vortex agitated for 2 min for complete lysis of macrophages. The macrophage lysate was briefly sonicated for 5 s (power output, 2.5 W/s) to disperse bacterial clumps and permit reproducible pour plate quantitation. As a control for osmotic stability, mycobacteria without macrophages were submitted to the same procedure, and quantitative colony counts were determined after plating onto 7H10 agar or TSA. Bacteria were 100% viable. The macrophage lysate was serially diluted, and 0.1 ml of the final suspension was plated onto 7H10 agar or TSA. The plates were allowed to dry at room temperature for 15 min and incubated at  $37^\circ\text{C}$  for 3 days (*B. subtilis* and *L. monocytogenes*) or 2 weeks (*M. avium*). The results are reported as mean CFU per milliliter of macrophage lysate. Duplicate plates were prepared for each well.

Uptake of *M. avium* by macrophages was also determined by staining macrophage monolayers established in Lab-Tek tissue culture chamber (Nunc Inc., Naperville, Ill.). After infection, monolayers were washed with HBSS and stained with acid-fast staining (5, 6). The number of intracellular bacteria was determined by counting 200 cells and the intracellular bacteria.

The intracellular killing assay were performed as previously described (7). Briefly, macrophage monolayers were incubated sequentially with sterile water and 0.025% SDS. The lysate was obtained and vortex agitated to ensure complete lysis of macrophages. The lysate was then mixed with equal volume of 20% bovine serum albumin to neutralize the SDS, serially diluted, and plated onto 7H10 medium. The plates were incubated for 10 days, and the colonies were quantitated.

**Reagents.** Anti-CR3 mouse antibody (CD11b monoclonal antibody) clone D12 was purchased from Becton Dickinson (Mountain View, Calif.), and mouse anti-human CR3 antibody (clone LO-MO2) was purchased from Biosource International (Camarillo, Calif.). Anti-CR1 rat monoclonal antibody (clone LO-MO1) and anti-B1 integrin mouse antibody (anti-CD29) were purchased from Biosource International (Irvine, Calif.). Recombinant tumor necrosis factor alpha (TNF- $\alpha$ ) was a gift from Genentech (South San Francisco, Calif.). Anti-transferrin receptor mouse monoclonal antibodies were purchased from Serotec (Oxford, England) and Biosource (clone B-624). Fucoidin (scavenger receptor inhibitor),  $\alpha$ -methylmannoside, and catalase were purchased from Sigma. Irrelevant mouse immunoglobulin IgG2 (IgG2a) and IgG1 (anti-fibroblast growth factor receptor and anti-insulin-like growth factor II) were purchased from Biosource. Dextran sulfate (molecular weight, 5000) and heparin were purchased from Sigma.

**Apoptosis assays.** The presence of apoptosis in infected macrophage monolayers in comparison with uninfected monolayers was examined by two methods:

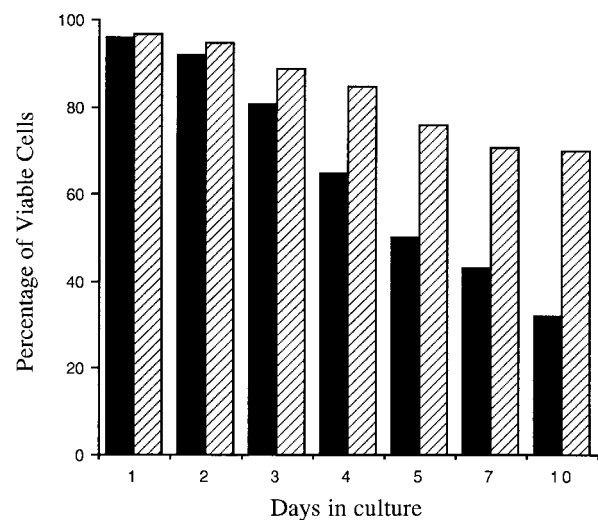


FIG. 1. Comparison of the percentage of viable macrophages in infected (■) and uninfected (▨) monolayers. The MOI was 10:1.

Cell Death Detection ELISA (enzyme-linked immunosorbent assay) (Boehringer Mannheim, Germany) for the detection of DNA fragmentation and detection of cell death in individual cells by the use of the TUNEL assay (Boehringer Mannheim). The in situ cell death detection assay detected cell death (apoptosis) by labeling DNA strand breaks in individual cells. The method uses terminal deoxynucleotidyltransferase to label free 3' OH ends in DNA with fluorescein-dUTP. This enzymatic labeling allows the detection of a very early apoptotic event. Detection of apoptosis by ELISA was carried out by coating the microtiter plate with an antihistone antibody that captures mono- and oligonucleosomes present in the cell lysate. Then anti-DNA antibody that binds to DNA in the mono- and oligonucleosomes was added. The anti-DNA antibody-peroxidase conjugate cleaves ABTS, a colorimetric peroxidase substrate. Both assays were used as directed by the manufacturers. For the ELISA, macrophage monolayers were infected with *M. avium* 101 and the apoptosis assay was performed with the lysate of the monolayer at various time points. For the individual-cell detection assay, macrophage monolayers were fixed with 3% paraformaldehyde (Sigma) for 1 h at several time points after infection and used in the assay as directed by the manufacturer. A bacterial control without macrophages was maintained in RPMI 1640 for several periods of time and subjected to the ELISA.

**Electron microscopy.** Electron microscopy was performed as previously described (3). EG and IG for transmission electron microscopy were fixed with 1% glutaraldehyde for 1 h in ice and postfixed in 1% aqueous osmium tetroxide for 1 h at  $4^\circ\text{C}$ . Then the sample was dehydrated in 50 and 80% ethanol at room temperature, embedded in resin, and polymerized at  $52^\circ\text{C}$ . Ultrathin sections were cut and mounted on carbon-coated grids.

**Statistical analysis.** Each experiment was carried out in triplicate and repeated at least three times. The results are shown as means  $\pm$  standard deviations. The significance of the results between control and experimental groups was determined either by Student's *t* test or by analysis of variance.

## RESULTS

**Viability of macrophage monolayer over time.** To examine the viabilities of infected and uninfected macrophages, macrophage monolayers were infected with *M. avium* at multiplicities of infection (MOIs) of both 10:1 and 100:1. Cell viability was monitored daily by acridine orange staining, trypan blue exclusion assay, and counting of 400 cells in 10 fields (47). As shown in Fig. 1, at an MOI of 10:1 after 4 days of infection, a significant difference in macrophage viability was observed between infected and uninfected monolayers. When an MOI of 100:1 was used, a significant difference ( $P < 0.05$ ) in viability between infected and uninfected monolayers was observed as early as the second day after infection (92% versus 81%) and in the subsequent days as well (day 3, 89% versus 63%; day 4, 88% versus 51%; day 5, 71% versus 39% [ $P < 0.01$  for all comparisons]).

