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IN VITRO INTERACTION OF MYCOBACTERIUM AVIUM WITH INTESTINAL EPITHELIAL CELLS

THE UNIVERSITY OF ARIZONA M.S. 1982

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IN VITRO INTERACTION OF MYCOBACTERIUM AVIUM
WITH INTESTINAL EPITHELIAL CELLS

by

Mary Elizabeth Mapother

A Thesis Submitted to the Faculty of the
DEPARTMENT OF ANIMAL PHYSIOLOGY
In Partial Fulfillment of the Requirements
For the Degree of
MASTER OF SCIENCE
In the Graduate College
THE UNIVERSITY OF ARIZONA

1982
STATEMENT BY AUTHOR

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SIGNED: Mary E. Mapother

APPROVAL BY THESIS DIRECTOR

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12/13/82 Date
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ABSTRACT

An \textit{in vitro} model was developed to study the infection of cultured Henle intestinal epithelial (HIE) cells with pathogenic \textit{Mycobacterium avium} and saprophytic mycobacteria.

Results indicate that uptake of pathogenic \textit{M. avium} occurred after 2 to 3 hours of incubation. Saprophytic mycobacteria did not attach to or penetrate the host cells of this model. Conditions necessary for uptake of \textit{M. avium} serotype 2 by HIE cells include viability, expression of surface molecules, and normal metabolic activity of the tubercle bacilli. Furthermore, results indicate that the mechanism of uptake is an endocytic process induced by virulent mycobacteria.
Swine tuberculosis is a disease problem that has affected the pork industry of the United States since the early 1900's. The prevalence of tuberculosis in swine has been estimated at 0.85% of all swine slaughtered under federal inspection, representing an almost $6 million annual loss to the pork industry.

The incidence of tuberculosis in swine is related to the opportunity for direct or indirect contact with tuberculous primates, cattle, avian species, and numerous environmental sources. Over the years most tuberculous lesions in U.S. swine have been found to be of avian origin. U.S.D.A. statistics have shown that chickens in the north-central region of the United States have the highest percentage of condemnations due to lesions of tuberculosis. This coincides with reports that states of the west north-central and east north-central accounted for more than 83% of swine condemnations and for more than 63% of carcasses retained for tuberculosis in 1971. Nationally, the incidence of swine lymphadenitis has declined. Most of this decline is largely attributable to an increase in the practice of maintaining all-pullet farm flocks among poultry producers. Control of tuberculosis among cattle, through tuberculin testing and slaughter of reactors, is also thought to have decreased the loss among hogs. Of the 31,843,375 cattle slaughtered under federal
inspection in 1972, only 246 carcasses (0.0008%) had tuberculous lesions. In contrast, of 83,126,396 swine slaughtered during the same year, 712,227 carcasses (0.85%) had lesions suggestive of tuberculosis, nearly 1000 times more than were found in cattle. The above figures indicate that tuberculosis may no longer be considered such a serious problem in swine in the U.S., but the decrease of the disease has not been as pronounced as for bovine and avian tuberculosis.

In 1972, the Food Safety and Inspection Service of the U.S.D.A. instituted new rules and regulations concerning tuberculous lesions in swine. Before 1972 when these granulomatous lesions were found at slaughter, they were excised and discarded, and the remainder of the carcass was processed as usual. Following new regulations, swine carcasses, and/or parts, are labeled as condemned, passed for cooking (PFC), or passed without restriction for human food. The entire carcass is condemned when the character of the lesions is not indicative of a localized condition. Portions of carcasses and whole carcasses are labeled PFC if they reveal lesions indicative of a localized condition. Such carcasses or parts must be cooked at 76.7°C for 30 minutes after removal of the infected part to be considered fit for human consumption. Portions of carcasses and carcasses of swine are passed without restriction provided the carcass is found free of lesions or when lesions are found confined to one primary site of infection. The economic devastation due to condemned carcasses is obvious. Cooked carcasses lose about 2/3 of
their original value. This loss is felt by the packing industry and the consumer, but mostly by the pork producer, since he not only obtains a lower price for his hogs, but may lose his market altogether.

**Etiology**

Historically, the etiologic agents of tuberculosis in swine have been the same agents which have caused tuberculosis in the animals with which swine have had contact. Before the start of attempts to eradicate both human and bovine tuberculosis, isolations of tubercle bacilli from lesions in swine were often shown to be of either bovine (Mycobacterium bovis) or human (Mycobacterium tuberculosis) type. However, Mycobacterium avium causes most mycobacterial disease in swine in the U.S. today. Additional evidence implies that M. avium serotypes 1 and 2, which also cause disease in chickens, are the organisms most frequently associated with tuberculous lesions in swine, although many mycobacteria in the M. avium complex are also involved. Other species of mycobacteria less frequently isolated in connection with swine tuberculosis include M. bovis, M. tuberculosis, M. scrofulaceum, M. xenopi, M. kansasii, M. microti, M. aquae, and M. chelonei. Corynebacterium equi has also been isolated from lymph nodes of swine with tuberculosis-like lesions. The organism produces localized lesions and cannot be easily differentiated from tuberculous processes macroscopically or histologically.
It has been reported that *Corynebacterium equi* is often found in association with tubercle bacilli in swine.\(^1\), \(^{14}\), \(^{21}\) Other workers have reported that it occurs with equal frequency in normal and *M. avium*-infected lymph nodes.\(^{20}\) Although the relationship between *C. equi* and *M. avium* and the possible role of *C. equi* in producing tuberculosis-like lesions in swine needs further investigation, its effect on occurrence of the disease is probably minimal.

**Taxonomy**

Koch's report of the tubercle bacillus in 1882 was followed in 1889 by Rivolta's description of the avian type bacillus, and in 1898 by Theobold Smith's differentiation of the bovine type bacillus.\(^{20}\) However, in the 100 years since, the taxonomy of mycobacteria has become more awkward and complex. This is especially true for organisms now included in the *M. avium* complex.

Schaefer is the person considered instrumental in taxonomy of what is now the *M. avium* complex. In 1935, he began his study of the serological properties of the "atypical" mycobacteria.\(^{117}\) He found that all avian strains were of 2 serotypes, characterized by their reactions with type-specific antisera. He also found that other "atypical" mycobacteria (Battey type) could also be typed serologically.\(^{117}\) *M. intracellulare* and *M. scrofulaceum* were named as species separate from *M. avium*, although biochemical differences among these 3 are so minimal that they cannot be reliably differentiated by this means. Almost 20 serotypes have been established among cultures identified as *M. intracellulare*, and several among
those identified as *M. scrofulaceum*.

In spite of objections by Runyon, who believed *M. intracellulare* should remain a distinct species, these organisms have now been grouped into the *M. avium* complex. The problem has been resolved somewhat by the new scheme proposed by Wolinsky and Schaefer in 1973. Individual species names are dropped and the 3 are grouped together in the "Mycobacterium avium" complex." Twenty numbers are reserved in the scheme after the former *M. intracellulare* group for possible new types related to this group or to *M. avium*, and it is believed this new scheme will be a great aid in the study of these organisms.

**Epidemiology**

Because most swine tuberculosis in the United States is of the avian type, and appears to be most prevalent in those geographic regions where tuberculosis in chickens is greatest, it might be assumed that infected chickens are the source of this disease. However, it should be kept in mind that this data comes from meat inspection records which may be misleading. Swine are often transported to abattoirs at great distance from their origin and, in addition, diagnosis is made on the basis of the macroscopic appearance of lesions. Some lesions may not be macroscopically visible and, therefore, some infections are missed. Also, there are reports of tuberculosis in hogs in areas where there is no association with chickens. Although tuberculous chickens may be the primary source of the disease in swine, it seems very probable that other aspects of the swine
environment also have a role in the origin and transmission of the
disease.

