

Mycobacterium avium Infection of Epithelial Cells Results in Inhibition or Delay in the Release of Interleukin-8 and RANTES

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Mycobacterium avium is an opportunistic pathogen in AIDS patients, who acquire the infection mainly through the gastrointestinal tract. Previous studies in vitro have shown that *M. avium* invades epithelial cells of both intestinal and laryngeal origin. In addition, *M. avium* enters the intestinal mucosa of healthy mice. Because *M. avium* invasion of the intestinal mucosa in vivo initially is not accompanied by significant influx of inflammatory cells, we sought to determine whether *M. avium* would trigger chemokine release upon entry into epithelial cells by using HT-29 intestinal and HEp-2 laryngeal epithelial cell lines. Chemokine synthesis was measured both by the presence of specific mRNA and protein secretion in the cell culture supernatant as determined by enzyme-linked immunosorbent assay. Infection of HT-29 intestinal cells with *M. avium* did not induce the release of interleukin-8 (IL-8) or RANTES for up to 7 days postinfection. However, infection of HEp-2 cells resulted in the release of IL-8 and RANTES at 72 h. Similar findings were observed with other AIDS *M. avium* isolates belonging to different serovars. Secretion of IL-8 by HEp-2 cells was dependent upon bacterial uptake. In addition, prior infection with *M. avium* suppressed IL-8 production by HT-29 cells infected with *Salmonella typhimurium*. Our results suggest that *M. avium* infection of epithelial cells is associated with a delay in IL-8 and RANTES production which, in the case of HT-29, is prolonged up to 1 week. These findings may explain the weak inflammatory response after intestinal mucosa invasion in mice and are probably related with the ability of the bacterium to evade the host's immune response.

Infections caused by organisms of the *Mycobacterium avium* complex are the most common bacterial infection in patients with AIDS (11, 14). In AIDS patients, in contrast to individuals without AIDS, where the pulmonary infection is more frequent, the gastrointestinal (GI) tract appears to be the primary route of *M. avium* infection (7, 15, 22). Colonization of the intestine is usually observed months prior to the diagnosis of disseminated infection (7, 22, 26), and it is assumed to be a risk factor for the disseminated disease.

We have shown that *M. avium* is able to bind to and invade intestinal and laryngeal epithelial cells in vitro (3, 4). In addition, when given orally to mice, *M. avium* enters the intact intestinal mucosa and subsequently disseminates (2).

Epithelial cells of the intestinal mucosal surface form a major mechanical barrier that separates the host's internal milieu from the external environment. Mucosal surfaces have a highly specialized immune system, and mucosal epithelial cells produce an array of cytokines and chemokines in response to different stimuli. The existence of mucosal cytokine response to infection was first shown to occur in mice as well as in patients after urinary tract colonization by *Escherichia coli* (10). In addition, secretion of interleukin-6 (IL-6), IL-1 β , and IL-8 have been demonstrated in a number of studies in vitro and in vivo after infection of mucosal epithelial cells with *E. coli* (9), *Helicobacter pylori* (6, 13, 24), *Salmonella* spp. (16, 27), and *Shigella* spp. (16), as well as other microorganisms (23).

In mice, infection of the intestinal mucosa by *M. avium* is followed by little inflammatory response, with neutrophil infiltration of the Peyer's patches being observed approximately 1 week after infection and migration of mononuclear cells to the

site of infection thereafter (17). Because the release of chemokines, such as IL-8 (a potent T-cell and neutrophil recruitment factor) has been reported in a number of inflammatory conditions of the mucosal layer, we investigated, using two different cell lines, whether *M. avium* infection of epithelial cells results in the induction of chemokine secretion.

MATERIALS AND METHODS

Bacteria. *M. avium* strains 101 (serovar 1), 104 (serovar 1), and 100 (serovar 8) were isolated from the blood of AIDS patients. *Mycobacterium smegmatis* 11727 and *Salmonella typhimurium* 15277 were purchased from The American Type Culture Collection (ATCC), Rockville, Md. *E. coli* DH5 α was a gift from Raul Barletta (University of Nebraska, Lincoln, Neb.).

M. avium strains were cultured in Middlebrook 7H10 agar supplemented with oleic acid, albumin, dextrose, and catalase (Difco Laboratories, Detroit, Mich.) for 10 days and harvested in phosphate-buffered saline (pH 7.4), whereas *M. smegmatis* was harvested after 3 days in culture as described previously (3). Only colonies of *M. avium* expressing the transparent morphotype were used in the experiments. *S. typhimurium* and *E. coli* were cultured in Luria-Bertani broth for 24 h under static conditions.

Because of the tendency of mycobacteria to form clumps, the inoculum was carefully prepared to ensure a dispersed preparation as described earlier (1). The viability of the inoculum was determined by both plating onto 7H10 agar and by visualization under fluorescent microscopy by using the LIVE-DEAD assay (Molecular Probes, Eugene, Oreg.) as previously reported (1).