TABLE 1. Phagocytosis of EG and IG by human macrophages<sup>a</sup>

Phenotype	Inoculum	No. of ingested <i>M. avium</i> after 1 h	% of inoculum
EG	$(1.1 \pm 0.3) \times 10^6$	$(6.5 \pm 0.3) \times 10^4$	5.9 <sup>c</sup>
EG + Mo lysate <sup>b</sup>			
1 day	$(1.2 \pm 0.3) \times 10^6$	$(6.6 \pm 0.4) \times 10^4$	5.5 <sup>c</sup>
2 days	$(1.0 \pm 0.3) \times 10^6$	$(6.1 \pm 0.3) \times 10^4$	6.1 <sup>c</sup>
3 days	$(1.1 \pm 0.2) \times 10^6$	$(6.8 \pm 0.3) \times 10^4$	6.1 <sup>c</sup>
5 days	$(1.2 \pm 0.5) \times 10^6$	$(6.4 \pm 0.2) \times 10^4$	5.3 <sup>c</sup>
7 days	$(9.9 \pm 0.4) \times 10^5$	$(6.5 \pm 0.4) \times 10^4$	6.5 <sup>c</sup>
10 days	$(1.0 \pm 0.3) \times 10^6$	$(6.6 \pm 0.3) \times 10^4$	6.6 <sup>c</sup>
IG			
1 day	$(1.1 \pm 0.2) \times 10^6$	$(7.8 \pm 0.4) \times 10^4$	7.0 <sup>c</sup>
2 days	$(1.2 \pm 0.3) \times 10^6$	$(3.6 \pm 0.3) \times 10^5$	30 <sup>d</sup>
3 days	$(9.8 \pm 0.4) \times 10^5$	$(4.4 \pm 0.4) \times 10^5$	44.9 <sup>d</sup>
5 days	$(1.0 \pm 0.2) \times 10^6$	$(5.1 \pm 0.3) \times 10^5$	51.0 <sup>d</sup>
7 days	$(9.7 \pm 0.3) \times 10^5$	$(5.6 \pm 0.2) \times 10^5$	57.7 <sup>d</sup>
10 days	$(1.0 \pm 0.4) \times 10^6$	$(5.5 \pm 0.3) \times 10^5$	55 <sup>d</sup>

<sup>a</sup> EG and IG were obtained as described in Materials and Methods.

<sup>b</sup> EG plus macrophage (Mo) lysate was obtained by incubating EG with lysate of  $10^5$  Mo for 1 h at 37°C.

<sup>c</sup>  $P > 0.05$  compared with EG.

<sup>d</sup>  $P < 0.05$  compared with EG.

**Macrophage uptake of EG and IG.** To determine whether *M. avium* grown intracellularly in human macrophages once released was able to invade other human macrophages more efficiently than *M. avium* grown in culture medium, we infected macrophage monolayers with an MOI of 100:1 and at different days after infection lysed the monolayers, using IG to infect a second macrophage monolayer. Since it was possible that macrophage proteins would bind to IG and influence bacterial uptake, we carried out assays in which EG was mixed with lysates of uninfected macrophage monolayers cultured for different periods of time for 1 h and used in the macrophage invasion assay.

As observed in Tables 1 and 2, intracellular growth of *M. avium* resulted in a significant increase in ability to infect human macrophages. Bacteria retrieved from macrophages 3 days after infection were capable of infecting macrophages with an efficiency sevenfold greater than that of EG or EG mixed with macrophage lysate. The increase in uptake was observed by day 2 of infection and peaked at day 3 or 4 after infection.

Infection of the first macrophage monolayer with *M. avium* for a number of days could have selected for an invasive phenotype. Therefore, it was important to establish whether the increased invasiveness of IG was secondary to phenotype selection or environmentally mediated induction of invasive phenotype. IG was recovered from the second infection, cultured

TABLE 2. Phagocytosis of EG and IG by human macrophages as determined by microscopic evaluation<sup>a</sup>

Phenotype	Inoculum ( $10^5$ )	% of infected macrophages <sup>b</sup>	No. of bacteria/infected cell
EG	$8.9 \pm 0.2$	$46 \pm 6$	$2 \pm 3$
IG			
1 day	$7.4 \pm 0.3$	$51 \pm 5$	$2 \pm 1$
2 days	$8.6 \pm 0.4$	$61 \pm 3$	$6 \pm 5^c$
3 days	$9.1 \pm 0.3$	$70 \pm 5$	$9 \pm 4^c$
7 days	$7.2 \pm 0.4$	$73 \pm 5$	$11 \pm 4^c$

<sup>a</sup> EG and IG were obtained as described in Materials and Methods.

<sup>b</sup> Uptake of *M. avium* after 1 h at 37°C.

<sup>c</sup>  $P < 0.05$  compared with EG.

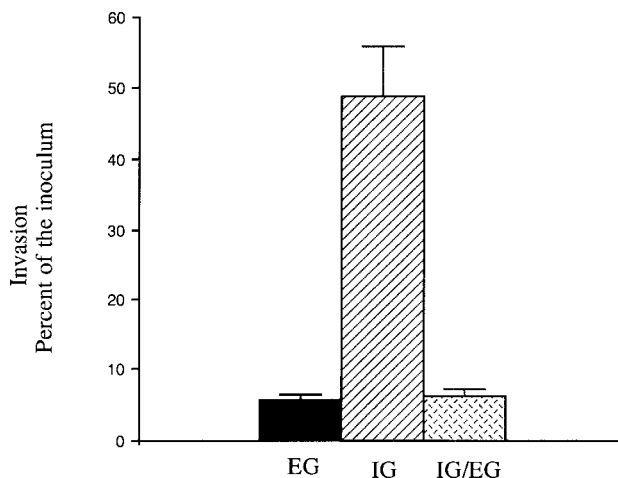


FIG. 2. Phagocytosis of EG in comparison with both IG and IG that was cultured in vitro before use in invasion assay. The result rules out selection of the invasive phenotype as an explanation of the increased ability of IG to invade macrophages.

in vitro in 7H9 broth for 5 days (in vitro passage), and used to infect macrophages. As shown in Fig. 2, simultaneous infection of human macrophages with EG, IG, and IG after passage in vitro demonstrated that intracellular growth in macrophages induces an invasive phenotype that is not expressed if the bacteria are subsequently cultured in vitro.

**Receptors associated with EG and IG invasion.** We and others have shown in previous studies that *M. avium* as well as *M. tuberculosis* and *M. leprae* invade macrophages through the CR3, CR1, and mannose receptors (8, 22, 42, 44–47). Two possible explanations for the finding that IG invades macrophages with greater efficiency than EG are that (i) there is an increase in the number of ligands on the outer layer of *M. avium* in the intracellular environment and (ii) invasion of macrophages by IG occurs by means other than the CR3 receptor and mannose receptor.

As shown in Fig. 3, in contrast to EG, *M. avium* antibodies to CR3 (two different antibodies were used with similar results, and the results shown are those obtained with D12) and CR1 at 30  $\mu\text{g/ml}$  as well as 2%  $\alpha$ -methylmannoside could not block uptake of IG by macrophages. Antibodies against transferrin receptor and  $\beta 1$  integrin (CD29) were also used in concentrations from 1 to 30  $\mu\text{g/ml}$ . Incubation with both anti- $\beta 1$  integrin and antitransferrin (two different antibodies) (Serotec) was able to block the uptake of IG by 66%  $\pm$  5% and 43%  $\pm$  8% (the two antibodies gave comparable results), while both antibodies failed to inhibit the uptake of EG (Fig. 4). As shown in Fig. 5, incubation of macrophages with fucoidin at 7  $\mu\text{g/ml}$  (0.5, 3, and 7  $\mu\text{g/ml}$  were tested), a potent inhibitor of scavenger receptors on macrophages, significantly blocked the uptake of both EG ( $P < 0.01$ ) and IG ( $P < 0.05$ ) by macrophages. Negative controls were carried out with irrelevant mouse IgG1 and IgG2a. Control antibodies did not block *M. avium* uptake (data not shown).

