In Vitro Interaction of *Mycobacterium avium* with Intestinal Epithelial Cells

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Human intestinal epithelial cell monolayers were inoculated with cultures of *Mycobacterium avium* serotype 2, 8, or 10 that were viable, autoclaved, Formalin killed, exposed to UV light, or suspended in anti-*M. avium* serotype 2 serum. The effects of four reagents known to block phagocytosis or endocytosis (cytochalasin B, dibutyryl cyclic adenosine monophosphate, iodoacetate, and 2,4-dinitrophenol) on the bacteria-cell interaction were also studied. The maximum uptake of pathogenic *M. avium* by human intestinal epithelial cells occurred after 2 to 3 h of incubation. Serotype 2 was taken up in greater quantity than serotype 8 or 10. Saprophytic mycobacteria did not attach to or penetrate the host cells. The data showed that viable mycobacteria are ingested by host cells, whereas dead organisms are not. Components of the bacterial cells are partially, but not solely, responsible for the phagocytosis of *M. avium* serotype 2 by human intestinal epithelial cells. Furthermore, uptake of *M. avium* by human intestinal epithelial cells was suppressed by reagents which inhibit uptake by known phagocytic cells, suggesting that the mechanism of uptake is an endocytic process induced by virulent mycobacteria.

Tuberculosis in pigs is caused primarily by serotypes of *Mycobacterium avium* (37). This disease is characterized by lesions in the lymph nodes of the digestive tract (8, 9, 20, 35, 37, 39), and it seldom becomes progressive. If, however, gross lesions are found at slaughter by meat inspectors, all or part of the carcass must be cooked at 170°F for 30 min or in some cases condemned due to potential public health problems (3, 9, 35, 37). Thus, although *M. avium* infection seldom affects the health of the pig, producers can experience a substantial economic loss due to the presence of these lesions. This loss is estimated at $6 million annually to the swine industry in the United States (3, 13).

Infection of pigs occurs mainly by ingestion (27, 33, 36, 37), but little is known about the passage of the organism from the gut lumen to the lymph nodes. The adult intestine has been thought to be impermeable to macromolecular penetration. Recently, however, studies have shown that gut-associated lymphoid tissue of the small intestine, primarily of Peyer's patches, are stimulated upon entrance of antigenic material into the lumen of the small intestine (7, 39). Further studies have suggested that the epithelial cells overlying this lymphoid tissue internalize the molecules and release them into the interstitial space, probably by a process of exocytosis (5, 24–26). From there the material is processed by lymphocytes circulating through the lymphoid tissue and is carried to regional lymph nodes (24). It seems likely that properties of both bacteria and intestinal epithelial cells are involved in the infection process. The objectives of this study were to examine the interaction of mycobacteria with intestinal epithelial cells in vitro to determine whether pathogens, represented by *M. avium*, possess unique properties of invasiveness as compared with saprophytes and to determine the role of the epithelial cell in the uptake of *M. avium*.

MATERIALS AND METHODS

Bacterial strains and maintenance of culture. Cultures of *M. avium* serotype 2 were obtained from C. O. Thoen, Iowa State University, Ames. Cultures of serotypes 8 and 10 were isolated from pigs with mycobacterial lymphadenitis in Arizona; *Mycobacterium phlei* and *Mycobacterium smegmatis* were obtained from the mycobacterial culture collection at the Trudeau Institute, Saranac Lake, N.Y. Dubos broth (Difco Laboratories, Detroit, Mich.) containing 1% horse serum (GIBCO Laboratories, Grand Island, N.Y.) was inoculated with the appropriate culture and incubated at 37°C until a density equivalent to a McFarland tube no. 1 was reached. The suspensions were aseptically transferred to sterile vials to a final volume of 2.0 ml and frozen at −20°C.

When needed, vials of culture were thawed in a 37°C water bath, and 1.0 ml of the stock culture was inoculated into 8.0 ml of Dubos broth containing 1% horse serum. After 7 days of incubation at 37°C, each bacterial culture was centrifuged at 2,000 × *g* for 20 min and suspended in 40.0 ml of minimum essential medium (MEM; GIBCO).

