Exposure to Low Oxygen Tension and Increased Osmolarity Enhance the Ability of *Mycobacterium avium* To Enter Intestinal Epithelial (HT-29) Cells

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Received 7 April 1997/Returned for modification 30 April 1997/Accepted 11 June 1997

Current evidence indicates that *Mycobacterium avium* infection in patients with AIDS is acquired mostly through the gastrointestinal (GI) tract and that *M. avium* binds to and invades GI mucosal cells in vitro. Since *M. avium* is exposed to specific environmental conditions in the GI tract such as changes in pH, low oxygen ($O_2$) tension, increased osmolarity, and low concentration of iron, we investigated the effects of these conditions on the bacterium’s ability to enter HT-29 intestinal cells. *M. avium* 101 (serovar 1) was cultured in 7H9 broth and then exposed to pH 4.5 to 8.0, low $O_2$ tension, 0.1 to 0.3 M dextrose, and absence of iron for 2 h. After washing, bacteria (10^7/ml) were used in the invasion assay. Confluent HT-29 cells were exposed to 10^9 bacteria for 1 h and then treated with amikacin (200 µg/ml) for 2 h to selectively kill extracellular but not intracellular *M. avium*. The supernatant was then removed, the monolayer was lysed, and the lysate was plated onto 7H10 agar. The invasion of intestinal epithelial cells, low $O_2$ tension and increased osmolarity enhanced invasion 11- and 9-fold, respectively, compared with the control. Exposure of *M. avium* to the combination of low $O_2$ concentration and hyperosmolarity resulted in an approximate 10- to 15-fold increase in penetration of HT-29 cells. Hypersomolarity and low $O_2$ tension induced the invasive *M. avium* phenotype and can be useful for the identification of genes associated with *M. avium* invasion of intestinal mucosa.

*Mycobacterium avium* is primarily a bird pathogen that can be found free-living in the environment and is commonly isolated from water (12, 17, 18, 28). In contrast to non-AIDS patients, in whom *M. avium* is often associated with pulmonary disease that resembles tuberculosis, in patients with AIDS, *M. avium* colonizes the intestine and causes disseminated disease (15, 16, 22).

Following ingestion by both in mice and humans, *M. avium* is found within the intestinal mucosa and submucosa from the stomach to the colon (3, 16). However, in mice the great majority of organisms are encountered in the terminal ileum and ascending colon (3). Previous studies in vitro had established that *M. avium* strains are capable of binding and invading intestinal epithelial cells (4, 5, 26). In addition, it was demonstrated that the invasion process was dependent on both the growth phase of the bacterium and the temperature (5). At temperatures that reproduce the temperature outside the host (23 and 30°C), *M. avium* was significantly less efficient in invading intestinal cells than at 37°C (temperature inside the host), which suggests that genes associated with virulence may be regulated by temperature and possibly other environmental stimuli to facilitate host-pathogen interaction.

Similar observations have been reported for *Yersinia pseudotuberculosis* (21), *Vibrio cholerae* (31), and *Salmonella* spp. (24, 25). *Y. pseudotuberculosis*, for example, upregulates the expression of the *inv* gene (associated with invasion of epithelial cells) when exposed to acid pH (21). Likewise, the *Salmonella* invasive phenotype is induced by low concentrations of oxygen in the environment (25).

*M. avium* causes infection in AIDS patients primarily after invasion of the intestinal mucosa (34). Therefore, it was of interest to investigate whether environmental conditions encountered within the intestinal tract, such as pH, low oxygen tension, increased osmolarity, and low iron concentration, would regulate the *M. avium* gene(s) associated with entry into the intestinal mucosa.

**MATERIALS AND METHODS**

*Mycobacterium*. *M. avium* 101 (serovar 1) was used for the experiments. Previous work determined that this is an invasive strain of *M. avium* (5). It was isolated from the blood of a patient with AIDS, and its ability to cause disseminated infection in mice has been established in this as well as other laboratories. For the assays, *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 through an 18-gauge needle 10 times. The suspension was then placed in a 15-ml polystyrene tube and vortex agitated for 2 min. The tube was placed to rest for 5 min, after which the top 1 ml was removed and used as a source of bacteria. The bacterial inoculum was stained by the Ziehl-Neelsen technique and observed under a light microscope. Only an inoculum with dispersed bacteria was used in the assays. The bacterial inoculum was also plated onto 7H10 agar for quantitation. Bacterial viability in the inoculum was determined to be between 90 and 92% by using the LIVE-DEAD assay (Molecular Probes, Portland, Oreg.) as previously reported (1).