Because inhibition of *M. avium* binding could be a nonspecific effect of the treatment of macrophages with fucoidin, we use *B. subtilis* as a negative control and *L. monocytogenes* (which has been shown to be a phagocytized by scavenger receptors in the absence of serum [21]) as a positive control. As shown in Fig. 5, treatment of macrophages with fucoidin resulted in significant inhibition of the uptake of *L. monocyto-*

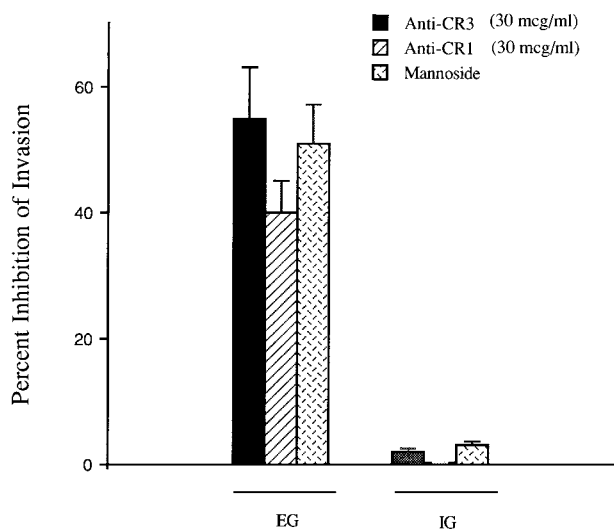


FIG. 3. Effects of antibodies to CR3 and CR1 and of 2%  $\alpha$ -methylmannoside on the uptake of EG and IG by human macrophages. Antibodies were tested at concentrations of 1, 10, and 30  $\mu$ g/ml (only results with 30  $\mu$ g/ml are shown).  $\alpha$ -Methylmannoside was used at concentrations of 1 and 2% (only results with 2% are shown). Irrelevant antibodies (30  $\mu$ g/ml) did not inhibit uptake of *M. avium* by macrophages.

genes but had no effect on the uptake of serum-opsonized *B. subtilis*.

Experiments were also carried out to determine if an effective competitor (dextran sulfate) and a control (ineffective competitor) for the scavenger receptors had any influence on the phagocytosis of IG. Table 3 shows that IG uptake by macrophages was inhibited 51 and 62% by fucoidin and dextran sulfate, respectively, at concentrations of  $\geq 1.0$   $\mu$ g/ml. In contrast, heparin (control) at up to 10 U/ml did not inhibit significantly the phagocytosis of IG by macrophages.

**Response to TNF- $\alpha$  stimulation.** Because a number of laboratories have demonstrated both in vitro and in vivo the ability of TNF- $\alpha$  to induce antimycobacterial activity in macrophages (5, 7), we used recombinant TNF- $\alpha$  to treat macrophage monolayers infected with either EG or IG. As shown in Table 4, TNF- $\alpha$  induced macrophages infected with EG to

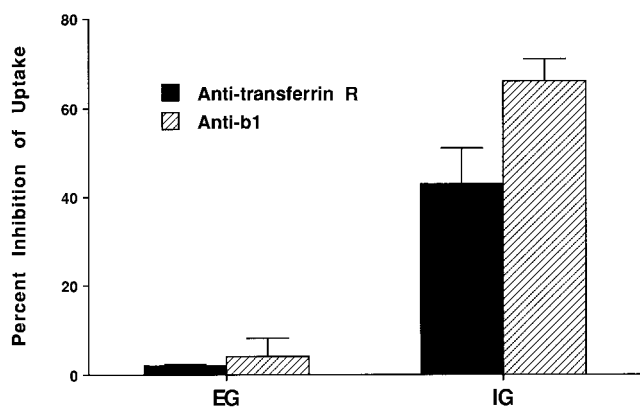


FIG. 4. Effects of antibody to transferrin receptor (R) (CD71) at 1, 10, and 30  $\mu$ g/ml (only results for 30  $\mu$ g/ml are shown) and of antibodies against  $\beta$ 1 integrin (CD29) (anti-b1) (two different antibodies were used with comparable results) at 1, 10, and 30  $\mu$ g/ml (only results with 30  $\mu$ g/ml are shown) on the uptake of EG and IG by human macrophages. Irrelevant antibodies (30  $\mu$ g/ml) did not inhibit uptake of *M. avium* by macrophages.

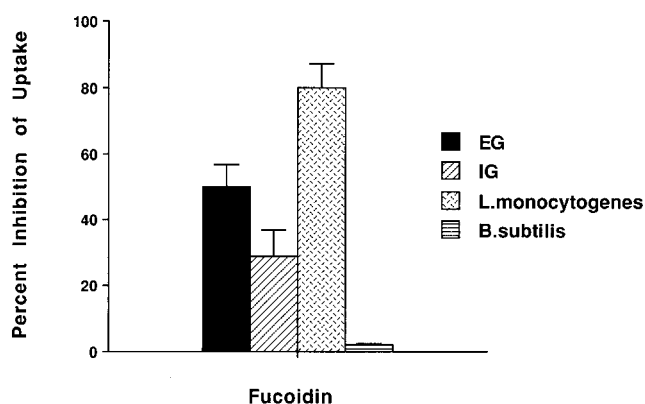


FIG. 5. Fucoidin, an inhibitor of the scavenger receptors, at 0.5, 3, and 7  $\mu$ g/ml (only results with 7  $\mu$ g/ml are shown) on EG and IG phagocytosis by human macrophages. *L. monocytogenes* and serum-opsonized *B. subtilis* were used as positive and negative controls, respectively.

inhibit intracellular replication by 40% compared with the untreated control at the same time point. Thus, intracellular bacteria grew from  $(5.7 \pm 0.3) \times 10^5$  (initial inoculum) to  $(2.4 \pm 0.3) \times 10^6$  in the untreated control, compared with  $(9.6 \pm 0.4) \times 10^5$  in monolayers treated with TNF- $\alpha$ . However, stimulation with TNF- $\alpha$  failed to stimulate mycobacteriostatic or mycobactericidal activity in macrophages infected with IG. IG grew from  $(3.0 \pm 0.4) \times 10^5$  to  $(1.2 \pm 0.3) \times 10^6$  in the untreated control, compared with  $(2.9 \pm 0.3) \times 10^6$  in monolayers treated with TNF- $\alpha$ , suggesting that the IG site within macrophages is protected against the TNF- $\alpha$ -induced bactericidal mechanisms.