Tissue culture methods and infection procedure. Henle 407 human intestinal epithelial (HIE) cells (ATCC strain CCL-6) were maintained in MEM containing 10% fetal calf serum (GIBCO), 200 IU of penicillin per ml, 200 μg of streptomycin per ml, and 5 μg of amphotericin B per ml. Cells were cultured routinely in 25.0-cm² tissue culture flasks (Bellco Glass, Inc., Vineland, N.J.) in an atmosphere of 5% CO_2. Confluent stock cultures were trypsinized, and six-well tissue culture plates (Bellco Glass) containing sterile cover slips were seeded and incubated in 5% CO_2 until confluency was again reached (usually about 4 days).

On the day an assay was to be performed, the tissue culture medium with antibiotics was aspirated, and monolayers were washed three times with antibiotic-free medium. Two milliliters of inoculum (ca. 0.25 mg per mg [wet weight] of bacteria) was then added to each well. After incubation for the appropriate time at 37°C, supernatant fluid was removed by aspiration, and monolayers were washed four times with 0.01 M phosphate-buffered saline (PBS; pH 6.2).

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Antisera production. Antisera against *M. avium* serotypes 2, 8, and 10 were prepared by the methods used to develop typing sera (22). Dilutions of 1:64, 1:160, and 1:320 were used for serotypes 2, 8, and 10, respectively, except where otherwise stated.

Staining techniques. The indirect fluorescent antibody test (IFAT) was performed in a manner similar to that of Kihlstrom (15), allowing differentiation between intracellular bacteria and those adherent to the cell surface. This is based upon the fact that immunoglobulin proteins do not cross the intact plasma membrane (38) but diffuse freely into methanol-fixed cells (31). After washing with PBS, infected cell monolayers on cover slips were either fixed with methanol or left unfixed. They were incubated with the appropriate dilution of homologous antiserum for 20 min at 37°C, washed with PBS, and incubated for 20 min at 37°C with a 1:32 dilution of fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin (Miles Laboratories, Inc., Elkhart, Ind.). Monolayers were then washed again with PBS, and the cover slips were mounted with 40% glycerol on glass slides. Cultures stained with auramine O-acridine orange or IFAT stains were examined by incident light fluorescence microscopy at ×1,000 with a Leitz photomicroscope with exciter filter II and a 400-nm barrier filter.

Kinyoun acid-fast stain (30) was employed in initial kinetics studies with cell cultures infected with *M. phlei* and *M. smegmatis* and those with *M. avium* treated with antiserum. The auramine O-acridine orange stain (40) was employed in all studies with inhibitors of phagocytosis or endocytosis. Data from these assays were reported as the number of associated bacteria per cell since it was impossible to differentiate between intracellular and extracellular bacteria.

Kinetics studies. Kinetics of the interaction of *M. avium* serotypes 2, 8 and 10, *M. phlei*, and *M. smegmatis* with HIE cells were studied. The bacterial inoculum was added to each monolayer at random, and plates were incubated at 37°C for 30 to 300 min. The inoculum was removed as described previously, and monolayers were stained for microscopic examination.

Studies involving altered *M. avium* serotype 2. A 7-day culture of *M. avium* serotype 2 was autoclaved (121°C, 15 lb/in², 20 min) and centrifuged at 2,000 × g for 20 min. The pellet was washed with PBS and suspended in MEM. A portion of this suspension was inoculated onto Middlebrook 7H10 agar plates (Difco) to determine whether any viable mycobacteria remained after autoclaving. Five randomly selected monolayers cultured on cover slips were inoculated with the autoclaved bacterial suspension. A sixth monolayer was inoculated with a suspension of viable *M. avium* serotype 2 and served as a control. After 3 h of incubation at 37°C, the cover slips were removed from each well and washed four times with PBS. Monolayers were either fixed in methanol or were left unfixed.

A 7-day culture of serotype 2 was centrifuged at 2,000 × g for 20 min and suspended in 5% phosphate-buffered Formalin for 15 min. This suspension was centrifuged as described above, washed once in PBS, and applied to monolayers in the same manner as the autoclaved cells.