The opportunity for transmission of tuberculosis among
tuberculous poultry, cattle, and swine has been documented by many
workers. Bovine tubercle bacilli are not a
cause of tuberculosis in swine in areas where the disease in
cattle is controlled. Where tuberculosis does occur in cattle,
the infection may be transmitted to swine by feeding of unpasteurized
milk and dairy by-products. Also, feces of tuberculous cattle may
contain viable tubercle bacilli, which makes the maintenance of
swine and cattle in common feedlots impractical.

Tuberculosis in chickens is found in the pectoral muscles, eggs,
and reproductive organs. The feeding of offal of tuberculous
chickens, and the raising of swine in areas previously occupied by
tuberculous chickens have been shown to be of great significance in
the transmission of avian type tuberculosis. Schalk and co-
workers found viable and pathogenic avian tubercle bacilli in the
soil and litter of a chicken coop after 4 years with no chickens.
They concluded that soil contaminated by feces of tuberculosis fowl is
one of the most important sources of infection for swine, and recom-
mended that young birds be reared on clean ground and that all fowl
more than 1 year old be disposed of regularly.

The transmission of tuberculosis from pig-to-pig has been
attributed to ingestion of contaminated feces, intrauterine infec-
tion, and aerosol infection from pigs showing pulmonary lesions.
However, work being done in our laboratory indicates that fecal shedding of the organism does not occur, and that transmission from pig-to-pig may be rare.

Human tubercle bacilli have been isolated from lesions in swine. People known to have active TB should not be permitted to have contact with domestic animals.

Soil, water, and house-dust have also been found to be possible sources of mycobacterial infection. Sawdust is often used as bedding for swine and has recently been shown to be a source of mycobacterial infection for swine. In a study by Beerwerth and Popp, M. avium complex organisms were isolated from more than 3% of sawdust and wood shavings samples examined. Reznikov and Leggo found sawdust to be an especially favorable medium for the multiplication of these organisms due to the relatively high temperatures that develop in the sawdust piles. Because swine often ingest sawdust, this reservoir could be an important source of infection and could account for many of the cases of M. avium complex disease in swine.

Wildlife is another possible source of the infection for pigs. Many wild birds have been shown to be infected. As early as 1940, Hignett and McKenzie reported the occurrence of avian tuberculosis in starlings to be 1.3 - 4.8%. They suggested the possibility of the starling acting as the vehicle of infection in the spread of avian tuberculosis. Tubercle bacilli could have been introduced into the swine herd by these birds. Other wild birds found infected with
members of the *M. avium* complex include a trumpeter swan, a sandhill crane, and a white-headed tree duck. This emphasizes the importance of adequate quarantine and test procedures to detect diseased birds before importation.

There has been an increased interest in tuberculosis in captive exotic species following recent reports of the disease in animals in zoos, primate centers, animal colonies at universities, and animal and game parks. The importance of these occurrences of tuberculosis is emphasized due to the public health hazard, the economic losses, and the difficulty of replacing rare and endangered exotic animals. Thoen et al. summarize results of mycobacteriologic examinations conducted on 474 specimens submitted from exotic animals suspected of having tuberculosis. The widespread occurrence of tuberculosis in exotic animals maintained in captivity emphasizes the importance of these infections.

The exact source of tuberculosis for swine is not always known. The organisms may be present in many areas of the swine environment or originate from only 1 source common to all animals. Many workers agree that the key lies in more accurate and reliable procedures for detection of *M. avium*.

**Diagnosis**

Clinical signs are of limited value in the diagnosis of *M. avium* infection in swine. Animals with progressive disease very seldom show symptoms of an infectious disease. If symptoms are present, they are not sufficiently characteristic to establish a diagnosis of
tuberculosis. In cases of rapidly disseminating disease, a circum-
stance which is not characteristic of *M. avium* infection, there may
be clinical evidence of infection including an elevation in body
temperature, anorexia, and weight loss. 17, 109

Tuberculous lesions in swine are usually limited to the lymph
nodes of the pharyngeal and cervical regions and of the mesentery. 19,
108 However, the lesions may be found to involve such organs as
liver, lung, spleen, and kidneys in widely disseminated or generalized
cases. 14, 17, 19, 109

As stated previously, tuberculosis in swine may be caused by *M.
bovis*, *M. tuberculosis*, or *M. avium*. Differentiating between porcine
lymphadenitis caused by *M. avium* and that caused by *M. tuberculosis* or
*M. bovis* on the basis of the gross lesions they produce is difficult.
Thoen and Karlson described some gross features characteristic of
each. 109 In avian tuberculosis, the lymph nodes are enlarged and firm
with no discrete purulent foci; there may be 1 or more soft caseous
areas with indistinct borders. Calcification is seldom seen. The cut
surface of a lesion has a neoplastic appearance with a few caseous
foci. There may be diffuse fibrosis but no encapsulation; the granu-
lomas are not easily enucleated. Mammalian lesions, on the other hand,
are well encapsulated and easy to separate from surrounding tissue,
calcification is prominent, and individual foci are discrete and
caseous.

Microscopically, avian tubercle lesions demonstrate diffuse
proliferation of epithelioid cells and giant cells. There may be some
necrosis but calcification is seldom seen and only in older lesions. There is a proliferation of connective tissue elements, but there is little tendency to form a well-defined wall of fibrosis. Mammalian lesions become encapsulated by a well-defined zone of connective tissue. Early caseation and marked calcification are also prominent.6, 106, 109

It is generally agreed that it is impossible to identify the causative agent on the basis of gross or microscopic appearance. This is probably due to the fact that there is a great similarity between localized tuberculous lesions and those associated with C. equi and other bacteria. Furthermore, it may be difficult to differentiate among granulomatous lesions due to M. avium infection, parasitic nodules, and neoplasms.109 A definitive diagnosis can only be made on the basis of bacteriologic procedures designed for isolation, identification and typing of mycobacteria. However, there is 1 drawback. It is well known that tubercles can resolve into nodules of fibrous tissue failing to yield tubercle bacilli.83

Cultures should be incubated at 41°C in an atmosphere of 5-10% CO₂; room temperature and 37°C without CO₂ are also adequate. Cultures should be examined daily for the first week and then weekly for at least 8 weeks. Usually growth occurs in 14-21 days on solid media, but it may take up to 5 weeks. Colonies are round, smooth and glistening with a light buff-yellow color. On Middlebrook 7H10 colonies are flat, white and translucent. Morphologically, M. avium is a short, acid-fast rod in lesions, but in cultures it may develop as acid-fast branching filaments.20, 41, 53
Routine biochemical tests on *Mycobacterium* spp. include niacin production, hydrolysis of Tween 80, nitrate reduction, and tellurite reduction. *M. avium* is negative for all the above.²⁰

In the outer layer of their cell walls mycobacteria contain a substance known as Wax D that is responsible for the relative difficulty of staining of these organisms by conventional methods.⁴¹ This problem is overcome by using the classical acid-fast stain. With this stain, mycobacteria stain cranberry red.

Two fluorescence techniques are currently employed in mycobacterial staining: auramine O-acridine orange (AOAO) and the indirect fluorescent antibody technique (IFAT).²⁹, ³⁹, ⁷² The former is more simple and requires fewer and less costly reagents, but the latter may aid in speciating an isolate.