**Cell death.** The above-described results suggested either that intracellular *M. avium* detects the changes in the intracellular environment of macrophages indicating that cell lysis is imminent or that *M. avium* itself triggers cell death of the macrophages. To investigate these possibilities, we first attempted to determine whether *M. avium* infection induced apoptosis of infected macrophages. As shown in Table 4, using an ELISA to detect fragmented DNA, we determined that *M. avium*-infected macrophages suffer apoptosis with significantly greater frequency than uninfected macrophages. In addition, the apoptosis appears to be dependent on the degree of infection and duration of infection. We confirmed the observations obtained by ELISA by examining apoptosis of individual cells with the TUNEL assay (Table 5 and Fig. 6). Nonetheless, not

TABLE 3. Competitive inhibition of IG uptake by human macrophages<sup>a</sup>

Blocking agent	Concn	IG within macrophages (% of control $\pm$ SD)
Fucoidin	3 $\mu$ g/ml	40 $\pm$ 6 <sup>b</sup>
	7 $\mu$ g/ml	51 $\pm$ 9 <sup>b</sup>
Dextran sulfate	1 $\mu$ g/ml	45 $\pm$ 5 <sup>b</sup>
	5 $\mu$ g/ml	62 $\pm$ 11 <sup>b</sup>
	10 $\mu$ g/ml	76 $\pm$ 10 <sup>b</sup>
Heparin	1 U	5 $\pm$ 2
	5 U	7 $\pm$ 2
	10 U	7 $\pm$ 4

<sup>a</sup> Macrophages were incubated with blocking agent for 1 h and then incubated with  $10^6$  IG for an additional hour. Viability of the monolayer was monitored during the experiments, and the data are based on monolayers with more than 95% viable macrophages.

<sup>b</sup>  $P < 0.05$  compared with control.

TABLE 4. Apoptosis of *M. avium*-infected macrophages as determined by ELISA

MOI <sup>a</sup>	OD <sub>430</sub> <sup>b</sup>			
	1 <sup>c</sup>	3	5	10
0 (no bacteria)	0.004	0.038	0.192	0.457
1:1	0.014 <sup>d</sup>	0.424 <sup>d</sup>	0.449 <sup>d</sup>	1.356 <sup>d</sup>
10:1	0.024 <sup>d</sup>	0.832 <sup>d</sup>	1.205 <sup>d</sup>	1.749 <sup>d</sup>
100:1	0.020 <sup>d</sup>	1.362 <sup>d</sup>	2.076 <sup>d</sup>	2.573 <sup>d</sup>
Bacterial control (10 <sup>6</sup> ) (no macrophages)	0.002	0.002	0.003	0.002

<sup>a</sup> As described in Materials and Methods.

<sup>b</sup> Mean of three determinations of optical density at 430 nm (OD<sub>430</sub>) in two separate experiments.

<sup>c</sup> Day after infection.

<sup>d</sup> *P* < 0.05 compared with the control (no bacteria).

all infected macrophage showed signs of apoptosis as determined by the TUNEL assay. Approximately 45% ± 7% of the infected macrophages showed evidence of apoptosis.

**Effect of catalase on apoptosis.** Because a recent study (32) had shown that hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) can induce apoptosis of *M. avium*-infected macrophages, we repeated the apoptosis assay in the presence of 100 μM catalase. Catalase by itself was not toxic to the monolayer at the concentration used. Catalase showed no effect on *M. avium*-triggered apoptosis (data not shown), indicating that H<sub>2</sub>O<sub>2</sub> was not associated with the mechanism of apoptosis in this model.

**Light microscopy and electron microscopy of IG and EG.** To determine whether EG and IG are morphologically different, bacteria from both sources expressing the GFP protein were subjected to light microscopy and transmission electron microscopy. As seen in Fig. 7, EG is short, while IG is predominantly a long bacillus. There is no evidence in the data presented either supporting or ruling out the presence of host proteins associated with the IG cell envelope.

## DISCUSSION

*M. avium* is a frequent cause of disseminated infection in patients with AIDS and is associated with localized (primarily pulmonary) infection in individuals who do not have AIDS (24, 30, 36, 38). Tissue destruction and apoptosis are believed to have a substantive role in the pathogenesis of both *M. tuberculosis* and *M. avium* infections (16, 34). Although a number of studies have established the role of host factors in necrosis of granulomas and cavitation, almost no information exists about the possible participation of the bacteria in these processes.

After infection, mycobacteria replicate intracellularly in tissue macrophages, and it is likely that organisms released from macrophages infect a large number of surrounding macro-

TABLE 5. Apoptosis of individual *M. avium*-infected macrophages

MOI <sup>a</sup>	% Apoptosis/200 cells <sup>b</sup>	
	3 days	10 days
0 (no bacteria)	0.5 (1/200)	2.5 (5/200)
1:1	12.5 (25/200) <sup>c</sup>	31.5 (63/200) <sup>c</sup>
10:1	23.0 (46/200) <sup>c</sup>	35.5 (71/200) <sup>c</sup>
100:1	31.0 (62/200) <sup>c</sup>	47.0 (94/200) <sup>c</sup>

<sup>a</sup> As described in Materials and Methods.

<sup>b</sup> Mean of three different experiments.

<sup>c</sup> *P* < 0.05 compared with the control (no bacteria), as determined by analysis of variance.

phages (15). In this report, we showed that IG infects macrophages with greater efficiency (7- to 10-fold) than EG, indicating that the intracellular environment of macrophages triggered the expression of an invasive phenotype of *M. avium*. This finding also suggests that investigation of mycobacterial invasion of macrophages in the laboratory probably has technical limitations, since some of the laboratory models do not take into consideration environmental conditions encountered in the host. A recent study by McDonough and Kress (35) has shown that *M. tuberculosis* H37Rv has increased ability to become associated with A549 epithelial cells and become cytotoxic to A549 lung epithelial cells after passage in a macrophage cell line, indicating that phenotypic characteristics that might be important in vivo are not observed when laboratory conditions are used. Previous studies in our laboratory with *M. avium* (4) and in other laboratories with *Yersinia pseudotuberculosis* (27, 40) and *Salmonella typhimurium* (33) have shown that under conditions that resemble the in vivo environment in the host, bacteria can have a completely different behavior (for example, becoming more invasive) not observed with bacteria cultured under usual laboratory conditions.

The precise mechanism(s) by which IG invades macrophages is not known, but our results suggest that the uptake of IG and the uptake of EG by macrophages occur through different receptors. Both the complement receptors and mannose receptors that have been shown to be involved in the uptake of EG by macrophages (8, 42) appear not to have a significant role on the phagocytosis of IG. In contrast, the transferrin receptor and β1 integrin were associated with the uptake of IG but not EG. Transferrin receptor has been shown to be weakly involved in *M. avium* uptake by macrophages (42). It is plausible that *M. avium* grown intracellularly is exposed to an environment deficient in iron, which may in turn be a stimulus to express proteins involved in the acquisition of iron. In fact, it has been shown that mycobacteria have a 65-kDa protein that shares homology with transferrin and lactoferrin (1). However, the expression of this protein and its regulation have not been studied. It is also possible that a mycobacterial protein with some degree of homology with transferrin is synthesized under low-iron conditions and may recognize the transferrin receptor on the macrophage membrane. Studies aimed at addressing the hypothesis that low-iron conditions trigger the synthesis of *M. avium* proteins that are used to invade macrophages are currently under way in our laboratory. β1 integrin is recognized by the *Y. pseudotuberculosis* protein, *invasin*, and has been shown to be the receptor used by *Y. pseudotuberculosis* to invade epithelial cells (28, 29). In addition, *M. leprae* invades nasal epithelial cells by using the β1 integrin as a receptor (10). The β1 integrin intracellular domain is linked to the cytoskeleton and can also stimulate signal transduction pathways, two aspects that may be important to bacterial uptake (43). An additional possibility is that macrophage proteins bound to the IG outer layer facilitate the uptake of the bacterium by other uninfected macrophages. This hypothesis was not explored in this present work; however, the plausible existence of such a mechanism would represent a natural pathway of uptake for intracellular mycobacteria.