To determine the effect of UV irradiation on *M. avium* serotype 2 in this system, 4 ml of a 7-day bacterial culture was placed into each of six plastic petri dishes to form a shallow layer. The petri dishes were exposed to a UV light source (260 nm) for 0 to 60 s at a distance of 60 cm. After irradiation, the suspension from each petri dish was cultured on Middlebrook 7H10 agar to check viability. Monolayers,

grown on cover slips, were overlaid with each bacterial suspension and incubated for 3 h at 37°C. A control monolayer was infected with a viable culture of *M. avium* serotype 2. After incubation, cover slips were removed from wells and washed four times with PBS and either fixed in methanol or processed unfixed.

A 7-day culture of *M. avium* serotype 2 was centrifuged at 2,000 × g for 20 min and suspended in MEM containing rabbit anti-*M. avium* serotype 2 antisera. Serum was filtered through a 0.20-μm cellulose-acetate filter before being diluted 1:16, 1:64, and 1:256 in MEM. Kinyoun acid-fast stain was used in these assays, so monolayers were cultured in the wells of tissue culture plates without cover slips. Monolayers were inoculated with one of the above three suspensions or with a control bacterial suspension containing no antisera. Plates were incubated for 3 h at 37°C, washed four times in PBS, and fixed with Formalin for 30 min.

Treatment of cells with cytochalasin B. Cytochalasin B (Sigma Chemical Co., St. Louis, Mo.) was prepared as a 1.0-mg/ml stock solution in dimethyl sulfoxide (Sigma) and diluted in MEM to a concentration of 0.05 to 4.0 μg/ml. Two different infection protocols were used in these assays. In procedure A, the reagent was applied and plates were incubated at 37°C for 3 h. Monolayers were then washed four times with PBS, the bacterial inoculum was added, and plates were incubated an additional 3 h at 37°C. In procedure B, equal volumes of the bacterial suspension and reagent were applied simultaneously, and the plates were incubated at 37°C for 3 h.

Alteration of cyclic nucleotide levels in cells. Cell monolayers were exposed to 1.0 to 10.0 mM dibutyryl cyclic AMP (cAMP; Sigma) in MEM. The reagent was added to HIE cell monolayers either before or concurrent with suspensions of *M. avium* serotype 2 as described above.

Treatment of cells with metabolic inhibitors. Iodoacetic acid (Sigma) and 2,4-dinitrophenol (Sigma) were dissolved in MEM at concentrations of 0.01 to 0.3 mM and 0.001 to 2.0 mM, respectively. Monolayers were preincubated with the inhibitor (procedure A) or were incubated with the reagent and bacteria simultaneously (procedure B).

Data analysis. Five wells of each tissue culture plate were used as treatment groups, and the sixth was used as a control group. Individual plates and wells were randomly assigned to treatment groups. Sixty epithelial cells per well were counted at random, and the number of bacteria associated with each cell was recorded.

The data were distributed according to a Poisson distribution, with numerous small integer and zero counts. For this reason, a $\sqrt{X + 0.5}$ transformation was used on most bacterial counts.

To determine the percentage of intracellular bacteria, the following formula was used: [(mean number of bacteria per fixed cell − mean number of bacteria per unfixed cell)/(mean number of bacteria per fixed cell)] × 100.

The cells were divided into two groups, infected and noninfected. Cells with one or more associated bacteria were considered to be infected. This was done for each concentration of each reagent, and because each assay was performed three times, an average of the three was used to figure the percentage of cells which were infected. Tests of significance were conducted by using the Newman-Keuls mean separation test with a 95% confidence level.

RESULTS

Kinetics studies. Figure 1 shows the interactions of *M. avium* serotype 2, *M. phlei*, and *M. smegmatis* with HIE cell

monolayers. Primary assays involving *M. avium* serotype 2 were conducted with the intention of establishing the amount of time required for maximum infection of cultured epithelial cells. A linear increase in the number of host cells infected was found during a 3-h period, after which the infection rate decreased. The interactions of *M. phlei* and *M. smegmatis*, saprophytic mycobacteria, were studied to compare them with the pathogen *M. avium*. Neither saprophyte displayed a significant ($P = 0.05$) ability to attach to or penetrate the host cells in this model.