A special technique has been developed for detection of mycobacteria by electron microscopy.³²

The tuberculin test, used on a herd basis, seems to be a useful tool in the diagnosis of tuberculosis in swine. Tuberculins are mixtures of soluble components of mycobacteria grown in liquid media. Robert Koch, in an effort to find a treatment for tuberculosis developed old tuberculin, a concentrate of the broth culture filtrate. It produced a marked reaction when injected intradermally into people with tuberculosis. Old tuberculin was later shown to be unsuitable for treatment, but its usefulness as a diagnostic tool was quickly realized. Currently, a purified protein derivative (PPD) of mycobacteria is used. These reagents are fractions of the culture filtrate prepared by various methods of precipitation.³ PPD reagents
have been prepared from *M. tuberculosis*, *M. avium*, *M. fortuitum*, *M. scrofulaceum*, *M. marinum*, and *M. kansasii*. PPD is now produced from mycobacteria grown in synthetic liquid medium rather than in broth. This procedure yields a very pure product, free from nonmycobacterial protein.

The test is usually performed by injecting 0.1 cc of tuberculin intradermally into either the ear or vulva, and results are read 48 and 72 hours after administration. The size, shape, and consistency of the tissue response do not reflect the degree of infection. Unfortunately, reports indicate that results from tuberculin skin tests in pigs may be unreliable. As early as 1952, Luke reported that there was a large percentage of error due to non-specific sensitivity or to residual sensitivity from healed lesions. Animals with negative skin test reactions had lesions at slaughter which Luke ascribed to the ability of the pig to overcome and apparently sterilize existing lesions. There still exists some controversy over the reliability and reproducibility of the tuberculin skin test.

A peripheral blood lymphocyte stimulation test has been studied and compared with the tuberculin skin test. Results indicate that *in vitro* lymphocyte immunostimulation may be of considerable value in detecting tuberculosis in animals in which skin testing has been used previously. More work needs to be done on this test, especially a comparison between the 2 tests using naturally infected pigs.

Lastly, the enzyme-linked immunosorbent assay (ELISA) has recently been described for detecting antibodies in swine infected with
M. avium. Positive ELISA reactions were seen in experimentally and naturally infected pigs. It is a rapid, automated test, which may be of value on a herd basis.

Pathogenesis

The mechanism by which mycobacteria produce disease in man and animals is relatively unknown. It is not known which properties of the bacteria are responsible for virulence, and the fact that the pathogenicity varies within different species of animals makes this matter even more complex.

Different hosts seem to be susceptible to different types of mycobacteria. Rabbits, cattle, and cats are susceptible to M. bovis, but M. tuberculosis causes little problem in these animals. Man and guinea pig are very susceptible to M. tuberculosis, however, the rat is resistant to this species.18, 40 In man, differences in age have been reported to affect the incidence of M. tuberculosis infection. The disease is found to be more frequent in the very young and in the elderly, perhaps due to the occasional inadequate immune status of these age groups.139 Pregnant women seem to be more susceptible to tuberculosis for the same reason.

Other studies have shown that the body temperature of the host may also play a role in susceptibility. Chickens, whose body temperature is 42°C, are susceptible to infection with M. avium which grows well in vitro at 43°C. On the other hand, chickens are resistant to M. bovis and M. tuberculosis which require growth temperatures of 37°C.

It has been known for some time that immunity to infection by
facultative intracellular parasites, such as mycobacteria, is not mediated by an antibody. This fact is supported by the fact that in either the natural or experimental disease, there is no relationship between the amount of antibody formed and immunity to tuberculosis. Experimentally, workers have been unable to transfer immunity from immunized animals to nonimmunized animals by injecting serum, but have now shown that immunity can be transferred by using lymphoid cells taken from infected animals. About 2-4 weeks following infection with tubercle bacilli, the host's cell-mediated immune response attempts to destroy the organism. The macrophage seems to be the most important cell type involved in this destruction process. The bacillus attaches to the macrophage, and is engulfed. This results in the formation of a phagosome. These phagosomes then fuse with lysosomes containing hydrolytic enzymes. This fusion results in the formation of phagolysosomes in which hydrolytic enzymes destroy the microorganism. However, mycobacteria are resistant to intracellular destruction. In fact, the tubercle bacilli multiplies in and will usually kill the macrophage. It is not certain how mycobacteria accomplish this feat, but different theories exist. The tubercle bacilli may use the enzymes present in the phagolysosome, or it may block fusion of the phagosome and lysosome. It is generally agreed that the resistance of mycobacteria to lysozyme is due to the high lipid content of the cell wall.

One of the outstanding features of the host-tubercle relationship is the formation of granulomas. This granulomatous inflammation first becomes evident in 2-4 weeks, after cellular immunity develops.
Macrophages accumulate at the site of infection, and enlarge following ingestion of bacilli. Macrophages are modified, lose their phagocytic ability, and are termed "epithelioid" cells because of their resemblance to epithelial cells. Epithelioid-cell formation is accompanied by a tendency for the cells to be arranged in groups rather than in diffuse sheets. Frequently, when macrophages encounter insoluble material or organisms causing chronic inflammation, they coalesce to form giant cells. Therefore, in a tuberculoid reaction, in addition to epithelioid cells, a considerable number of giant cells are also present. Surrounding the mass of epithelioid cells, there is a diffuse zone of lymphocytes with a few plasma cells and fibrocytes. Within 2 weeks, caseation necrosis takes place, and the tissue forms a dry, firm, coagulated mass. This is thought to be caused by the development of hypersensitivity to the bacilli. The end result is a fully formed tubercle follicle, consisting of a central mass of caseation surrounded by epithelioid cells and giant cells. This, in turn, is surrounded by a zone of round cells. With swine tuberculosis, these tubercles can be found most commonly in cervical or mesenteric lymph nodes.

As previously stated, the properties which cause mycobacteria to be pathogenic are poorly understood. A factor noted early which may relate to pathogenesis of tubercle bacilli is the formation of cords by the organisms in liquid medium. It is thought that the cell wall and its lipid components, in particular a lipid identified as trehalose-6, 6'-dimycolate, are responsible for this phenomenon.
Cord factor can be extracted, and is known to inhibit the migration of lymphocytes.\textsuperscript{7} Experimentally, it was noted that some animals, inoculated with extracted bacilli, developed disease more slowly than those inoculated with unextracted bacilli. Further studies showed that the level of succinic dehydrogenase in the animal decreased with an increase in the severity of tuberculosis.\textsuperscript{7} Cord factor apparently inhibits host production and/or secretion of succinic dehydrogenase and, therefore, the host is less able to prevent, or more likely control, advancement of the infection.\textsuperscript{45}

Cord factor has also been shown to cause swelling and lysis of mouse liver mitochondria.\textsuperscript{47} The effect of cord factor on other organelles has also been studied. It has been found to induce separation of the ribosomes from the rough endoplasmic reticulum of liver cells in mice.\textsuperscript{24} The nature of these actions is not well understood.

Other compounds isolated from virulent mycobacteria are sulfolipids.\textsuperscript{27} In fact, many species are known to have common sulfolipids. Sulfatides have been shown to prevent fusion of phagosomes and lysosomes.\textsuperscript{47} This would allow the bacilli another means, aside from cord factor, of escaping and/or prolonging digestion by lysosomal enzymes. These sulfolipids, like cord factor, are known to affect mitochondria. In fact, it appears the 2 compounds may act synergistically, as sulfolipid was found to drastically increase the toxicity of cord factor.\textsuperscript{46}

During phagocytosis, there is an increased production of superoxide by the host tissue, another attempt at stopping infection.
An iron-containing superoxide dismutase has been isolated from *M. tuberculosis*, and has been found to protect the organism from the toxic effect of superoxide.\textsuperscript{52}

With *M. avium*, colony morphology is correlated with virulence.\textsuperscript{113} Those colonies which are flat, transparent, and colorless are usually more virulent than those that are dome-shaped, smooth, and opaque. These "virulent" colonies can also be identified by their growth requirement for palmitic acid; nonpathogenic *M. avium* has no such requirement.\textsuperscript{64}

*M. avium* infection in swine is primarily a disease of the lymph nodes associated with the gastrointestinal tract, more specifically the small intestine. The following is a brief discussion of the anatomy and physiology of the small intestine with an emphasis on those structures involved in the uptake and transport of macromolecules from the gut.