**Cells.** Colon carcinoma cell line HT-29, a well-differentiated cell line with marked characteristics of human intestinal cells, was purchased from the American Type Culture Collection (Rockville, Md.) and maintained in McCoy 5A medium without glucose (Gibco Laboratories, Detroit, Mich.) supplemented with 1% galactose, 2 mM L-glutamine, and 5% fetal bovine serum (Sigma Chemical Co., St. Louis, Mo.). For the assays, 10^7 HT-29 cells were seeded in each well and left to grow for 4 days to 80% confluence in a 24-well tissue culture plate. The cell line was used between passages 45 and 56 in all assays.

**Invasion assay.** The invasion assay was carried out as previously described (5). It was adapted for mycobacteria from a method previously described by Isberg and Falkow (20). Briefly, prior to the assay, the culture medium of the monolayers was removed and replaced with prewarmed (37°C) culture medium. Bacterial samples were added, and the monolayers were incubated at 37°C in a 5% CO_2 incubator for 1 h. Assays were terminated by replacing the overlying medium with 1 ml of tissue culture medium supplemented with 200 µg of amikacin.
Effect of environmental conditions. An attempt to reproduce the conditions encountered by mycobacteria in the intestines in vivo, we tested the effects of a number of environmental conditions, both individually and in combination, on the ability of *M. avium* to invade HT-29 intestinal cells.

Bacteria were washed, resuspended in 7H9 broth without oleic acid, albumin, dextrose, and catalase (OADC), and then exposed to different conditions for 1 to 24 h at 37°C. Usual laboratory conditions (controls) were defined at 37°C, 20% O₂, and pH 7.4. Different pHs were established by using 0.1 N HCl and 0.1 N NaOH.

The effect of iron was tested aerobically in complete 7H9 as well as 7H10 agar medium (pH 7.4) by supplementing the medium with FeCl₃ or by removing iron with 100 μM 2,2-dipyridyl (Sigma).

**Effect of increased osmolarity.** We tested the ability of *M. avium* to invade HT-29 intestinal cells under anaerobic conditions at 37°C and in the presence of 1, 5, and 20% oxygen tension.

The effect of increased osmolarity was determined by electron microscopy. We found that increased osmolarity was associated with increased ability to invade HT-29 intestinal cells. A direct relationship was observed between decreasing in oxygen tension and efficiency of invasion. Uptake of *M. avium* exposed to anaerobiosis for 2 h was approximately ninefold greater than the uptake of *M. avium* incubated in the presence of atmospheric oxygen tension (20%) (P < 0.001).

**Effect of exposure to low oxygen tension on invasion.** We found that similar studies using anaerobic bacteria showed different abilities to invade HT-29 intestinal cells. A direct relationship was observed between decreasing in oxygen tension and efficiency of invasion. Uptake of *M. avium* exposed to anaerobiosis for 2 h was approximately ninefold greater than the uptake of *M. avium* incubated in the presence of atmospheric oxygen tension (20%) (P < 0.001).

**Effect of exposure to increased osmolarity on invasion.** We found that increased osmolarity was associated with increased ability to invade HT-29 intestinal cells. A direct relationship was observed between decreasing in oxygen tension and efficiency of invasion. Uptake of *M. avium* exposed to anaerobiosis for 2 h was approximately ninefold greater than the uptake of *M. avium* incubated in the presence of atmospheric oxygen tension (20%) (P < 0.001).

**Effect of exposure to the combination of acid pH with either hyperosmolality or anaerobiosis.** We found that incubation of *M. avium* in pH 5.0 and hyperosmolality (0.3 M) did not result in an increase in bacteria uptake compared with exposure to hyperosmolality alone. Incubation under hyperosmolar conditions for 1 h prior to exposure to HT-29 cells resulted in a small but significant increase in invasion, while incubation in hyperosmolar conditions for 1 h followed by incubation at acidic pH was not associated with increased ability to invade HT-29 cells (Table 5).