The majority (60%) of *M. avium* serotype 2 associated with HIE cells after 3 h of incubation were in fact intracellular (Fig. 2). Figure 2 also includes data for serotypes 8 and 10, which are less commonly encountered as causes of porcine mycobacterial lymphadenitis (35). After a 3-h incubation, intracellular residence of serotype 8 reached a maximum of only 42.9%, 13% less than the maximum uptake of serotype 2. Maximum uptake of serotype 10 was even less and occurred after a 2-h incubation.

The interaction of *M. avium* serotype 2 with epithelial cells was examined in greater detail by studying separately the contributions of HIE cells and bacteria to the uptake process.

Studies with altered bacteria. Initial studies used bacteria killed by autoclaving or by suspension in Formalin. The interactions were assessed quantitively by the IFAT.

Figure 3 shows the results of inoculation of HIE cell monolayers with autoclaved, Formalin-killed, and viable *M. avium* serotype 2. A significant decrease ($P = 0.05$) in uptake
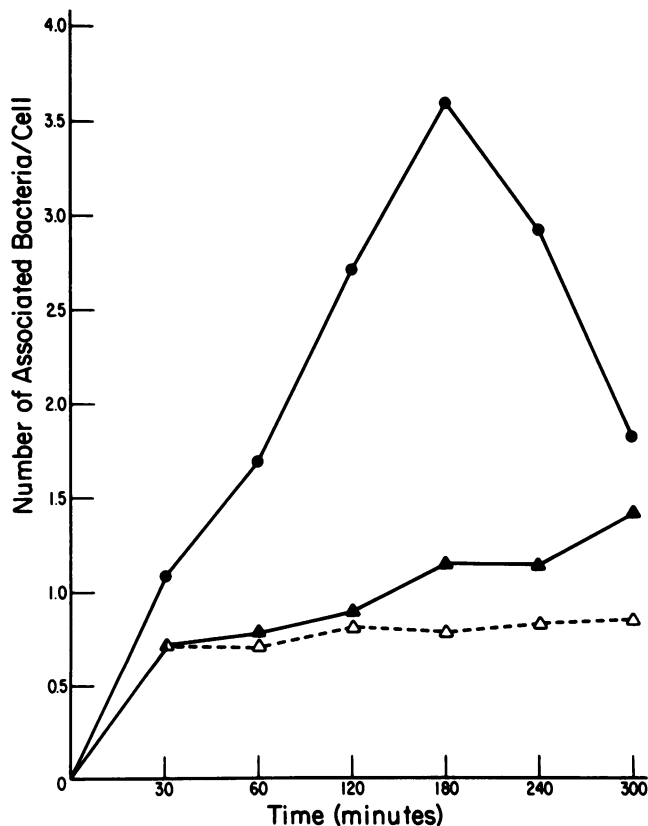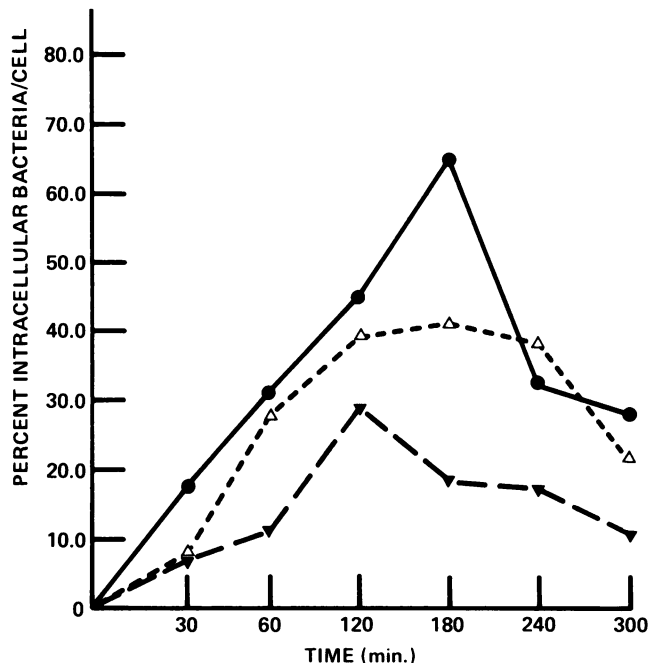
FIG. 2. Percentage of *M. avium* serotypes 2 (●), 8 (△), and 10 (▼) shown to be intracellular when associated with HIE cells.