The small intestine has 4 main functions: secretion, motility, digestion, and absorption.\textsuperscript{87, 94} Here, we are mostly concerned with absorption or the mechanisms by which molecules transverse the intestinal wall. Through an understanding of these mechanisms involved in absorption, a better understanding of the potential mechanisms by which *M. avium* may travel from the intestinal lumen to the regional lymph nodes may be obtained.

The wall of the small intestine is composed of 4 layers: the mucosa, submucosa, muscularis externa, and serosa. The mucosa is composed of an epithelial cell layer with its filamentous basement
membrane. The basement membrane is a lamina propria containing blood vessels, lymphatics, smooth muscle cells, nerve fibers, plasma cells, lymphocytes, fibroblasts, eosinophils, macrophages, reticular cells, mast cells, collagen, and reticular fibrils and is separated from the submucosa by the muscularis mucosa. The submucosa contains larger blood vessels, lymphatics, more connective tissue, nerves and ganglia, and more lymphoid elements. The muscularis externa is divided into an inner circular layer and an outer longitudinal layer of smooth muscle, with the myenteric plexus between the two.87, 94

The small intestine is characterized by 3 structures which increase its surface area and aid in absorption. First, there are circumferential folds of mucosa and submucosa. They are responsible for the "feathery" appearance of the small intestine. Second, the villi are projections of mucosa which are usually fingerlike. The individual cells of the villi possess filamentous microvilli, which in turn are coated with a more fine filamentous structure. Lastly, the mucosal crypts lie between the villi and extend to the muscularis mucosa. The surfaces of the villi and the linings of the crypts are covered by a layer of columnar cells.87, 94

The microvillus plasma membrane is covered with an adherent, filamentous structure called the glycocalyx. It is composed of sulfated, weakly acidic mucopolysaccharide which appears to be continuously synthesized by the Golgi apparatus of the epithelial cell, especially at the villous tips. The plasma membrane and its associated glycocalyx constitute a digestive-absorptive unit.87, 94
The process of absorption is initiated in the lumen of the small intestine and is completed by the specialized absorptive cells, the plasma membrane of which maintains composition and electrical charge between the lumen and inside the cell by regulating the rate at which molecules enter the cell. The difference between the function of this membrane at the cell's apical and basal surfaces results in a net movement of molecules through the cell or absorption. Because a main function of the small intestine is the absorption of lipids, and *M. avium* has a high lipid content, a special interest was taken in the mechanism by which lipids are absorbed. Fats are continuously broken down and emulsified into very small complexes called chylomicrons. These enter the lacteals by reverse pinocytosis, and electron microscopy has demonstrated that pinocytic vesicles appear to occupy approximately 15% of the endothelial cell cytoplasm.

The GI tract of mammals is complete with immunologically competent tissue. It is capable not only of experiencing but also of mounting an immune response to antigenic stimulation. Two of the most important components of this system are the Peyer's patches and specialized cells, the microfold or M cells.

The Peyer's patches lie in the lamina propria and submucosa throughout the small intestine, usually on the antimesenteric wall. They occur more frequently in more distal areas of the intestinal tract, but some are always found in the duodenum. Light microscopy has shown that regardless of how many follicles a patch contains, each
patch is only one follicle thick, thus preserving the close relationship between the follicle and the overlying epithelium. This tissue is also commonly called gut-associated lymphoid tissue or GALT.

The surfaces of Peyer's patches are characterized by specialized epithelium which differs from that of adjacent areas and possesses features which enhance trapping and uptake of antigenic and particulate material.

M cells appear responsible for antigen transport within the epithelium. These variably surfaced cells with their scattered, short protuberances are interspersed among the ordinary epithelial cells. They form cytoplasmic bridges between ordinary epithelial cells, and separate the lumen from underlying nests of lymphoid cells. The reticulum formed by M cells allows cells from the lymphoid series (above) to approach the gut lumen very closely without being lost in the lumen. At the same time, the M cells maintain tight junctions with adjacent epithelial cells so that the integrity of the gut epithelium is preserved. M cells transport antigens to the lymphocytes through a tubulovesicular system, represented by pits in the luminal cell surface. The underlying lymphoid cells then migrate into mucosal nodules, or are carried away by afferent lymphatics to mesenteric lymph nodes. In a study by Owens and Jones, they concluded that 1) the lymphoid cells contained within M cell pockets could be responding there to antigenic material transported from the intestinal lumen via the M cells, or 2) M cells may
couple IgA to secretory components and transport the immunoglobulin complex into the lumen.  

All of these lymphoid "structures" maintain a lymphoepithelial relationship which is similar to that of the thymus and the avian bursa of Fabricius. The former is responsible for T cell immunity and the latter, in avian species at least, regulates the development of B cell immunity. Most immunoglobulin-containing cells in the small intestine are mature plasma cells, although some immunoglobulins are found within immature plasma cells and lymphocytes. It is well established that IgA is the predominant immunoglobulin in the lymphoid cells and secretions of the small intestine. IgM, IgE, and IgD are also present.  

Secretory IgA has been demonstrated to possess antiviral and antibacterial activity as well as the properties of isohemagglutinins.  

Aside from these immunospecific mechanisms, any bacteria colonizing the intestinal tract would have to contend with the non-specific resistance mechanisms functioning in the tract. This would require bypassing the low pH of the stomach, and the peristalsis and villous action in the small intestine. They would have to resist the toxicity of conjugated or unconjugated bile acids. However, those organisms sensitive to bile acids may avoid this by taking up residence in the upper small intestine, where the concentration of bile is low.  

Mucin is another barrier against microbial invasion in the small intestine. Mucin traps particles, such as bacteria, and then
clears them away through the villous action and peristalsis in the stomach and intestines. However, certain bacteria are known to overcome this. In fact, they may colonize mucins and use them as sources of carbon and energy.  

Indigenous microorganisms also may block, or limit, colonization and growth of pathogenic bacteria in the GI tract. However, this normal flora changes under conditions such as antibiotic treatment, stress, diet, etc. Pathogens may take advantage of these circumstances, and infect only "altered" hosts.  

Although the neonatal small intestine has long been known to have the ability to absorb macromolecules, until recently it was thought that the adult mammalian gut maintained a complete barrier against such absorption.  