Cell cultures. The human intestinal epithelial cell line HT-29 was obtained from the ATCC. Cells were grown in a medium consisting of modified McCoy-5A medium (Difco) with 10% of galactose (Sigma Chemicals, St. Louis, Mo.) and 10% fetal bovine serum (Sigma). The HEp-2 laryngeal cell line was also obtained from the ATCC, and it was maintained in RPMI 1640 supplemented with 5% fetal bovine serum as described previously (4).

Cell lines were used between passages 15 and 20. HT-29 and HEp-2 cells were seeded in 24-well or 12-well tissue culture plates (Costar, Cambridge, Mass.) at 1×10^5 or 2×10^5 cells per well, respectively, in a volume of 1 ml, and then cultured to 90 to 100% confluence. HT-29 cells were also seeded on transwell membrane (3- μ m pore size) and allowed to achieve confluence (ca. 1 week). The integrity of the polarized monolayers on transwell was determined by measuring the transmembrane resistance. Monolayers were used when the resistance reached approximately 400 Ω /cm².

Monolayers were infected with bacteria by removing the medium and replacing it with 1 ml of medium containing 10^6 organisms to each well in the 24-well

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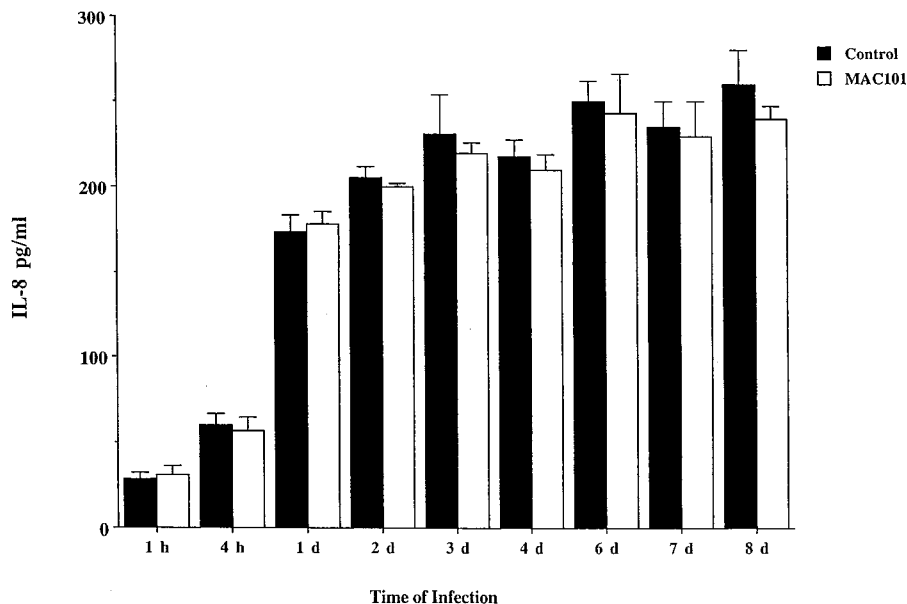


FIG. 1. Production of IL-8 by infected HT-29 cells overtime. HT-29 cells (confluent monolayers) were infected with *M. avium* 101 (MAC101) (MOIs of 1, 10, or 100) for several days, and IL-8 production was measured in the supernatant after 1, 2, 3, 4, and 7 days. Results are shown for the assays by using an MOI of 10. Cells infected with *S. typhimurium* produced 343 ± 29 pg of IL-8 per ml after 24 h (data not shown).

tissue culture plate and transwell monolayers or 2×10^6 in each well in the 12-well plates.

Invasion assay and intracellular growth. The invasion assay was performed as previously described (3, 4), with the modification that bacteria were allowed to invade for 1 h at 37°C instead of 2 h as originally described (4). After 1 h, cells were washed to remove unattached bacteria and incubated with 200 μ g of amikacin per ml for 2 h (4). Then, cells were lysed by adding 1% Triton X-100 (Sigma), and the lysate was subsequently diluted and plated onto 7H10 agar to quantitate the viable intracellular bacteria. Results were reported as a percentage of the initial inoculum that entered both HT-29 and HEP-2 cells. The viability of the infected monolayers versus uninfected control was monitored during the experiments by trypan blue exclusion and did not show any differential cytotoxic effect related to the presence of the bacteria.

Infected transwell monolayers were maintained for up to 1 week, and the integrity of the monolayer was verified daily by measuring the resistance between both membranes.