FIG. 1. Kinetics of the interactions of *M. avium* serotype 2 (●), *M. phlei* (▲), and *M. smegmatis* (△) with HIE cells.

was evident with both autoclaved and Formalin-killed *M. avium* compared with the control. An exposure of 40 s or more to UV irradiation significantly reduced ($P = 0.05$) the infectivity of serotype 2 (Fig. 4). Results of viability tests on the *M. avium* suspensions paralleled those shown for infectivity. After 8 weeks of incubation at 37°C, Middlebrook 7H10 agar plates inoculated with suspensions exposed for 10, 20, or 30 s showed growth similar to that on plates inoculated with nonirradiated *M. avium*. Plates inoculated with bacterial cultures exposed for 40 s demonstrated a 65% reduction in growth compared with cultures containing nonirradiated bacilli. Plates inoculated with cultures exposed to more than 50 s of UV irradiation exhibited no growth.

Figure 5 shows the effect of anti-*M. avium* serotype 2 serum on the interaction of the tubercle bacilli with HIE cells. Inhibition of infection or attachment was inversely proportional to the concentration of antiserum.

Studies of inhibitors of phagocytosis. Cytochalasin B significantly inhibited uptake of serotype 2 ($P = 0.05$) at concentrations ≥1.0 µg/ml in both procedures A and B (Table 1). Higher concentrations virtually eliminated uptake.

Experiments were conducted to test the effect of dibutyryl cAMP upon the susceptibility of HIE cells to mycobacterial infection. Results indicated that dibutyryl cAMP significantly ($P = 0.05$) inhibited the infection of host cell monolayers at different concentrations dependent upon the infection procedure used (Table 1). It was found that concentrations ≥1.0 mM caused significant inhibition in procedure A. Under procedure B, however, significant inhibition did not occur at concentrations less than 4.0 mM.

Table 1 also shows results when iodoacetate and 2,4-dinitrophenol were used as inhibitors. In both cases it was found that infection was significantly reduced ($P = 0.05$) at concentrations ≥0.10 mM regardless of the infection procedure used (A or B).
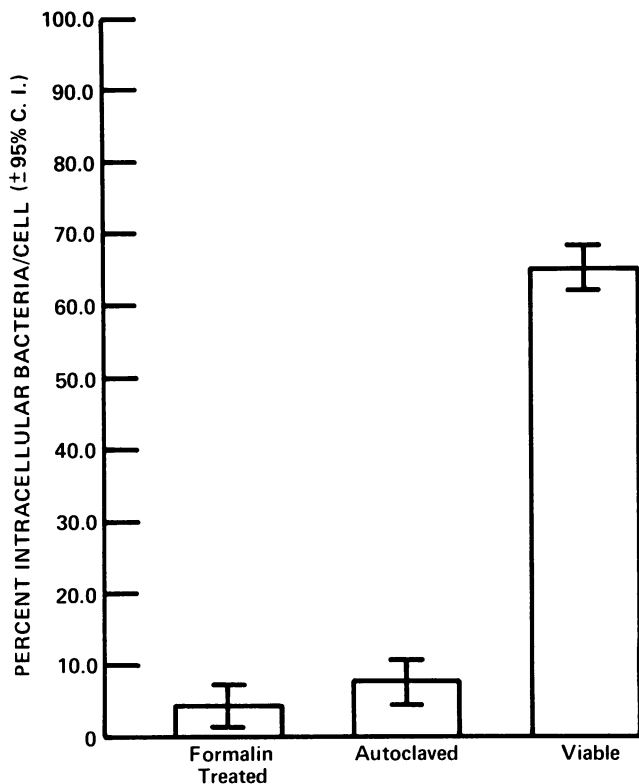
FIG. 3. Difference in uptake of Formalin-killed, autoclaved, and viable *M. avium* serotype 2 by HIE cells.

killed cells remain intact, with surface molecules altered but preserved. These data indicate that viability of the invading bacteria is a prerequisite for entrance into the host cells.