Today, 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. Initially, there is an interaction between these large molecules within the intestinal lumen and components of the microvillus membrane of intestinal absorptive cells. When a sufficient quantity of molecules comes in contact with the cell membrane, invagination occurs, and small vesicles are formed. This process of uptake is energy-dependent and it has been shown to be inhibited by metabolic inhibitors of both glycolysis and oxidative phosphorylation. After invagination, macromolecules travel within
membrane-bound vesicles (phagosomes) to the supranuclear region of the cell where the vesicles fuse with lysosomes to form phagolysosomes. In these structures intracellular digestion occurs. However, small quantities of macromolecules escape breakdown and may be deposited in the intercellular space by reverse endocytosis or exocytosis. These molecules or antigens may come in contact with local lymphoid tissue or may pass into the systemic circulation. Numerous studies using macromolecules such as HRP (horseradish peroxidase), PVP, insulin, and vitamin B\textsubscript{12} have confirmed this finding.\textsuperscript{12, 50, 53, 58, 70, 89, 93, 113}

The pathogenic mechanism involved in the uptake and processing of \textit{M. avium} from the small intestine is not known. Through studies involving other intracellular parasites, it is believed that this process may be similar to the endocytic-type uptake discussed in relation to macromolecular uptake from the gut.\textsuperscript{76} However, after the formation of phagolysosomes, intracellular digestion does not occur. Instead, tubercle bacilli reside and multiply within the involved macrophages. By escaping breakdown, bacilli may be deposited in the intercellular space by exocytosis, and come in contact with local lymphoid tissue. At this time, the cellular immune response of the host, and the corresponding granulomatogenesis, would be initiated to form the typical lesions found upon postmortem examination and slaughter.

If \textit{M. avium} does penetrate into intestinal epithelial cells by a phagocytic process, the various events in this process may be
elucidated by studying them in vitro using cultured intestinal epithelial cells. The objectives of this investigation are:

1) To determine the maximum rate of infection of HIE cells by *M. avium* serotype 2.

2) To evaluate any differences in the interaction of pathogenic versus saprophytic mycobacteria with HIE cells.

3) To assess the properties of *M. avium* that may be responsible for its invasiveness.

4) To determine what role, if any, the host epithelial cell plays in uptake of *M. avium*. 
CHAPTER 2

MATERIALS AND METHODS

Experimental Design

Each assay consisted of Henle intestinal epithelial (HIE) cells grown to confluency in cluster 6 well tissue culture dishes. Each well was inoculated with 0.25 mg (wet weight) of the appropriate organism suspended in minimum essential medium (MEM). Following incubation, plates were washed in PBS, stained, and examined microscopically under oil immersion (100X).

For all studies, 5 wells on each plate were used as treatment groups and the sixth as the control. Individual plates and wells were randomly assigned to treatment groups. Thirty cells per well were counted at random, and the number of bacteria associated with each cell recorded.

Each assay was performed 3 times and the results were averaged.

Bacterial Cultures

Cultures of M. avium serotype 2 were obtained from Dr. C. O. Thoen at Iowa State University. Those of serotype 8 and serotype 10 originated from pigs with mycobacterial lymphadenitis in Arizona, and cultures of M. phlei and M. smegmatis from the mycobacterial collection

b. Gibco. Grand Island, N.Y.
at the Trudeau Institute, Saranac Lake, NY. Stock solutions of each organism were prepared and stored at 0°C for future use to avoid an excessive number of passages from the original slants. Throughout this entire study, no bacterial culture exceeded 4 passages from the original slant. Tubes of Dubos broth\(^c\) containing 1.0% horse serum\(^d\) were inoculated with the appropriate culture, and incubated at 37°C until a density equivalent to a McFarland Tube 1 was reached. The suspensions were aseptically transferred to sterile vials containing 2.0 ml final volume and frozen at 0°C.

Seven days prior to assay, the vials were thawed in a 37°C water bath. One ml of the stock culture was inoculated into 8.0 ml of Dubos broth containing 1.0% horse serum.

Following 7 days incubation at 37°C, each bacterial culture was centrifuged at 2000 rpm for 20 minutes, and resuspended in 40.0 ml of MEM. Two mls of this suspension were overlayed on each well. This resulted in approximately 0.25 mg (wet weight) of bacteria per well.

Cell Cultures

Henle 407 human intestinal epithelial (HIE) cells\(^e\) were cultured in MEM containing 10.0% fetal calf serum\(^f\) (FCS), 200 IU of

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c. Difco. Detroit, Michigan.
d. Gibco. Grand Island, N.Y.
e. American Type Culture Collection (CCL 2). Rockville, Maryland.
f. Gibco. Grand Island, N.Y.
penicillin per ml, 200 ug of streptomycin per ml, and 5 ug of fungizone per ml in 25.0 cm² tissue culture flasks. All media were filter-sterilized using a 0.20 μ cellulose-acetate filter. Once a monolayer was established, cultures were either divided 1:3 into new flasks to facilitate new growth, or transferred 1:1 into cluster 6 well tissue culture plates for assay. The procedure for passage of cells is outlined below.

1. Flasks containing monolayers were washed twice with MEM without FCS.
2. 1.0 ml of 0.25 percent trypsin -0.10 percent EDTA solution was added to each flask.
3. Flasks were incubated at 37°C with 5% CO₂ until gentle tapping detached the monolayer from the vessel (about 2 minutes).
4. A 2.0 ml solution of MEM containing 10% FCS was added to each flask to neutralize the action of trypsin and to wash the cells from the surface of the flask.
5. The contents of each flask were dispensed into 15.0 ml centrifuge tubes, and centrifuged at 800 rpm for 10 minutes.
6. The supernatant fluid was discarded, and the pellet was resuspended in MEM containing 10% FCS and 2.0% antibiotic. It was at this point that a bifurcation

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g. Bellco. Vineland, New Jersey.
in the procedure occurred dependent upon whether the passage was into another flask or into a 6-well plate for assay.

<table>
<thead>
<tr>
<th>FLASK</th>
<th>PLATE</th>
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<tbody>
<tr>
<td>7a. The pellet was resuspended in 1.5 ml of MEM containing 10% FCS and 2% antibiotic.</td>
<td>7b. The pellet was resuspended in 12.0 ml MEM containing 10% FCS and 2% antibiotic.</td>
</tr>
<tr>
<td>8a. 0.5 ml of this solution was dispensed into each of three 25.0 cm² flasks.</td>
<td>8b. A sterile coverslip was placed in each well of the tissue culture plate for assays using the IFAT.</td>
</tr>
<tr>
<td>9a. The total volume was adjusted in each flask to 5.0 ml with MEM containing 10% FCS and 2% antibiotic.</td>
<td>9b. 2.0 ml of the bacterial suspension in (7b) were added to each well.</td>
</tr>
<tr>
<td>10a. Flasks were incubated at 37°C with 5% CO₂ until confluent. (4 days)</td>
<td>10b. Plates were incubated at 37°C until confluent. (3-4 days)</td>
</tr>
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**Antiserum Preparation**

Antiserum against *M. avium* serotype 2 was prepared for use with the indirect fluorescent antibody stain. Antisera against serotypes 8 and 10 were previously prepared by Dr. J. G. Songer, and were available.
for testing. Antisera against M. phlei and M. smegmatis were not available, and alternative staining techniques were employed for assays involving these bacteria.

The preparation of hyperimmune serum against serotype 2, the immunization of rabbits, and the collection of serum was as follows:

1. Tubes of Dubos broth containing OADC and Tween 80 were inoculated with M. avium serotype 2, and incubated at 37°C for 14 days.

2. One ml of this suspension was transferred to each of several plates of Middlebrook 7H10 Agar containing OADC enrichment and 4.1 mg of sodium pyruvate per ml of medium. Plates were incubated at 37°C for 14 days.

3. Cultures were checked daily for contamination. If pure, phenolized phosphate buffered saline (PPBS) was added to each plate, and the cells harvested.

4. Cells remained in PPBS, at a density equivalent to a McFarland III Standard, for 5 days.

5. Phenolized cell suspensions were centrifuged and the pellet was resuspended in 2.0 ml of PBS. This was used to prepare a cell suspension of 0.4 optical density at 525 nm in PBS.

h. Difco. Detroit, Michigan.

i. Sigma. St. Louis, Missouri.
6. The marginal ear vein of New Zealand White rabbits was injected with 1.0 ml of the final suspension on days 0, 4, 8, and 12.