Coinfection experiments. To determine whether *M. avium* infection of HT-29 cells would suppress IL-8 production by the cells after uptake of *S. typhimurium*, we infected a 100% confluent HT-29 cell monolayer with 10^7 or 10^8 *M. avium* 101 for 1 h; this was then washed and treated with amikacin for 2 h as described above. After 18 h the monolayers were infected with *S. typhimurium* (10^5 cells) for 2 h and then treated with gentamicin (20 μ g/ml) for 1 h. Monolayers were then washed, and 24 h later the supernatants were obtained to measure IL-8 concentration. Controls of cells without infection, infected with *M. avium* or *Salmonella* were run in parallel.

To determine whether *M. avium* and *Salmonella* were coinfecting the same cells, we performed similar assays in LabTek chamber slides as reported (1, 4).

After *M. avium* and/or *Salmonella* infection and treatment with antibiotics, the monolayers were fixed with 2% paraformaldehyde for 30 min at room temperature. After being washed with Hank's balanced salt solution (HBSS), monolayers were incubated with Triton X-100 (Sigma) for 10 min and then washed three more times with HBSS. Rabbit anti-*M. avium* antibody (1:40) and fluorescein isothiocyanate (FITC)-labelled anti-rabbit antibody (1:400), as well as mouse anti-*S. typhimurium* (1:30) antibody and Texas Red anti-mouse antibody (1:800), were used to identify the bacteria. After a washing, the slides were mounted and observed by using a light microscope (Nikon). Intracellular bacteria in 100 cells per field in 10 fields were numbered by using a source of UV light and specific filters for FITC and Texas Red.

Assays for chemokine release. Monolayers were infected with bacteria or incubated with lipopolysaccharide (LPS). Infection was allowed to occur for 1 h at 37°C, and then the wells were washed with HBSS to remove noninternalized bacteria. Fresh medium was then added, and at 4, 24, 48, and 72 h after infection, supernatants were obtained, filtered through a 0.22-pore-size- μ m filter to remove residual bacteria and cell debris, and frozen at -20°C .

Concentration of human IL-8 and RANTES, as well as IL-1 α , IL-1 β , transforming growth factor β (TGF- β), and IL-6 were measured in the supernatant by enzyme-linked immunosorbent assay (ELISA) by using kits purchased from R &

D Systems, Minneapolis, Minn., and BioSource, Camarillo, Calif. The assays were carried out according to directions provided by the manufacturers.

RNA preparation and RT-PCR analysis. To determine the relative level of IL-8 mRNA transcription, total RNA was obtained with the RNeasy Mini Kit (QIAGEN, Santa Clarita, CA), following the supplier's instructions, from 12-well infected monolayers. It was then retrotranscribed with the Ready-To-Go T-Primed First-Strand Kit (Pharmacia, Piscataway, NJ), and quantitated by reverse transcriptase PCR (RT-PCR) with previously described primers (ATG ACT TCC AAG CTG GCC GTG GCT and TCT CAG CCC TCT TCA AAA ACT TCT C) for IL-8 and GADPH (glyceraldehyde-3-phosphate dehydrogenase) as a control (TGA AGG TCG GAG TCA ACG GAT TTG CT and CAT GTG GGC CAT GAG GTC CAC CAC) (16).

The samples were heated at 94°C for 4 min and then subjected to 30 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s in a GeneAmp PCR System 2400 (Perkin-Elmer, Foster City, Calif.). Then, 10 μ l of the PCR products was separated on 1.5% agarose gels and visualized by staining with ethidium bromide.

Statistical analysis. Each experiment was repeated at least three times, and the results were expressed as the mean \pm the standard deviation. The significance of the differences between experimental groups was analyzed by the Student's *t* test. A *P* value of <0.05 was considered significant.

RESULTS

Chemokine and cytokine production by uninfected cells. HT-29 intestinal cells do not produce IL-1 α , IL-1 β , IL-6, or RANTES constitutively. However, IL-8 and TGF- β (ca. 800 and 700 pg, respectively) were observed in uninfected HT-29, confirming results shown by Eckmann and colleagues (8). Uninfected HEP-2 produced approximately 1,600 pg of IL-8 and 1,400 pg of RANTES per ml.

Chemokine and cytokine production after infection in vitro. After infection with *M. avium* at a ratio of 10:1, HT-29 cells secreted IL-8 at similar levels of noninfected monolayer at 24, 48, 72, 96, and 168 h (Fig. 1). In addition, no production of other inflammatory cytokines (IL-1 α , IL-1 β , and IL-6) was observed (data not shown). In contrast, HT-29 cells infected with a 10:1 ratio of *S. typhimurium* produced $6,400 \pm 150$ pg of IL-8 by 24 h.