Hale and Bonventre (10) found that treatment with homologous antiserum enhanced infection of HIE cells by *Shigella flexneri*. They attributed this to agglutination of the bacteria, which promoted efficient contact between the host cell and bacteria. They point out, however, that conditions in vitro are unlike those in vivo, since agglutination of bacteria in the gut by secretory immunoglobulin A (IgA) would very likely provide protection. The role of secretory IgA in *M. avium* infection in swine is unknown. Porcine anti-*M. avium* IgA was not available, so rabbit anti-*M. avium* serum was used. Contrary to the findings of Hale and Bonventre (10), a decrease was found in infection of HIE cells in the presence of antiserum. In fact, the rate of infection was shown to be inversely proportional to the concentration of antiserum used. This may have resulted from the masking of surface molecules which are required for attachment. The role of surface molecules in this process must be evaluated further.

The data also show that partial integrity of metabolic function may play an important role in the infectivity of *M. avium*. The effects of short-term irradiation (less than 60 s) on bacteria are thought to be confined to the nucleic acids (10). Based on this, our results suggest that chemical or heat-sensitive surface antigens are not exclusively responsible for infectivity since these would remain intact after brief UV exposure.

Although the neonatal small intestine has long been known to have the ability to absorb macromolecules, until recently

DISCUSSION

The IFAT was useful in distinguishing attached from internalized bacteria. With Kinyoun acid-fast stain, this was impossible since nearly all cells were shown to have associated bacteria. With the IFAT, over 60% of the associated bacteria were shown to be intracellular. The decrease in the number of intracellular bacteria after a 3-h incubation may be due to exocytosis (17).

The kinetics of the interaction of serotypes 8 and 10 with HIE cells were studied to determine whether they differed from the supposedly more pathogenic serotype 2. Serotype 8 showed a 13% reduction in infection as compared with serotype 2, and serotype 10 exhibited a 27% reduction. The literature suggests that there may be differences in pathogenicity for pigs between serotypes of *M. avium*. These data suggest that there is a decrease in uptake of serotypes 8 and 10 as compared with serotype 2. Whether this is due to decreased pathogenicity is not known.

Saprophytic mycobacteria, although not usually associated with any disease process, are widespread in the environment and are thus available for infection of swine. *M. phlei* and *M. smegmatis* have occasionally been isolated from tuberculous swine, but if found are usually with *M. avium*. Neither saprophyte displayed a significant ability to attach to or penetrate HIE cells. If an equivalent response occurs in vivo, it may explain the rare occurrence of these, as compared with *M. avium*, in porcine lymph nodes.

The inability of killed bacteria to invade host cells was confirmed in assays utilizing autoclaved and Formalin-killed *M. avium* serotype 2. It is possible that disruption of the cell wall during autoclaving causes lysis. However, Formalin-
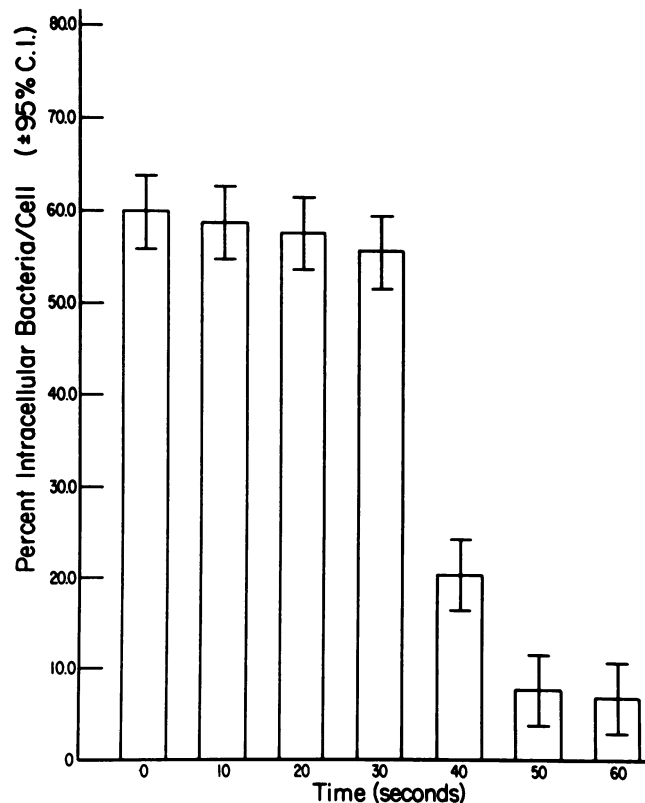