It is possible that the bacterial route into the cells influences the outcome of infection. This has been demonstrated with *Toxoplasma gondii* in the presence and absence of serum (31). In addition, *Legionella pneumophila* can invade eukaryotic cells with increased efficiency following association with *Acanthamoeba castellanii* (11). One benefit of the bacteria using alternative pathways of cellular entry is the possibility that the intracellular compartments associated with alternate receptors differ regarding the ability to fuse with lysosomes, acidify, transport proteins across the vacuolar membrane, and respond

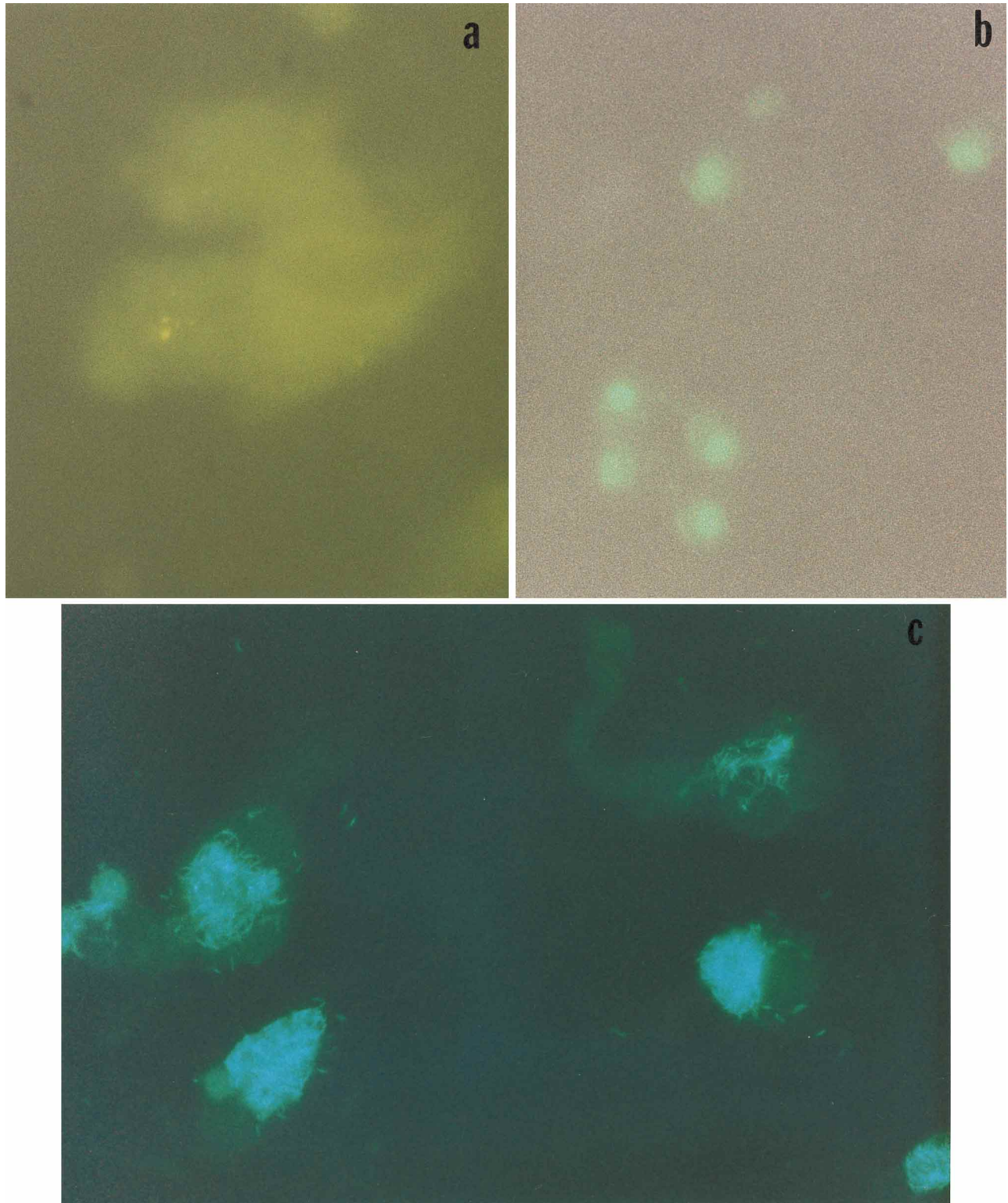


FIG. 6. Fragmentation of chromatin following *M. avium* infection of macrophages as determined by the TUNEL assay. (a) Uninfected macrophages cultured for 5 days and incubated with fluorescein isothiocyanate-labeled antibody against free-end DNA; (b) macrophages infected with strain 101 for 5 days (free-end DNAs are visualized by fluorescein isothiocyanate-conjugated antibodies in several cells in the monolayer); (c) macrophages infected with the strain 104 expressing GFP for 5 days. Three of four macrophages are observed in the process of apoptosis. The picture was taken with the GFP filter.