Table 6 shows the results of similar studies using anaerobiosis and acidic pH as environmental cues. Our observation was that incubation of *M. avium* under anaerobiosis at an acidic pH had an effect on *M. avium* uptake by HT-29 cells similar to that of hyperosmolality and acidic pH. Acidic pH does not seem to influence the bacterial response to either anaerobiosis or hyperosmolality.

**Effect of low iron concentrations on *M. avium.*** We found that the availability of iron can be low in the intestinal tract (29). However, *M.
M. avium cultured for 2 h in the absence of iron did not differ in its ability to enter HT-29 cells compared with M. avium grown in medium with iron (using 80% confluent monolayers [data not shown]).

**RNA synthesis.** Because M. avium responded to both hyperosmolar and anaerobic conditions with increased ability to enter intestinal epithelial cells (Tables 5 and 6), we examined M. avium RNA synthesis following exposure to acidic pH, hyperosmolarity, or anaerobiosis. As shown in Fig. 3, incubation of M. avium in the presence of acidic pH, hyperosmolarity, or low O2 tension for 2 and 4 h led to a significant incorporation of [3H]uracil. The fact that exposure to acidic pH is not associated with increased invasion of HT-29 cells but resulted in rapid incorporation of [3H]uracil probably means that acidic pH triggers expression of genes not linked with entry of M. avium into epithelial cells.

**Effect of inhibition of protein synthesis on anaerobiosis- and hyperosmolarity-mediated increase in M. avium uptake by HT-29 cells.** In an attempt to determine whether the effects of conditions resembling environmental cues on M. avium uptake

![FIG. 1. Electron micrographs showing HT-29 cells after 1 h of incubation with control bacteria (A) and M. avium incubated for 2 h in the presence of hyperosmolar conditions (B) and anaerobiosis (C). Magnification, ×9,548.](http://iai.asm.org/)

![FIG. 2. Effects of different pHs on the ability of M. avium to invade HT-29 intestinal cells.](http://iai.asm.org/)

**TABLE 3. Effect of exposure to low tension of oxygen and increased osmolarity on the ability of M. avium to invade HT-29 intestinal cells**

<table>
<thead>
<tr>
<th>Environmental conditionsa</th>
<th>% of initial inoculum resistant to amikacinb (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control O2 (20% O2 tension)</td>
<td>0.53 ± 0.09</td>
</tr>
<tr>
<td>Low O2, at O2 tension of:</td>
<td></td>
</tr>
<tr>
<td>10%</td>
<td>3.7 ± 0.08c</td>
</tr>
<tr>
<td>5%</td>
<td>6.9 ± 0.6c</td>
</tr>
<tr>
<td>0%</td>
<td>9.3 ± 1.1c</td>
</tr>
<tr>
<td>Control osmolarity</td>
<td>0.52 ± 0.08</td>
</tr>
<tr>
<td>Increased osmolarity</td>
<td></td>
</tr>
<tr>
<td>0.1 M</td>
<td>3.1 ± 0.9c</td>
</tr>
<tr>
<td>0.2 M</td>
<td>6.7 ± 1.0c</td>
</tr>
<tr>
<td>0.3 M</td>
<td>8.6 ± 0.6c</td>
</tr>
</tbody>
</table>

* M. avium was cultured in Middlebrook 7H9 broth for 5 days and was exposed to the indicated conditions for 2 h.

b The invasion assay was carried out as described in Materials and Methods, and then extracellular bacteria were exposed to amikacin (200 μg/ml) for 2 h. Initial inoculum, (1 ± 0.3) × 10⁶ bacteria.

c P < 0.01 compared with control.
by intestinal epithelial cells were due to induction of a phenotypic change in the bacterium, we carried out studies in which \textit{M. avium} was incubated under acidic pH, hyperosmolarity, or anaerobiosis for 2 h in the presence or absence of amikacin at 12 \textmu g/ml (subinhibitory concentration that inhibits protein synthesis). As shown in Fig. 4, incubation in the presence of amikacin significantly suppressed the increase in invasion observed when \textit{M. avium} is exposed to hyperosmolar and anaerobiosis conditions.