FIG. 4. Difference in uptake of *M. avium* serotype 2 irradiated with UV light (260 nm) for various times from 0 to 60 s.

it was thought that the adult mammalian gut maintained a complete barrier against such absorption (4, 6, 24–26, 41). There is increasing evidence that the normal adult intestine is permeable to macromolecules, not in sufficient quantities to be of nutritional importance, but in quantities that may be antigenic or biologically active. The process employs an endocytic mechanism (6, 41), is energy dependent, and has been shown to be inhibited by compounds which inhibit glycolysis or oxidative phosphorylation (6). Numerous studies with macromolecules such as horseradish peroxidase, polyvinylpyrrolidone, insulin, and vitamin B_{12} have confirmed this finding (6, 16, 19, 21, 28, 32, 41). Our data support this hypothesis. Internalization of *M. avium* serotype 2 by HIE cells was significantly ($P = 0.05$) reduced by compounds known to inhibit uptake of particles by phagocytic cells.

Cytochalasin B, a fungal metabolite, inhibits phagocytosis by polymorphonuclear leukocytes and macrophages (1, 2). Many investigators have reported the disruption of actin polymers of microfilaments in both phagocytic and nonphagocytic cells treated with 1 to 3 μg of cytochalasin B per ml (1, 2, 7, 12, 29, 43). In the present studies, significant inhibition of endocytosis occurred at a concentration of 1.0 μg/ml, regardless of which procedure was applied (Table 1). This suggests that infection of epithelial cells in vitro is dependent upon functional host cell microfilaments and that the mycobacteria were unaffected by cytochalasin B.
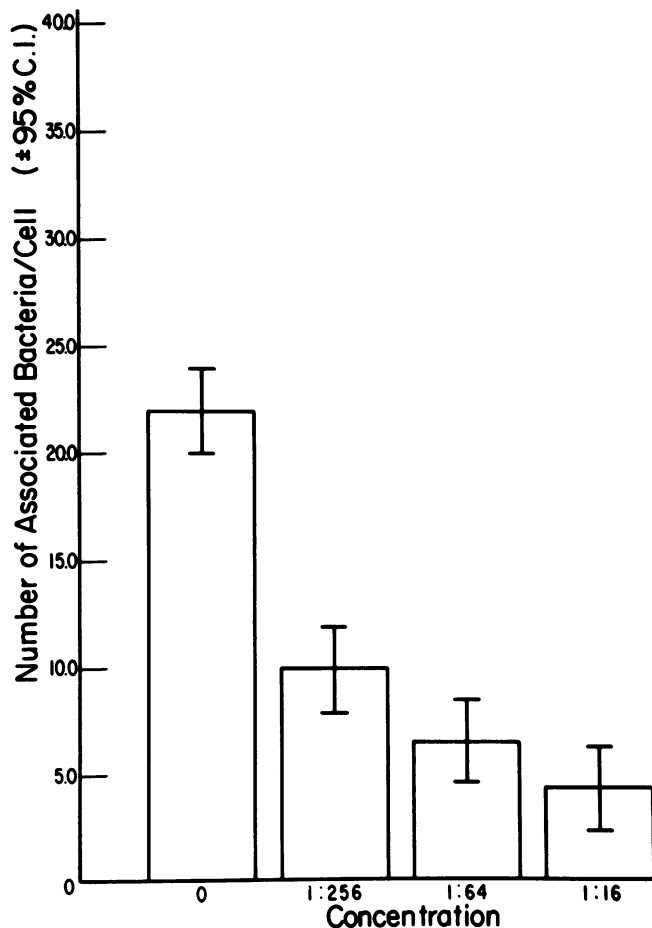
TABLE 1. Effect of cytochalasin B, dibutyryl cAMP, iodoacetate, and 2,4-dinitrophenol on interaction of *M. avium* serotype 2 with HIE cells