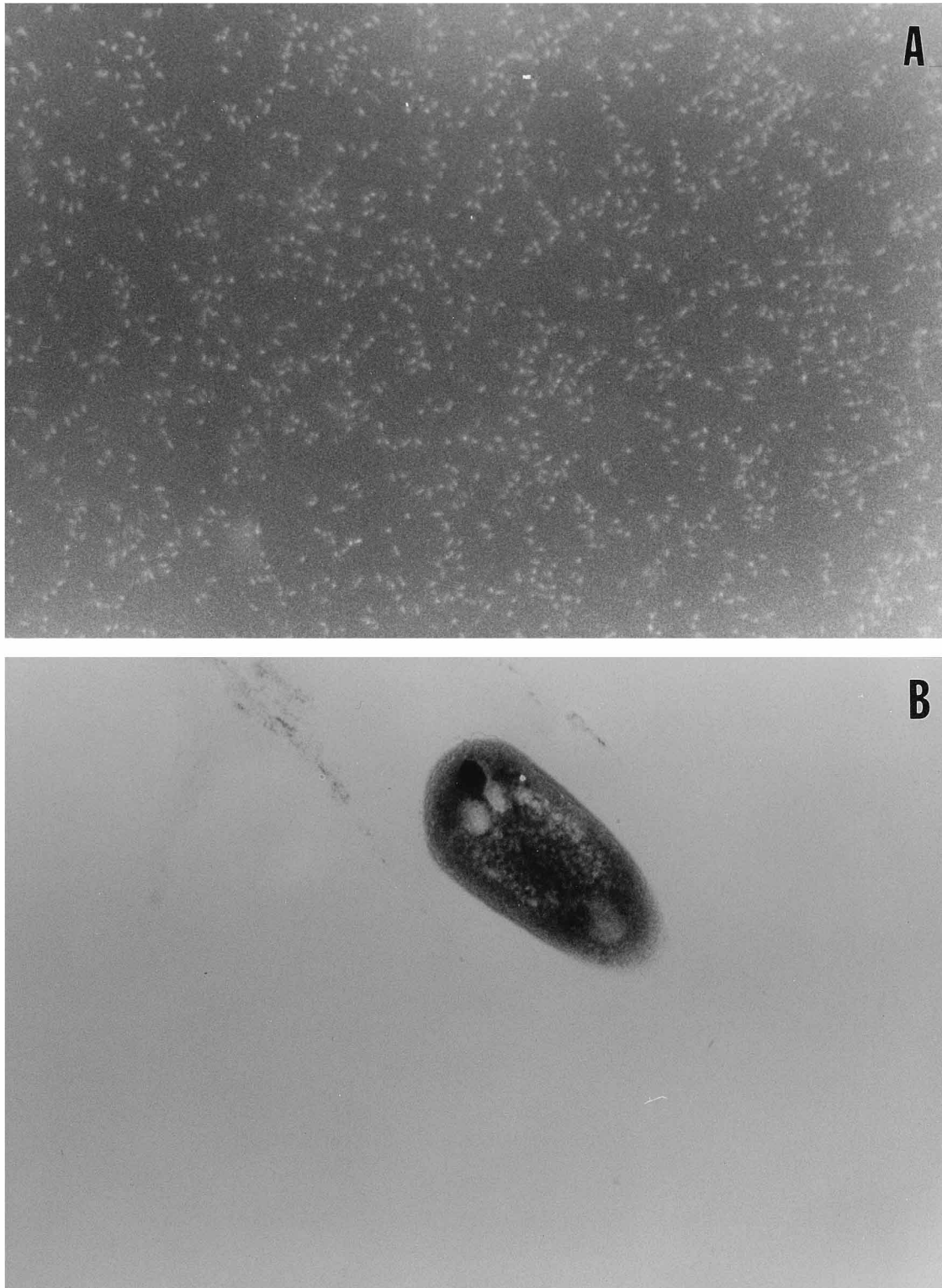


FIG. 7. Light microscopy and transmission electron microscopy of EG (A and B) and IG (C and D). IG, in contrast to EG, has a predominantly long shape. Apparently there is no structural difference between EG and IG (B and D). Magnification,  $\times 82,500$ .

to stimulation by cytokines with the activation of bactericidal mechanisms. Our results showing that TNF- $\alpha$  (a cytokine produced primarily by macrophages and shown to stimulate mycobacterial and/or mycobacteriostatic activity in both human

and murine macrophages [5, 7]) could induce anti-*M. avium* activity in macrophages infected with EG but not with IG support this hypothesis. While activated macrophages ingest and destroy *M. avium* (6), macrophages that surround infected

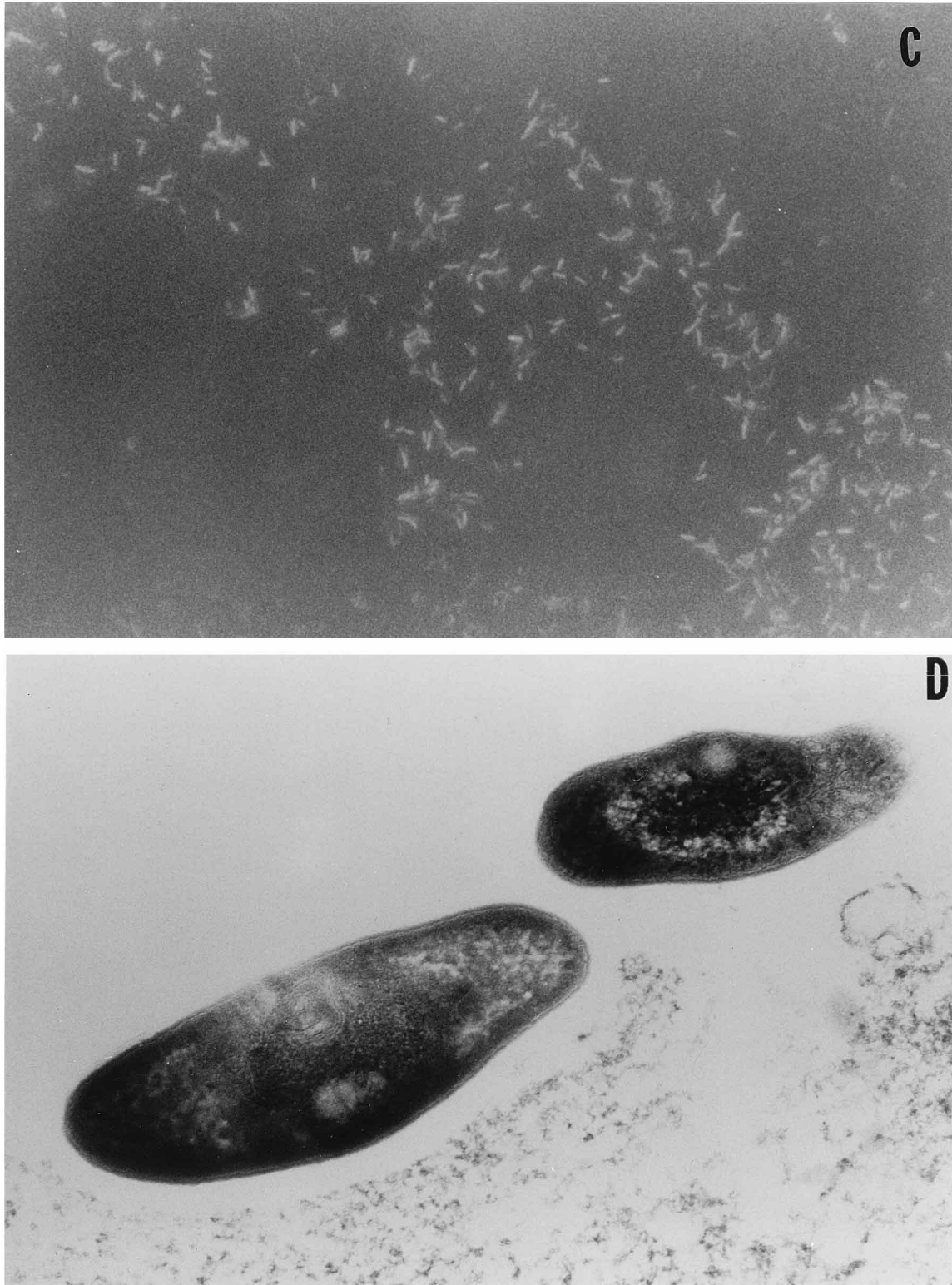


FIG. 7—Continued.

cells may have their bactericidal mechanisms bypassed by the IG phenotype.