HEP-2 cells infected with *M. avium* did not synthesize increased amounts of chemokines (IL-8 and RANTES) for the first 48 h, but a significant increase of IL-8 and RANTES was

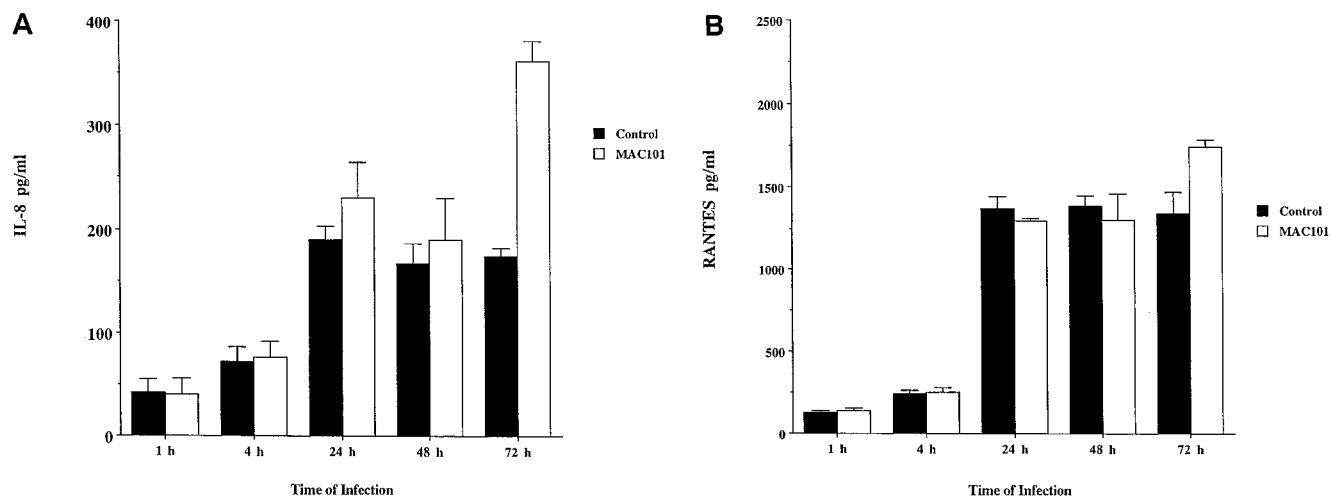


FIG. 2. Production of chemokines by HEP-2 cells infected with *M. avium* 101 (MAC101). (A) IL-8. (B) RANTES. Cells were infected with an MOI of 10. *, $P < 0.05$ compared with the uninfected control.

observed at 72 h (Fig. 2). Greater ratios of infection than 10:1 (100:1) resulted in production of IL-8 after 24 h (data not shown).

Production of IL-8 and RANTES did not change independently of the *M. avium* strains (100, 104, and 101) used in the experiment. As shown in Fig. 3, in contrast to *M. avium* and *E. coli* DH5 α , a noninvasive strain of *E. coli*, infection of HEP-2 cells with *S. typhimurium* triggered a significant release of IL-8 after 24 h.

The viability of HEP-2 and HT-29 monolayers was not affected by *M. avium* and remained $96 \pm 2\%$ after *M. avium* infection.

M. avium infection of polarized HT-29 monolayer in transwell plates did not result in either IL-8 or RANTES production for up to 72 h after infection, a result similar to that observed in assays with cell monolayers on plastic. The concentration of chemokines was measured in both the apical and basal supernatants.

To confirm the level of infection of the monolayers, HEP-2 monolayers infected with bacterial strains were lysed, and the percentage of initial inoculum within cells was determined. Cells infected for 1 h with *M. avium* 101 had taken up 3.6% of the inoculum, while cells infected with *M. avium* strains 104 and 100 had taken up 3.2 and 1.8% of the inoculum, respectively. *M. smegmatis* had 0.04% of the inoculum taken up by HEP-2 cells. The uptake of *E. coli* DH5 α was 0.0018%, and that for *S. typhimurium* was approximately 26% of the inoculum (Table 1). Because *Salmonella* invades HEP-2 cells more efficiently than *M. avium*, we repeated the assay with a 100:1 ratio of *M. avium* and a 10:1 ratio *Salmonella* per cell. After 2 h of uptake, the number of intracellular bacteria was determined and shown to be similar. We then measured IL-8 production by the monolayers after 24 h. As shown in Fig. 4, *Salmonella* still induced significantly more IL-8 production, while *M. avium* uptake did not induce IL-8 at 24 h, which can be explained by the shorter replication time of *Salmonella* compared with *M. avium*.

mRNA. As shown in Fig. 5A and B, RT-PCR with total RNA extracted from HT-29 and HEP-2 cells confirmed the results shown by ELISA that *M. avium* does not trigger IL-8 production by epithelial cells.