Inhibitor	Concn[a]	% Infected cells by procedure:[b]	
		A[c]	B[d]
Cytochalasin B	0	93.8	96.1
	0.05		84.4
	0.1	75.5	67.2
	0.5	65.5	57.7
	1.0	42.2*	36.1*
	2.0	37.2*	27.7*
	4.0	26.6*	11.1*
Dibutyryl cAMP	0	96.1	98.8
	1.0	35.5*	77.2
	2.0	23.8*	55.5
	4.0	24.4*	44.4*
	10.0	16.6*	19.4*
Iodoacetate	0	98.8	98.3
	0.01	96.1	87.7
	0.03	91.6	73.3
	0.05	59.4	66.1
	0.1	43.3*	45.0*
	0.2	32.7*	30.0*
	0.3	25.5*	14.4*
2,4-Dinitrophenol	0	98.3	98.8
	0.001	72.7	76.1
	0.01	58.3	53.8
	0.1	48.8*	49.4*
	1.0	46.1*	41.6*
	2.0	19.4*	34.4*

[a] Concentrations are millimolar except for cytochalasin B, which is in micrograms per milliliter.
[b] Determined from the mean number of cells infected per a total of 60 cells observed. This was repeated three times. Values which differed significantly ($P = 0.05$) from the control (no inhibitor) are marked with an asterisk.
[c] Bacteria applied after removal of reagent.
[d] Bacteria and reagent applied simultaneously.

FIG. 5. Interaction of *M. avium* serotype 2 suspended in various concentrations of homologous antiserum with HIE cells.

Dibutyryl cAMP inhibits particle ingestion by polymorphonuclear leukocytes and mouse peritoneal macrophages at a concentration of 2.0 mM (34, 42). Our results indicated that dibutyryl cAMP significantly inhibited the infection of HIE cell monolayers at different concentrations dependent upon the infection procedure used (Table 1). Dibutyryl cAMP is soluble in the lipids of the host cell membrane and thereby inhibits phagocytosis (11). It may, therefore, be readily absorbed by *M. avium* due to the high lipid content of its cell membrane (14) as indicated by the fact that in procedure B, in which the reagent and bacteria were added simultaneously, the tubercle bacilli may have absorbed the reagent, impairing its ability to affect the HIE cells at lower concentrations. In procedure A, however, the effective concentration of dibutyryl cAMP was the same as that concentration found to inhibit phagocytosis by polymorphonuclear leukocytes and mouse peritoneal macrophages (34, 42).

Because phagocytosis is an energy-dependent process, experiments were done to determine whether disruption of carbohydrate metabolism affected the interaction between *M. avium* serotype 2 and HIE cells. Iodoacetate inhibits phagocytosis in polymorphonuclear leukocytes (40), blood monocytes (5), and peritoneal macrophages (23); in these cells, the energy required for phagocytosis has been linked to glycolysis, a process known to be inhibited by iodoacetate. 2,4-Dinitrophenol inhibits Krebs cycle activity, and thus phagocytosis, by alveolar macrophages (23). Both compounds significantly reduced the infection rate at concentra-

tions of 0.10 mM or greater. These data support other studies involving cytochalasin B and dibutyryl cAMP, indicating that the uptake of *M. avium* into intestinal epithelial cells is at least partially dependent upon the endocytic activity of the host cell.

We conclude from these data that pathogenic mycobacteria participate in the infection process in an active manner. Bacterial surface properties are not solely responsible for invasiveness; metabolic activity on the part of the organism is also required. The exact nature of this phagocytic signal remains unknown, but it appears that it is only found in pathogenic mycobacteria, since saprophytic species failed to infect HIE cells. Studies involving the disruption of host cell energy metabolism and modulation of cyclic nucleotide levels have shown that the host cell also plays an important role in mycobacterial infection in vitro. Coupled with the fact that cytochalasin B also inhibited infection, it is suggested that infection of HIE cells in vitro is accomplished by an endocytic process very similar to the uptake of particles by phagocytic cells. An inducing stimulus originating from the mycobacteria apparently initiates membrane activity and phagocytosis.

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