**DISCUSSION**

The expression of a number of bacterial genes is regulated by environmental conditions. For example, oxygen tension regulates the expression of the \textit{ail} gene, and low pH affects the regulation of the \textit{inv} gene in \textit{Y. enterocolitica} (32, 33). Iron regulates the expression of diphtheria cytotoxin (11). Temperature affects invasion of \textit{Shigella flexneri} (27), \textit{Y. tuberculosis} (19), \textit{Bordetella pertussis} (30), and \textit{M. avium} (5) into epithelial cells. Osmolarity has been noted to be an environmental signal controlling the expression of genes associated with virulence of several organisms, among them the \textit{toxR} gene of \textit{V. cholerae} (31) and the \textit{ompR} genes of \textit{Salmonella} and \textit{Shigella} species (8, 9).

Present evidence supports the gastrointestinal tract as the most important route of \textit{M. avium} infection in AIDS patients. Organisms found in the intestinal lumen, and not the respiratory tract, have been associated with disseminated disease by using DNA fingerprinting techniques (36). In addition, biopsies of the intestinal tracts of AIDS patients have documented a large “leprosy-like” number of \textit{M. avium} organisms in the lamina propria of the mucosa (18, 22). Furthermore, studies in which healthy mice were given a number of AIDS-related strains of \textit{M. avium} have shown that the bacterium can penetrate intact intestinal mucosa and cause disseminated disease in 100% of either immunocompetent or immunocompromised mice (3).

\textit{M. avium} ingested by a host will transiently encounter a reduced pH in the stomach and acid and basic pH in the intestines. Osmolarity in the intestines is high, with a high NaCl concentration (0.31 OsM). In addition, the terminal ileum, one of the sites of preference for \textit{M. avium} invasion (3), is normally anaerobic (33). Therefore, the results of our study demonstrated that \textit{M. avium} is well adapted to the environmental conditions encountered within the intestines and that both hyperosmolarity and anaerobiosis significantly increase the efficiency of \textit{M. avium} to enter HT-29 cells in culture. In contrast, conditions such as acidic or basic pH and low iron have no impact on \textit{M. avium} invasion. It was also demonstrated that acidic pH does not change the response to either hyperosmolarity or anaerobiosis.

In previous studies, we have shown that temperature influences \textit{M. avium} interaction with epithelial cells (3). However, it was determined that temperature (37°C) by itself was re-

### TABLE 4. Effect of exposure to combination of hyperosmolarity and anaerobiosis on \textit{M. avium} invasion of HT-29 cells

<table>
<thead>
<tr>
<th>Conditions (duration of exposure)</th>
<th>% of initial inoculum amikacin resistant* (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>0.51 ± 0.07</td>
</tr>
<tr>
<td>Hyperosmolarity, 0.3 M (2 h)</td>
<td>8.7 ± 0.46</td>
</tr>
<tr>
<td>Anaerobiosis (2 h)</td>
<td>9.2 ± 0.95</td>
</tr>
<tr>
<td>Hyperosmolarity (1 h) + anaerobiosis (1 h)</td>
<td>10.04 ± 1.10</td>
</tr>
<tr>
<td>Anaerobiosis (1 h) + hyperosmolarity (1 h)</td>
<td>9.6 ± 1.26</td>
</tr>
<tr>
<td>Anaerobiosis + hyperosmolarity (2 h)</td>
<td>13.5 ± 2.1</td>
</tr>
</tbody>
</table>

* Percentage of the initial inoculum that was intracellular.

### TABLE 5. Effect of exposure to combination of acid pH and hyperosmolarity on \textit{M. avium} invasion of HT-29 cells

<table>
<thead>
<tr>
<th>Conditions (duration of exposure)</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>0.51 ± 0.07</td>
</tr>
<tr>
<td>pH 5.0 (2 h)</td>
<td>0.53 ± 0.06</td>
</tr>
<tr>
<td>Anaerobiosis (2 h)</td>
<td>8.9 ± 1.1</td>
</tr>
<tr>
<td>pH 5.0 (1 h) + anaerobiosis (1 h)</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>Anaerobiosis (1 h) + pH 5.0 (1 h)</td>
<td>0.58 ± 0.06</td>
</tr>
<tr>
<td>Anaerobiosis + pH 5.0 (2 h)</td>
<td>9.3 ± 1.0</td>
</tr>
</tbody>
</table>

* Percentage of the initial inoculum that was intracellular.