Apoptotic cell death plays a critical role in developmental biology, inflammation, and autoimmune disease. Regardless of

the cell type involved, the hallmark of apoptosis is internucleosomal DNA degradation which is mediated by  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -dependent endonucleases (39). Traditionally, the presence of apoptosis is inferred by gel electrophoresis of the



fragmented DNA or electron microscopy changes of the cell nuclei. Recently, enzymatic methods were adapted to study apoptosis at the cellular level, making possible the detection of apoptotic phenomenon much earlier than before. These enzymatic reactions detect only apoptotic DNA, not random fragmentation that occurs in cellular necrosis (20). Our results of both an ELISA and in situ detection of cell death showed that apoptosis more frequently occurs in cells infected by *M. avium*. In addition, the apoptotic event in infected cells is dependent on the period of infection and the number of bacteria within the cell. Whether *M. avium* triggers the apoptotic event or it is a passive participant in the cell death is unknown. However, the data shown suggest that independent of the mechanism that provokes apoptosis of infected cells, intracellular bacteria sense the environmental changes and prepare themselves to leave the dead cell and to invade a surrounding macrophage with increased efficiency. In infected monolayers, there is a direct relationship between rate of apoptosis and ability of the intracellular bacteria to invade another macrophage.

IG and EG exhibit different morphologies, as observed by light and electron microscopy. While EG is a classical short bacillus, IG has a predominantly long shape. Although our data suggest that both morphologies are associated with different pathways of entry into macrophages, more studies are needed to understand the changes observed in the IG strain.

Our results with *M. avium* are in agreement with the observation made by McDonough and Kress (35) indicating that the intracellular environment can regulate the invasiveness of mycobacteria. The role of invasion in the pathogenesis of *M. avium* infection is complex. Several laboratories have identified putative receptors for *M. avium* on macrophages, such as CR3 and CR1 receptors, fibronectin receptor, mannose receptor, and vitronectin receptor (8, 42). However, it appears that some invasion pathways, such as the one(s) linked to binding to the  $\beta$ 1 integrin and transferrin receptors, may affect virulence.

The results presented here indicate that the mechanisms used to enter macrophages may differ in vivo. Further studies to improve the understanding of the molecular interactions between the bacteria and host cells are clearly necessary.

#### ACKNOWLEDGMENTS

We are in debt to Karen Allen for the preparation of the manuscript. We also thank Jeffrey Cirillo for discussions in the course of this work.

This work was supported by grant AI 25769 from the National Institutes of Health.

#### REFERENCES

- Águas, A. P., N. Esaguy, C. E. Sunkel, and M. T. Silva. 1990. Cross-reactivity and sequence homology between the 65-kilodalton mycobacterial heat shock protein and human lactoferrin, transferrin, and DR $\beta$  subsets of major histocompatibility complex class II molecules. *Infect. Immun.* **58**:1461–1470.
- Bermudez, L. E. 1993. Differential mechanisms of intracellular killing of *Mycobacterium avium* and *Listeria monocytogenes* by activated human and murine macrophages. The role of nitric oxide. *Clin. Exp. Immunol.* **91**:277.
- Bermudez, L. E., and J. Goodman. 1996. *Mycobacterium tuberculosis* invades and replicates within type II alveolar cells. *Infect. Immun.* **64**:1400–1406.
- Bermudez, L. E., and M. Petrofsky. 1995. Exposure to low concentration of oxygen (low O $_2$ ) and hyperosmolarity (Ho) increase the ability of *Mycobacterium avium* (MAC) to enter epithelial cells, abstr. U-123, p. 138. *In Abstracts of the 95th General Meeting of the American Society for Microbiology*. American Society for Microbiology, Washington, D.C.
- Bermudez, L. E., P. Stevens, P. Kolonoski, M. Wu, and L. S. Young. 1989. Treatment of disseminated *Mycobacterium avium* complex infection in mice with recombinant interleukin-2 and tumor necrosis factor. *J. Immunol.* **143**:2996–3002.
- Bermudez, L. E., and L. S. Young. 1987. Phagocytosis and intracellular killing of *Mycobacterium avium* complex by human and murine macrophages. *Braz. J. Med. Biol. Res.* **20**:191–200.
- Bermudez, L. E., and L. S. Young. 1988. Tumor necrosis factor, alone or in combination with IL-2, but not IFN- $\gamma$ , is associated with macrophage killing of *Mycobacterium avium* complex. *J. Immunol.* **140**:3006–3013.
- Bermudez, L. E., L. S. Young, and H. Enkel. 1991. Interaction of *Mycobacterium avium* complex with human macrophages: roles of membrane receptors and serum proteins. *Infect. Immun.* **59**:1697–1702.
- Bertram, M. A., C. B. Inderlied, S. Yadegar, P. Kolonoski, J. K. Yamada, and L. S. Young. 1986. Confirmation of the beige mouse model for study of disseminated infection with *Mycobacterium avium* complex. *J. Infect. Dis.* **154**:194–195.
- Byrd, S. R., R. Gelber, and L. E. Bermudez. 1993. Roles of soluble fibronectin and  $\beta$ 1 integrin receptors in the binding of *Mycobacterium leprae* to nasal epithelial cells. *Clin. Immunol. Immunopathol.* **69**:266–272.
- Cirillo, J. D., S. Falkow, and L. S. Tompkins. 1994. Growth of *Legionella pneumophila* in *Acanthamoeba castellanii* enhances invasion. *Infect. Immun.* **62**:3254–3261.
- Collins, F. M. 1989. Mycobacterial disease, immunosuppression, and acquired immunodeficiency syndrome. *Clin. Microbiol. Rev.* **2**:360–367.
- Crowle, A., R. Dahl, E. Ross, and M. H. May. 1991. Evidence that vesicles containing living, virulent *Mycobacterium tuberculosis* or *Mycobacterium avium* in cultured human macrophages are not acidic. *Infect. Immun.* **59**:1823–1827.
- Crowle, A. J., A. Y. Tsang, A. E. Vatter, and M. H. May. 1986. Comparison of 15 laboratory and patient-derived strains of *Mycobacterium avium* for ability to infect and multiply in cultured human macrophages. *J. Clin. Microbiol.* **24**:812–821.
- Dannenberg, A. M., Jr. 1993. Immunopathogenesis of pulmonary tuberculosis. *Hosp. Pract.* **68**:51–58.
- Dannenberg, A. M., Jr., and G. A. W. Rook. 1994. Pathogenesis of pulmonary tuberculosis: an interplay of tissue-damaging and macrophage-activating immune responses—dual mechanisms that control bacillary multiplication, p. 459–483. *In* B. R. Bloom (ed.), *Tuberculosis: pathogenesis, protection, and control*. ASM Press, Washington, D.C.
- Dannenberg, A. M., Jr. 1982. Pathogenesis of pulmonary tuberculosis. *Am. Rev. Respir. Dis.* **125**:25–30.
- Engbaek, H. C., B. Vergmann, and M. W. Bentzon. 1981. Lung disease caused by *Mycobacterium avium*/*Mycobacterium intracellulare*. An analysis of Danish patients during the period 1962–1976. *Eur. J. Respir. Dis.* **62**:72–83.
- Farhi, D. C., U. G. I. Mason, and C. R. J. Horsburgh. 1986. Pathologic findings in disseminated *Mycobacterium avium* intracellulare infection. *Am. J. Clin. Pathol.* **85**:67–72.
- Gold, R., M. Schmied, G. Giegerich, H. Breitschopf, H. P. Hartung, K. V. Toyka, and H. Lassman. 1994. Differentiation between cellular apoptosis and necrosis by the combined use of *in situ* tailing and nick translation techniques. *Lab. Invest.* **71**:219–225.
- Greenberg, J. W., W. Fischer, and K. A. Joiner. 1996. Influence of lipoteichoic acid structure on recognition by the macrophage scavenger receptor. *Infect. Immun.* **64**:3318–3325.
- Hirsch, C. S., J. J. Ellner, D. G. Russell, and E. A. Rich. 1994. Complement receptor-mediated uptake and tumor necrosis factor  $\alpha$ -mediated growth inhibition of *M. tuberculosis* by human alveolar macrophages. *J. Immunol.* **152**:743–747.
- Holsti, M. A., and P. M. Allen. 1996. Processing and presentation of an antigen of *Mycobacterium avium* require access to an acidified compartment with active proteases. *Infect. Immun.* **64**:4091–4098.
- Horsburgh, C. R., Jr. 1991. *Mycobacterium avium* complex in the acquired immunodeficiency syndrome (AIDS). *N. Engl. J. Med.* **324**:1332–1338.
- Horsburgh, C. R., Jr., B. G. Metchock, J. McGowan, and S. E. Thompson. 1991. Progression to disseminated infection in HIV-infected persons colonized with mycobacteria other than tuberculosis. *Am. Rev. Respir. Dis.* **143**:A279.
- Inderlied, C. B., C. A. Kemper, and L. E. Bermudez. 1993. The *Mycobacterium avium* complex. *Clin. Microbiol. Rev.* **6**:266–310.
- Isberg, R. R. 1989. Determinants for thermoinducible cell binding and plasmid-encoded penetration detected in the absence of the *Yersinia pseudotuberculosis* invasin protein. *Infect. Immun.* **57**:1998–2005.
- Isberg, R. R., and S. Falkow. 1985. A single genetic locus encoded by *Yersinia pseudotuberculosis* permits invasion of cultured animal cells by *Escherichia coli* K-12. *Nature* **317**:262–264.
- Isberg, R. R., D. L. Voorhis, and S. Falkow. 1987. Identification of invasin: a protein that allows enteric bacteria to penetrate cultured mammalian cells. *Cell* **50**:769–778.
- Jacobson, M. A., P. C. Hopewell, D. M. Yajko, W. K. Hadley, E. Lazarus, P. K. Mohanty, G. W. Modin, D. W. Feigal, P. S. Cusick, and M. A. Sande. 1991. Natural history of disseminated *Mycobacterium avium* complex infection in AIDS. *J. Infect. Dis.* **164**:994–998.
- Joiner, K. A., S. A. Fuhrman, H. M. Miettinen, L. H. Kasper, and I. Mellman. 1990. *Toxoplasma gondii*: fusion competence of parasitophorous vacuoles in Fc receptor-transfected fibroblasts. *Science* **249**:641–646.
- Laochumroonvorapong, P., S. Paul, K. B. Elkon, and G. Kaplan. 1996. H $_2$ O $_2$  induces monocyte apoptosis and reduces viability of *Mycobacterium avium*-*M. intracellulare* within cultured human monocytes. *Infect. Immun.* **64**:452–459.
- Lee, C. A., and S. Falkow. 1990. The ability of *Salmonella* to enter mamma-