7. Two ml of the same suspension were injected into the ear vein on days 16, 20, 24, and 28.

8. Rabbits were exsanguinated on day 35. The blood was allowed to clot and then centrifuged.

9. The serum was collected and stored at -20°C until needed.

Method of Assay

Kinetics Studies

Kinetic studies were performed on M. avium serotypes 2, 8, and 10, M. phlei, and M. smegmatis. Each assay required 6 tissue culture plates and 60 ml of the appropriate bacterial culture. Each well was washed twice with MEM and 2.0 ml of the bacterial suspension were overlaid onto the monolayer of each of 5 wells. The sixth well contained MEM only, and served as a control. Plates were incubated at 37°C for 30, 60, 120, 180, 240, or 300 minutes, after which the bacterial suspension was aspirated, and each well was washed 4 times with PBS. Cells were then fixed for 30 minutes, or processed without fixation, depending upon the staining technique to be used.

Assays Involving Phagocytic Inhibiting Reagents

These studies involved M. avium serotype 2 only. The procedures for preparing HIE cell monolayers and bacterial cultures were the same as previously described, except coverslips were not used. The
reagents used included cytochalasin B at concentrations of 0.05, 0.10, 0.50, 1.0, 2.0, and 4.0 mg/ml, dibutyryl cyclic AMP at concentrations of 1.0, 2.0, 4.0, and 10.0 mM, iodoacetic acid at concentrations of 0.01, 0.03, 0.05, .10, .20, and .30 mM and 2,4-dinitrophenol at concentrations of 0.001, 0.01, 0.1, 1.0, and 2.0 mM. All reagents were diluted in MEM except cytochalasin B which was prepared as a 1.0 mg/ml stock solution in dimethyl sulfoxide and then diluted in MEM. Two different infection protocols were used in these assays. In Procedure A, 2.0 ml of the appropriate concentration of reagent were applied to each well, and the plates were incubated at 37°C for 3 hours. Following incubation, monolayers were washed 4 times in MEM and infected with 2.0 ml of the appropriate bacterial suspension. After an additional incubation period of 3 hours at 37°C, plates were washed 4 times in PBS and fixed with formalin for 30 minutes. In Procedure B, equal volumes of the bacterial suspension and reagents were incubated together at 37°C for 3 hours. This suspension was used to infect monolayers by overlaying each well with 2.0 ml of the appropriate solution and incubating an additional 3 hours at 37°C. Each well was then washed 4 times in PBS and fixed with formalin for 30 minutes. All plates were stained with the acid-fast stain. One plate was run for each reagent per replication except in those instances where there were more dilutions than wells on each plate, and then it was necessary to run 2 plates for that reagent. Each assay incorporated 1 monolayer not subject to chemical treatment to serve as a

j. Sigma. St. Louis, Missouri.
control.

**Studies Involving "Altered" Bacteria**

These studies involved *M. avium* serotype 2 only. The procedure for preparing HIE cell monolayers and bacterial suspensions were the same as previously described.

A 7 day culture of *M. avium*, grown in Dubos broth, was autoclaved and centrifuged at 2000 rpm for 20 minutes. The supernatant fluid was discarded and the pellet was resuspended in 40 ml of MEM. Prior to infection of monolayers with this suspension, Middlebrook 7H10 Agar plates were inoculated to determine the viability of mycobacteria after autoclaving. Two confluent, 6-well plates of HIE cells were prepared per replication. After washing each well twice with MEM, the cells from randomly selected wells were infected with 2.0 ml of the above bacterial suspension. The sixth well was overlaid with 2.0 ml of viable *M. avium* serotype 2 suspended in MEM, and served as the control. Following 3 hours of incubation at 37°C, the coverslips were removed from each well, labeled, and washed 4 times in PBS. The monolayers, grown on coverslips from 1 plate were fixed in methanol for 30 minutes, while those from the second plate were processed without fixing.

Those experiments involving formalinized *M. avium* used the same protocol as above except the 7 day culture of *M. avium* was centrifuged and resuspended in 5% formalin for 15 minutes. Cells were washed twice in PBS before resuspension in 40 ml of MEM.

To determine the effect of UV irradiation on *M. avium* in this
system, a 7-day culture of *M. avium* was centrifuged at 2000 rpm for 20 minutes and resuspended in 40 ml of MEM. Four ml of the above suspension were placed in each of 6 100 x 15 mm petri dishes to form a shallow layer. The petri dishes were exposed to UV light for 10, 20, 30, 40, 50, or 60 seconds from a distance of 60 cm. After irradiation, the contents of each petri dish were cultured on Middlebrook 7H10 Agar to check viability. Using two 6-well tissue culture plates as treatment groups, each treated bacterial solution was randomly assigned to 1 of the 6 wells per plate. Two ml of this suspension were overlaid onto each monolayer. The third plate was used as the control, and cells from each well were infected with 2.0 ml of *M. avium* in MEM that had not been exposed to UV irradiation. All plates were incubated for 3 hours at 37°C. Coverslips, upon which monolayers had been grown, were removed from wells, labeled, and washed 4 times in MEM. The coverslips from 1 of the treated plates were fixed in methanol for 30 minutes, while coverslips from the other treated plates were processed without fixing. Three randomly selected coverslips from the control plate were also fixed in methanol for 30 minutes while the remaining 3 were not fixed.

*M. avium* was suspended in MEM containing various dilutions of antisera to observe the possible effect of masking surface molecules functioning as receptor sights in attachment. Serum was filtered through a 0.20 μ cellulose-acetate filter before being diluted 1:16, 1:64, and 1:256 in MEM. Each replication of this assay required

k. Falcon. Oxnard, California.
monolayers in three 6-well tissue culture plates, and three 7-day cultures of \textit{M. avium}. The acid-fast stain was employed in this assay so it was not necessary to incorporate coverslips in these tissue culture plates. Bacterial cultures were centrifuged at 2000 rpm for 20 minutes before suspension in 40 ml of 1 of 3 antibody-containing solutions of MEM. All monolayers were washed twice in MEM before infection. Two ml of the appropriate bacterial suspension were added to each of 5 wells. The monolayer in the sixth well was inoculated with \textit{M. avium} suspended in MEM not containing antisera. Plates were incubated for 3 hours at 37°C, washed 4 times in MEM, and fixed with formalin for 30 minutes.

The format followed in studies involving \textit{M. avium} cultures suspended in milk or colostrum was essentially the same as that described above, except the FA staining technique was used. Two confluent 6-well tissue culture plates with coverslips, and one 7-day culture of \textit{M. avium} were needed per replication for each of the 2 treatments (milk or colostrum). The unpasteurized milk and colostrum were obtained from the University of Arizona Dairy from cattle not receiving antibiotic therapy, and the pH was adjusted to 7.0. A culture of \textit{M. avium} was resuspended in 40 ml of milk or colostrum, and 2.0 ml of this suspension were overlaid onto each of 5 monolayers. The sixth monolayer, the control, was infected with \textit{M. avium} suspended in MEM. Plates were incubated 3 hours at 37°C, after which the coverslips, containing monolayers, were removed, labeled, and washed 4 times in MEM. The coverslips from 1 plate were fixed in methanol for
30 minutes, while those from the second plate were processed without fixing.