### TABLE 6. Effect of exposure to the combination of acid pH and anaerobiosis of \textit{M. avium} invasion of HT-29 cells

<table>
<thead>
<tr>
<th>Conditions (duration of exposure)</th>
<th>% of initial inoculum amikacin resistant* (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>0.51 ± 0.09</td>
</tr>
<tr>
<td>pH 5.0 (2 h)</td>
<td>0.53 ± 0.06</td>
</tr>
<tr>
<td>Anaerobiosis (2 h)</td>
<td>8.9 ± 1.1</td>
</tr>
<tr>
<td>pH 5.0 (1 h) + anaerobiosis (1 h)</td>
<td>1.9 ± 0.5</td>
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</tr>
<tr>
<td>Anaerobiosis + pH 5.0 (2 h)</td>
<td>9.3 ± 1.0</td>
</tr>
</tbody>
</table>

* Percentage of the initial inoculum that was intracellular.

FIG. 3. RNA synthesis as measured by incorporation of [3H]uracil following exposure to acidic pH, anaerobiosis, or hyperosmolarity (hyperosm), for 2 and 4 h, in comparison with bacteria maintained under standard conditions. +, statistically significant compared with control.
sponsible for less than 40% of the effects of both hyperosmolarity and anaerobiosis on \textit{M. avium} uptake (data not shown).

The regulation of the invasive phenotype by conditions such as hyperosmolarity and anaerobiosis is reversible, and the augmented ability to invade intestinal epithelial cells associated with them can be inhibited by amikacin in a subinhibitory concentration. This finding, added to the observation that RNA synthesis is induced in \textit{M. avium} exposed to hyperosmolarity and anaerobiosis, suggests that the increase in the efficiency of invasion is due to the synthesis and expression of proteins (or glycoproteins) associated with entry. Interestingly, the evidence that protein synthesis appears to occur very promptly in \textit{M. avium} supports the concept that a pathogen mechanism to explain how osmolarity regulates invasion of \textit{M. avium} into host cells. We are currently undertaking studies to identify proteins related to the invasive phenotype.

The sigma factor encoded by \textit{rpoS} is required under the conditions (27). Homologs of the \textit{rpoS} gene have been identified in \textit{S. flexneri} (33), \textit{Salmonella typhimurium} (13), \textit{Yersinia enterocolitica} (33), and more recently \textit{Mycobacterium tuberculosis} (37). We have identified the \textit{rpoS} gene homolog of the \textit{rpoS} gene (34a), and investigation is under way to determine whether expression of \textit{rpoS} is required under the conditions associated with increased binding and invasion of \textit{M. avium} to epithelial cells.

\textit{M. avium} binding to and invasion of HT-29 cells can be inhibited by DNA gyrase inhibitors both in vitro and in vivo (6). Alteration in DNA supercoiling has been suggested as the mechanism to explain how osmolarity regulates invasion of \textit{Salmonella typhimurium} through the expression of \textit{invA} (14). However, the role of supercoiling in regulating the expression of genes in \textit{M. avium} is unknown.

Finally, there is accumulating evidence that stress at the level of starvation, pH, and heat shock may control the expression of virulence genes (29). For example, the expression of listeriolysin, a protein involved in intracellular virulence of \textit{Listeria monocytogenes}, is induced by heat shock and oxidative stress (35). Although some of the proteins expressed by \textit{M. avium} upon entry into epithelial cells are heat shock proteins (2), we have no evidence to link the increase of invasion under both hyperosmolarity and anaerobiosis to the \textit{M. avium} response to stress. In addition, the failure of two stress factors, acidic pH and depletion of iron, to induce the expression of invasive phenotype supports the conclusion that the correlation (when it exists) is not always exclusive.

In summary, we have determined that hyperosmolarity and anaerobiosis are environmental signals for optimal entry of \textit{M. avium} to intestinal epithelial cells. Future studies will determine whether the ability to respond to environmental cues can distinguish between \textit{M. avium} strains that can cause disease and those that do not.

ACKNOWLEDGMENTS

We thank Karen Allen for preparing the manuscript and Jeffrey Cirillo for helpful discussions during the time the work was performed. This work was supported by grant AI25767 from the National Institutes of Health.

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Editor: S. H. E. Kaufmann