Uptake is necessary to induce IL-8 production. Previous studies have determined that *M. avium* entry into both HEP-2

and HT-29 cells was accompanied by cytoskeleton rearrangement and incubation of epithelial cells with cytochalasin prior to the exposure to the bacterium would block uptake (4). Cytochalasin D (5 μ M, a concentration that blocks $90 \pm 4\%$ of *M. avium* uptake) was then used to examine whether bacterium uptake was necessary to induce IL-8 production by HEP-2 cells. Cytochalasin was added for 30 min and then removed, and then *M. avium* 101 was used to infect HEP-2 monolayers (at a multiplicity of infection [MOI] of 10). After 2 h, extracellular bacteria was removed by washing, and amikacin (200 μ g/ml) was used for an additional 2 h to kill extracellular bacteria. As shown in Fig. 6, IL-8 production after 72 h of incubation did not occur if uptake was inhibited. Cytochalasin D, however, when added alone or 1 h after *M. avium* infection, did not have influence on chemokine production by HEP-2 cells. In addition, cytochalasin D does not block IL-8 production by HEP-2 cells stimulated with IL-1 β (data not shown).

***M. avium* blocks IL-8 production in response to *Salmonella* invasion.** HT-29 monolayers were infected with either 10^7 or 10^8 (to maximize the number of infected cells) *M. avium* 101 and 18 h later infected with *S. typhimurium* (10^5 /well) for 1 h. Preinfection with *M. avium* resulted in complete blockage of IL-8 release by 24 h after *S. typhimurium* infection (Fig. 7). To determine the percentage of cells that had the two bacteria, we stained intracellular bacteria with different colors and scored them. We found that $78 \pm 4\%$ of the cells had at least one *M. avium* bacillus, while $81 \pm 6\%$ of the cells had at least one *Salmonella* organism and that $72 \pm 5\%$ of the cells in the monolayer were coinfecting with *M. avium* and *Salmonella*.

DISCUSSION

Several studies have demonstrated that *M. avium* infection in AIDS patients is acquired primarily through the GI tract (7, 15, 22). When *M. avium* is given orally to healthy C57BL/6 mice, the bacterium invades the intact mucosa of the intestinal tract and secondarily causes disseminated infection (2). Epithelial cells are the first cell type to encounter bacteria at mucosal sites. It was proposed that epithelial cell cytokine-chemokine production could function as an "early warning system" of mucosal infection. In fact, a number of microorganisms have been shown to induce cytokine-chemokine synthesis upon contact with epithelial cells such as IL-8, MCP-1, gran-