- lian cells is affected by bacterial growth state. Proc. Natl. Acad. Sci. USA **87**:4304–4308.
34. **Lowrie, D. B.** 1990. Is macrophage death on the yield of battle essential to victory, or a tactical weakness in immunity against tuberculosis? Clin. Exp. Immunol. **80**:301–303.
  35. **McDonough, K., and Y. Kress.** 1995. Cytotoxicity for lung epithelial cells is a virulence-associated phenotype of *Mycobacterium tuberculosis*. Infect. Immun. **63**:4802–4811.
  36. **Nightingale, S. D., L. T. Byrd, P. M. Southern, J. D. Jochusch, S. X. Cal, and B. A. Wynne.** 1992. Disseminated MAC infection: an inevitable complication of HIV infection in children and adults? Incidence of *Mycobacterium avium-intracellulare* complex bacteremia in human immunodeficiency virus-positive patients. J. Infect. Dis. **165**:1082–1085.
  37. **Parker, A., and L. E. Bermudez.** Expression of the green fluorescent protein in *Mycobacterium avium* as a tool to study the interaction between mycobacteria and host cells. Microb. Pathog., in press.
  38. **Parker, B. C., M. A. Ford, H. Gruft, and J. O. Falkinham.** 1983. Epidemiology of infection by nontuberculous mycobacteria. IV. Preferential aerosolization of *Mycobacterium avium* from natural waters. Am. Rev. Respir. Dis. **128**:652–656.
  39. **Peitsch, M. C., B. Polzar, H. Stephan, T. Crompton, H. R. MacDonald, H. G. Mannherz, and J. Tschopp.** 1993. Characterization of the endogenous deoxyribonuclease involved in nuclear DNA degradation during apoptosis (programmed cell death). EMBO J. **12**:371–377.
  40. **Pepe, J., J. L. Badger, and V. L. Miller.** 1994. Growth phase and low pH affect the terminal regulation of the *Yersinia enterocolitica* *inv* gene. Mol. Microbiol. **11**:123–135.
  41. **Rao, S. P., K. Ogata, and A. Catanzaro.** 1993. *Mycobacterium avium-M. intracellulare* binds to the integrin receptor  $\alpha_v\beta_3$  on human monocytes and monocyte-derived macrophages. Infect. Immun. **61**:663–670.
  42. **Ratliff, T. L., R. McCarthy, W. B. Telle, and E. J. Brown.** 1993. Purification of a mycobacterial adhesin for fibronectin. Infect. Immun. **61**:1889–1894.
  43. **Ruoslahti, E.** 1991. Integrins. J. Clin. Invest. **87**:1–5.
  44. **Schlesinger, L. S.** 1993. Macrophage phagocytosis of virulent but not attenuated strains of *M. tuberculosis* is mediated by mannoside receptors in addition to complement receptors. J. Immunol. **150**:2920–2930.
  45. **Schlesinger, L. S., C. Bellinger-Kawahara, N. R. Payne, and M. A. Horwitz.** 1990. Phagocytosis of *M. tuberculosis* is mediated by human monocyte complement receptors and complement component C3. J. Immunol. **144**:2771–2780.
  46. **Schlesinger, L. S., and M. A. Horwitz.** 1990. Phagocytosis of leprosy bacilli is mediated by complement receptors CR1 and CR3 on human monocytes and complement component C3 in serum. J. Clin. Invest. **85**:1304–1314.
  47. **Shepard, C. C.** 1957. Growth characteristics of tubercle bacilli and certain other mycobacteria in HeLa cells. J. Exp. Med. **105**:39–48.
  48. **Sturgill-Koszycki, S., P. H. Schlesinger, P. Chakraborty, P. L. Haddix, H. L. Collins, A. K. Fok, R. D. Allen, S. L. Gluck, J. Heuser, and D. G. Russell.** 1994. Lack of acidification in *Mycobacterium* phagosomes produced by exclusion of the vesicular proton-ATPase. Science **263**:678–681.

---

Editor: S. H. E. Kaufmann