Staining Techniques

Kinyoun's Acid-Fast Stain

This procedure was employed initially with cultures infected with *M. avium* serotype 2 to determine if there was any attachment occurring, in all assays involving *M. phlei* and *M. smegmatis*, and in those incorporating *M. avium* suspended in antiserum. The procedure described below begins after fixation of cultures with formalin:

1. Carbol fuchsin
2. Rinse 70 percent alcohol 2 Times
3. Rinse running tap water 5 Minutes
4. Rinse acid alcohol 3 Times
5. Rinse running tap water 8 Minutes
6. Methylene blue
7. Rinse running tap water 10 Minutes

Auramine O-Acridine Orange Stain

This stain was used on all cultures involving the phagocytosis inhibiting reagents. After fixation in formalin, infected monolayers were treated as follows:

1. Auramine O (0.30% solution) 15 Minutes
2. Rinse running tap water 5 Minutes
3. Rinse acid alcohol 2 Times

4. Acridine orange\textsuperscript{m} 5 Minutes
5. Rinse running tap water 5 Minutes

**Indirect Fluorescent-ANTibody Stain**

Immunofluorescent techniques similar to those described by Kihlstrom\textsuperscript{49} and modified to incorporate a counterstain were used to differentiate intracellular and cell surface adherent bacteria in kinetic studies involving *M. avium* serotype 2, serotype 8, and serotype 10. Assays incorporating autoclaved, ultraviolet exposed, formalin treated, and milk or colostrum coated *M. avium* serotype 2 were also evaluated using this procedure. Monolayers, grown on cover-slips, were either fixed in methanol or processed without fixation according to the following protocol:

1. Monolayers were incubated with a 1:64 dilution of rabbit anti-*M. avium* serotype 2 antibody for 20 minutes at 37°C. A dilution of 1:160 was used for serotype 8 and a dilution of 1:320 was used for serotype 10.
2. Cells were washed 4 times in PBS.
3. Monolayers were incubated with 1:32 dilution of fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit 75 immunoglobulin\textsuperscript{n} for 20 minutes at 37°C.
4. Cells were washed 4 times in PBS.
5. A counterstain, consisting of 0.125 percent Evans Blue\textsuperscript{m} 

\textsuperscript{m} Sigma. St. Louis, Missouri.

\textsuperscript{n} Miles. Elkhart, Indiana.
diluted in PBS, was applied to each coverslip for 10 minutes.

6. Cells were washed twice in PBS and mounted in 60% glycerol.

Microscopy

Light

Those cultures stained with Kinyoun's acid-fast stain were examined under oil immersion using the 100 x objective.

Fluorescence

Cultures stained with the Auramine O-acridine orange stain and those involving the indirect fluorescent antibody stain were examined by incident light fluorescence microscopy at 100X (oil immersion) using a Leitz photomicroscope with exciter filter II and a 400 nm barrier filter.

Statistical Analysis

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

Two statistical computer programs, compiled by Kelley, were used for data analysis. The first included an analysis of variance and tests of significance between treatment groups ($\alpha = .05$) were performed with the F-test. A second program was devised to find the mean
count for each treatment group per assay. Therefore, each data point consisted of the average of 3 means corresponding to the 3 performances of each treatment.

When the percent of intracellular bacteria was needed, the means for fixed and unfixed monolayers were employed in the following formula:

\[
\frac{\text{Mean Number of Bacteria/Fixed HIE Cell} - \text{Mean Number of Bacteria/Unfixed HIE Cell}}{\text{Mean Number of Bacteria/Fixed HIE Cell}} \times 100
\]

All data from those assays involving reagents to inhibit phagocytosis were evaluated without transformation. The cells were arranged into 2 groups: infected and noninfected. Cells with 1 or more associated bacteria were defined as infected. This was done for each concentration of each reagent, and because each assay was performed 3 times, an average of the 3 was used in the following formula to give the percentage of HIE cells infected per monolayer:

\[
\frac{\text{Mean Number of Cells Infected}}{\text{Mean Number of Cells Infected} + \text{Mean Number of Cells Noninfected}} \times 100
\]

Tests of significance were conducted using the Newman-Keuls mean separation test and a 95% confidence level.

In some cases, it was necessary to look individually at fixed and unfixed HIE cell monolayers. Bacteria could be considered intracellular if the 2 preparations were found to stain differently, and extracellular if preparations stained alike. Using raw data, each HIE cell counted was placed in 1 of 7 groups depending upon the number of bacteria which were found in association with it. Groups included
0, 1-10, 11-20, 21-30, 31-40, 41-50, and 51-60 associated bacteria. The distributions for fixed and unfixed monolayers were then graphed for comparison.
CHAPTER 3

RESULTS AND DISCUSSION

Kinetics Studies

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. Data indicate that maximum cellular uptake occurred after 3 hours incubation time. (Figure 1.) Because mycobacteria are intracellular parasites, the validity of the data in Figure 1 is dependent upon the assumption that cell-associated bacteria seen in acid-fast stained monolayers were in fact intracellular and not merely adhering to host cell surfaces. A technique described by Kihlstrom was used to aid in distinguishing surface-associated from internal bacteria. This indirect fluorescent-antibody test is based upon the fact that immunoglobulin proteins do not cross the intact plasma membrane but diffuse freely into methanol-fixed cells. This principle was first applied to monolayers infected with M. avium serotype 2 for time intervals of 30 minutes to 5 hours. Figure 2 shows the percent intracellular bacteria at each time. Maximum infection of epithelial cells occurred after 3 hours of incubation, and at this time, over 60% of the bacteria seen in association with each epithelial cell were, in fact, intracellular.

The fact that the number of intracellular bacteria per HIE cell decreased as incubation time exceeded 3 hours, may be due to
Figure 1. Kinetics of the Interaction of *M. avium* Serotype 2 with HIE Cells.
Figure 2. Percent of *M. avium* Serotype 2 Shown to be Intracellular when Associated with HIE Cells.
exocytosis of tubercle bacilli. 53

The kinetics of the interactions of serotypes 8 and 10 with HIE cells were studied to determine if they differed from the supposedly more pathogenic serotypes 1 and 2. Serotype 8 exhibited maximum uptake of 42.94% after 3 hours incubation (Figure 3). This was 13% less than serotype 2 uptake. Figure 4 shows that cellular uptake is even less with serotype 10. Furthermore, this maximum uptake of only 28.60% occurred after only 2 hours incubation. The differences in kinetics of the M. avium:HIE cell interaction may partly explain the differences in rates of infection with various serotypes. Other factors, such as the relative prevalence in the environment of the different serotypes, may also play a role.

Saprophytic mycobacteria, which are not causally associated with a disease process, are widespread in the environment. Two of these, M. phlei and M. smegmatis, are occasionally isolated from tuberculous lesions of swine, often with M. avium. The interaction of these 2 organisms with HIE cells were studied to compare them with pathogenic M. avium. Results are shown in Figures 5 and 6, as the mean number of bacteria associated with each HIE cell. The lack of antisera against these species of bacteria did not allow use of IFAT. Neither organism displayed a significant (P = 0.05) 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 to M. avium, in porcine lymph nodes.
Figure 3. Percent of M. avium Serotype 8 Shown to be Intracellular when Associated with HIE Cells.
Figure 4. Percent of *M. avium* Serotype 10 Shown to be Intracellular when Associated with HIE Cells.
Figure 5. Kinetics of the Interaction of *M. phlei* with HIE Cells.
Figure 6. Kinetics of the Interaction of M. smegmatis with HIE Cells.
The interaction of *M. avium* serotype 2 with epithelial cells was examined in greater detail by studying separately the contributions of the HIE cells and the bacteria to the uptake process.