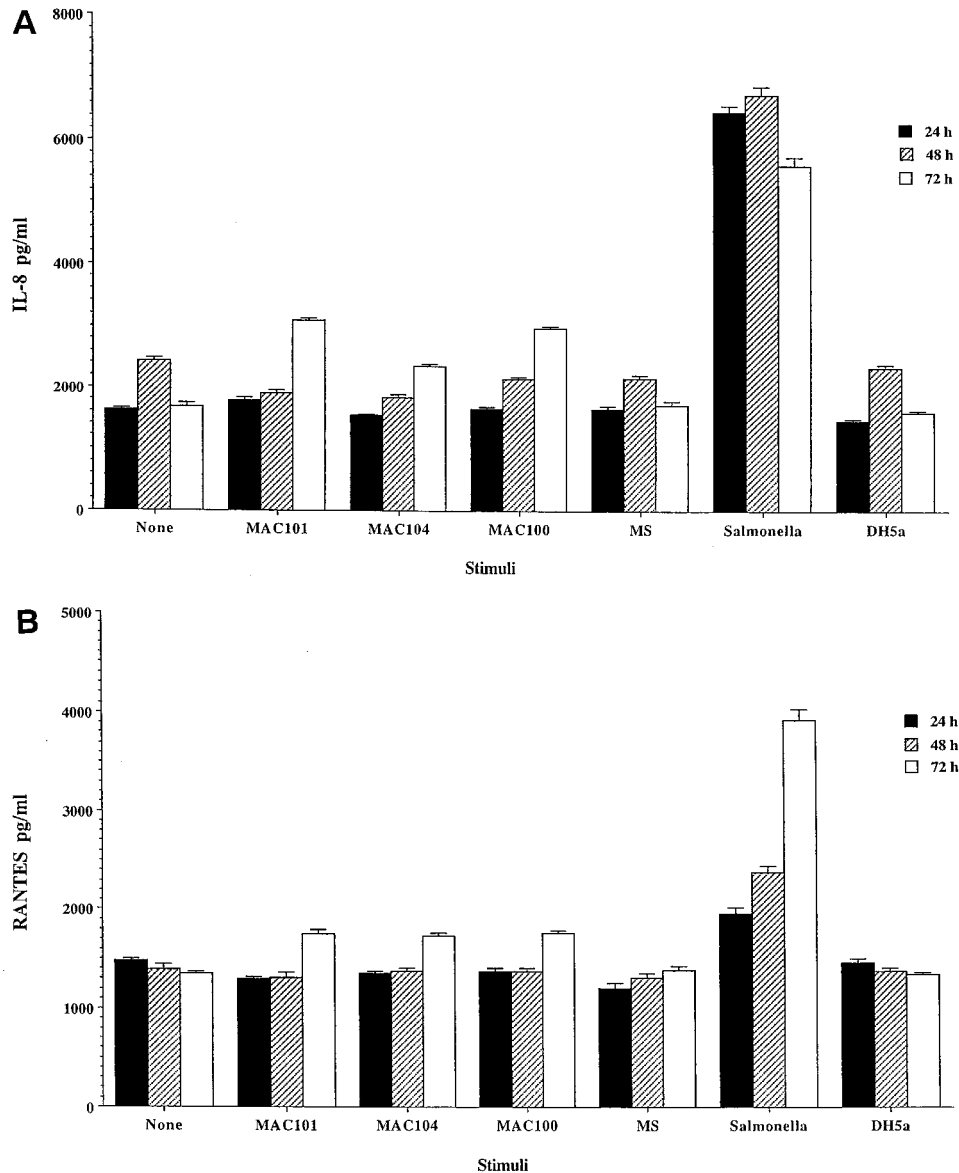


FIG. 3. Production of chemokines by HEP-2 cells infected with different *M. avium* strains, *M. smegmatis*, *Salmonella*, and *E. coli* DH5 α for 24 to 72 h. (A) IL-8. (B) RANTES. Monolayers were infected with an MOI of 10. For the 72-h time point of *M. avium*-infected cells compared with uninfected control and for *Salmonella* at the three time points, the *P* value is <0.05.

ulocyte-macrophage colony-stimulating factor, tumor necrosis factor alpha, IL-6, and IL-1 α (6, 8, 16, 20).

In this study we show that *M. avium* uptake by both HT-29 intestinal and HEP-2 laryngeal cell lines does not result in the release of chemokines for several days. We found that the ability to delay IL-8 and RANTES production was shared among the three strains of *M. avium* (representing the three most common serovars isolated from AIDS patients). It is interesting that previous studies by Yuanguang et al. (28) and Rhoades et al. (21) had shown that *Mycobacterium tuberculosis* infection of the A549 lung epithelial cell line and macrophages, respectively, trigger the release of great amounts of chemokines, including IL-8. In fact, Yuanguang et al. also showed that *M. avium* infection of A549 cells did not induce the synthesis of IL-8 and MCP-1. Although these authors argued that *M. avium*'s failure to induce chemokine production was due to

the inability to grow intracellularly, this is not the case with both HT-29 and HEP-2 cells, in which *M. avium* grows intracellularly (4). These findings suggest that the initial mechanisms of pathogenesis (i.e., contact with the epithelial layer) differ significantly between *M. avium* and *M. tuberculosis*. It is certainly plausible to hypothesize that, because of the decreased intrinsic virulence compared with *M. tuberculosis*, *M. avium* is required to prevent immune response early in the infection to survive. *M. avium* infection of the intestinal mucosa ultimately causes segmental necrosis of the intestinal villi followed by the influx of inflammatory cells (17). However, this is not observed until several weeks after infection, which suggests that *M. avium* infection of intestinal epithelial cells is a quiet process in its beginning.

While bacteria such as *Salmonella* trigger chemokine production soon after entering intestinal cells (27), it has been

TABLE 1. Bacterial uptake by HEp-2 and HT-29 cells^a

Strain	Inoculum (10 ⁶)	% Inoculum	
		HEp-2	HT-29
<i>M. avium</i> 101	2.4 ± 0.3	3.6	2.9
<i>M. avium</i> 104	4.1 ± 0.5	3.2	3.3
<i>M. avium</i> 100	2.1 ± 0.3	1.8	1.6
<i>M. smegmatis</i>	4.7 ± 0.7	0.04	0.02
<i>S. typhimurium</i>	2.7 ± 0.3	26	28
<i>E. coli</i> DH5α	3.1 ± 0.2	0.0018	0.0012

^a HEp-2 and HT-29 cells were incubated with bacteria for 1 h, and then monolayers were washed to remove extracellular bacteria. Monolayers were then lysed to determine the number of intracellular bacteria as described in Materials and Methods.

shown that organisms such as *Chlamydia* sp. also delay inflammation for several hours (20). Therefore, it may be important to some bacteria to maintain the immune system of the host “in check” while the infection is being established. Several of the cytokines and chemokines are potent chemoattractants and activators for neutrophils, monocytes, and T lymphocytes (8, 25). The fact that intestinal epithelial cells can participate in the network of cytokine-chemokine response and the observation that these cells can express human lymphocyte antigen class II molecules suggests a role for them in the mucosal defense against *M. avium* infection. Cytokines and chemokines in the intestinal mucosa provide bidirectional communication between inflammatory cells such as tissue macrophages, monocytes, T cells, and epithelial cells. Inhibition of chemokines production appears to be secondary to active interaction of the bacterium and epithelial cells. Although *M. avium* infection cannot inhibit RANTES and IL-8 production after LPS stimulation (data not shown), it significantly inhibits IL-8 production after stimulation with *Salmonella*, suggesting that the mechanisms of chemokine stimulation is probably different for LPS and *Salmonella*. This also implies that *M. avium* infection actively blocks some step the signal transduction pathway triggered by *Salmonella* and does not simply remain quiet in the host cell, thus resembling the case of *Yersinia*, which is able to

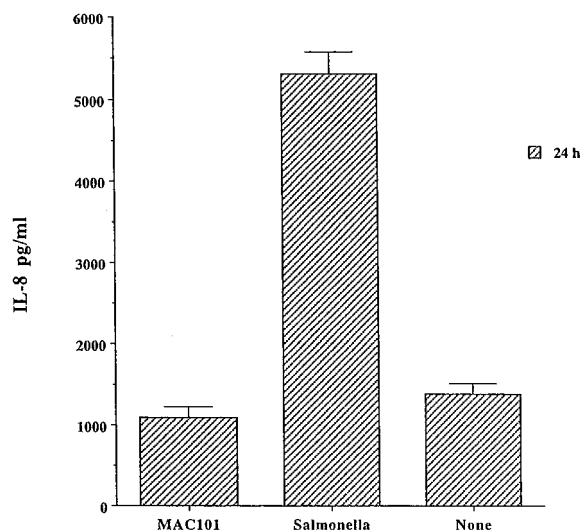


FIG. 4. Comparative production of IL-8 by HEp-2 cells after 24 h of infection with similar number of intracellular *M. avium* 101 (MAC101) and *S. typhimurium*. P values were <0.05 for the comparison between *Salmonella* and the control and <0.05 for the comparison between *M. avium* 101 and the control.

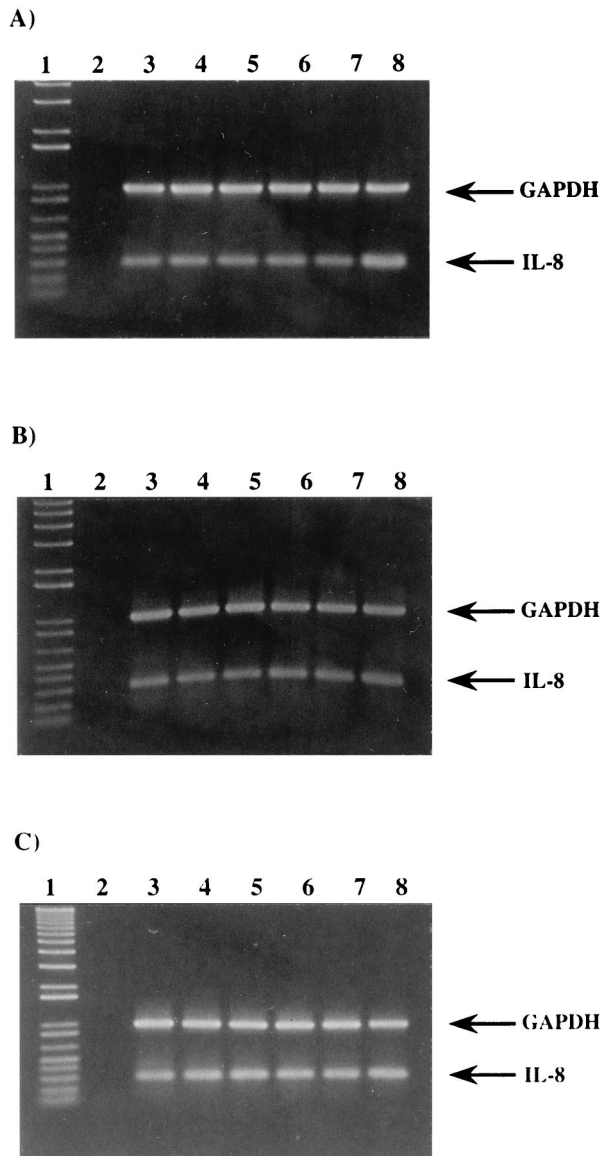


FIG. 5. IL-8 mRNA upon *M. avium* infection. (A) HT-29 cells infected with *M. avium* 101 at an MOI of 100. (B) HEp-2 cells infected with *M. avium* 101 at an MOI of 10. (C) HEp-2 cells infected with MAC101 at an MOI of 100. Lanes: 1, 1 kb plus molecular weight marker; 2, negative control; 3, uninfected control; 4, 4 h after infection; 5, 24 h after infection; 6, 48 h after infection; 7, 72 h after infection; 8, *Salmonella*-infected cells for 4 h. The amplified products are IL-8 (289 bp) and GAPDH (983 bp).

interrupt host cell pathways through the secretion of Yop proteins (19).

The findings that *M. avium* triggers different responses in HT-29 intestinal and HEp-2 laryngeal cells could be relevant for the pathogenesis of the infection and is currently being investigated. However, it also suggests either that the signal transduction pathways for chemokine synthesis differ between the cells or that the mechanism of *M. avium* invasion of HT-29 cells is probably different from the mechanism of invasion of HEp-2 cells.

It is interesting to speculate that in human immunodeficiency virus type 1 (HIV-1)-infected individuals, infection of the intestinal mucosa with HIV-1 prior to *M. avium* may be

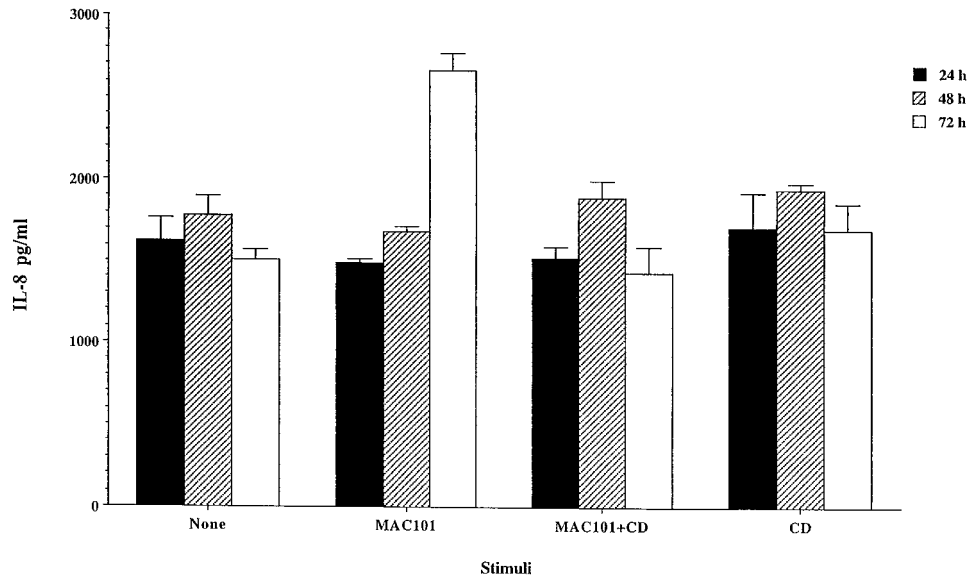


FIG. 6. Production of IL-8 by HEP-2 cell requires *M. avium* uptake. Cells were treated or not treated with cytochalasin D (CD) for 30 min (known to prevent *M. avium* uptake) and then exposed to *M. avium* 101 (MAC101) (MOI 10) after 2 h; extracellular bacteria were removed by washing. Supernatants were obtained after 24, 48, and 72 h, and the IL-8 concentration was determined. Production of IL-8 was not observed on cytochalasin D-treated monolayers.

associated with production of chemokines (18), although HIV-1 has been shown to enter the intestinal mucosa through M cells (5), whereas *M. avium* invades the intestinal mucosa primarily through enterocytes (12).

Our findings offer another perspective on *M. avium* infection of the host, with little initial inflammatory response followed, depending on the cell infected, by the release of inflammatory cytokines (17). However, it seems important for *M. avium* to be able to prevent cytokines and chemokine synthesis by infected cells, potentially creating a protective shield against the host immune response.

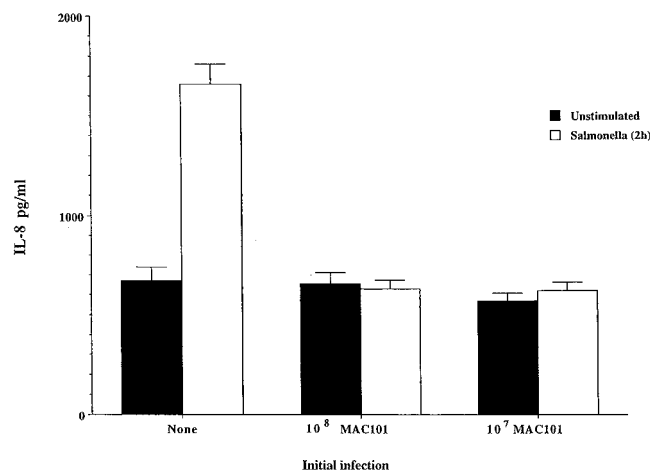


FIG. 7. IL-8 production by HT-29 cells coinfected with *M. avium* 101 (MAC101) and *S. typhimurium*. HT-29 monolayers were infected with *M. avium* (10^7 or 10^8 bacteria) for 1 h and after 18 h were coinfected with *S. typhimurium* (10^5 bacteria). Approximately 70% of the cells in the monolayers were coinfected as evidenced by light microscopy as described in Materials and Methods. *M. avium* infection suppresses IL-8 production after *Salmonella* invasion of HT-29 cells.

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