**Host Cell Effects On Uptake**

HIE cell monolayers were treated with 4 compounds known to inhibit the ability of phagocytic cells to engulf particles. Pre-incubation of host cells with the appropriate reagent (Procedure A) versus treatment of bacterial cultures with each reagent before infecting monolayers (Procedure B) was designed to assure that the effect of each reagent was upon the host cells and not upon the bacteria.

Cytochalasin B, a fungal metabolite, inhibits phagocytosis by polymorphonuclear leukocytes (PMN) and macrophages. Many investigators have reported the disruption of actin polymers of microfilaments in both phagocytic and nonphagocytic cells treated with cytochalasin B. According to these authors, concentrations of 1-3 mg cytochalasin B/ml disrupt microfilaments and suppress cell phagocytosis. Results in Table 1 indicate significant inhibition of phagocytosis at a concentration of 1.0 mg cytochalasin B/ml, regardless of which procedure was applied. This suggests that infection of epithelial cells *in vitro* is dependent upon functional host cell microfilaments.

Dibutyryl cAMP inhibits particle ingestion by PMN and mouse
Table 1. Effect of Cytochalasin B on Interaction of M. avium Serotype 2 with HIE Cells.

<table>
<thead>
<tr>
<th>PROCEDURE</th>
<th>A</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>B</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (µg/ml)</td>
<td>4.0</td>
<td>2.0</td>
<td>1.0</td>
<td>0.5</td>
<td>0.1</td>
<td>0.05</td>
<td>0</td>
<td>4.0</td>
<td>2.0</td>
<td>1.0</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>%</td>
<td>16</td>
<td>22.33</td>
<td>25.33</td>
<td>39.33</td>
<td>45.33</td>
<td>56.33</td>
<td>59.66</td>
<td>6.66</td>
<td>16.66</td>
<td>21.66</td>
<td>34.66</td>
<td>40.33</td>
</tr>
<tr>
<td>%</td>
<td>26.66</td>
<td>37.22</td>
<td>42.22</td>
<td>65.55</td>
<td>75.55</td>
<td>93.88</td>
<td>99.43</td>
<td>11.10</td>
<td>27.77</td>
<td>36.10</td>
<td>57.77</td>
<td>67.22</td>
</tr>
</tbody>
</table>

* significant (P = .05)
† bacteria applied after removal of reagent
‡ reagent and bacteria applied simultaneously
** mean number cells infected
   total number cells counted (3 observations)
peritoneal macrophages at a concentration of 2.0 mM. \textsuperscript{13, 97, 115}

Experiments were conducted to test the effect of this compound upon the susceptibility of HIE cells to mycobacterial infection. Results indicated that dibutyryl cAMP significantly inhibited the infection of host cell monolayers at different concentrations dependent upon the infection procedure used (Table 2). It was found that 1.0 mM solutions caused significant inhibition with Procedure A. However, under Procedure B, significant inhibition did not occur at concentrations less than 4.0 mM. Dibutyryl cAMP is soluble in the lipids of the host cell membrane, thereby allowing it to cross the intact cell membrane and inhibit phagocytosis. \textsuperscript{31} It may be readily absorbed by \textit{M. avium} due to the high-lipid content of its cell membrane, thereby impairing the ability of this compound to affect the HIE cells at lower concentrations.

Because phagocytosis is an energy-dependent process, experiments were done to determine if disruption of carbohydrate metabolism affected the interaction between \textit{M. avium} serotype 2 and HIE cells. Iodoacetate inhibits phagocytosis in PMN, \textsuperscript{115} blood monocytes, \textsuperscript{11} and peritoneal macrophages; \textsuperscript{74} in these cells, the energy required for phagocytosis has been linked to glycolysis which is known to be inhibited by iodoacetate. 2,4-dinitrophenol inhibits Krebs' cycle activity and, thus phagocytosis, by alveolar macrophages. \textsuperscript{74} These 2 compounds were tested for effects on mycobacterial infection of HIE cells. Tables 3 and 4 show results when iodoacetate and 2,4-dinitrophenol were employed, respectively. In both cases it was found that infection was
Table 2. Effect of Dibutyryl cAMP on Interaction of M. avium Serotype 2 with HIE Cells.

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>PROCEDURE</th>
<th>PROCEDURE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A†</td>
<td>B†</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>4.0</td>
</tr>
<tr>
<td>X **</td>
<td>10</td>
<td>14.66</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>%</td>
<td>16.67</td>
<td>24.43</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>X **</td>
<td>11.66</td>
<td>26.66</td>
</tr>
<tr>
<td></td>
<td>60</td>
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<td>44.43</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>60</td>
</tr>
</tbody>
</table>

* significant (P = .05).
† bacteria applied after removal of reagent
‡ reagent and bacteria applied simultaneously
** mean number cells infected / total number cells counted (3 observations)
Table 3. Effect of Iodoacetate on Interaction of *M. avium* Serotype 2 with HIE Cells.

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
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<th>PRODUCRE B ‡</th>
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</thead>
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<tr>
<td></td>
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<td>0.03</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>0.2</td>
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<td></td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
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</table>

<table>
<thead>
<tr>
<th><em>X</em> **</th>
<th>15.33</th>
<th>19.66</th>
<th>26</th>
<th>35.66</th>
<th>55</th>
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<th>59.33</th>
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<tbody>
<tr>
<td></td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
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<table>
<thead>
<tr>
<th>%</th>
<th>25.55</th>
<th>32.77</th>
<th>43.33</th>
<th>59.43</th>
<th>91.67</th>
<th>96.10</th>
<th>98.83</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
</tbody>
</table>

*significant (P = .05)
† bacteria applied after removal of reagent
‡ reagent and bacteria applied simultaneously
** mean number cells infected
    total number cells counted (3 observations)
Table 4. Effect of 2,4-Dinitrophenol on Interaction of *M. avium* Serotype 2 with HIE Cells.

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>PROCEDURE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A †</td>
</tr>
<tr>
<td>2.0</td>
<td>11.66</td>
</tr>
<tr>
<td>1.0</td>
<td>27.66</td>
</tr>
<tr>
<td>0.1</td>
<td>29.33</td>
</tr>
<tr>
<td>0.01</td>
<td>35</td>
</tr>
<tr>
<td>0.001</td>
<td>43.66</td>
</tr>
<tr>
<td>0</td>
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<tr>
<td>0</td>
<td>20.66</td>
</tr>
<tr>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>0</td>
<td>29.66</td>
</tr>
<tr>
<td>0</td>
<td>32.33</td>
</tr>
<tr>
<td>0</td>
<td>45.66</td>
</tr>
<tr>
<td>0</td>
<td>59.33</td>
</tr>
<tr>
<td>0</td>
<td>30</td>
</tr>
</tbody>
</table>

* significant (P = 0.05).

† bacteria applied after removal of reagent
‡ reagent and bacteria applied simultaneously

** mean number cells infected / total number cells counted (3 observations)
significantly reduced at a concentration of 0.10 mM or greater. Furthermore, there was no difference based upon the infection procedure used. These data support other studies using cytochalasin B and dibutyryl cAMP by showing that the uptake of *M. avium* into intestinal epithelial cells is at least partially dependent upon the endocytic activity of the host cell.

**Bacterial Effects On Uptake**

Uptake of *M. avium* into HIE cells may be partially explained by factors related to the bacterium, such as its cell surface characteristics (proteins or lipids) or metabolic products. This was studied using cultures of *M. avium* serotype 2 manipulated in various ways to alter these characteristics, and their subsequent interactions with HIE cells.

Initial studies used bacteria killed by autoclaving or by suspension in formalin. Killed suspensions were incubated with HIE cells for 3 hours and the interaction was assessed quantitatively using the indirect fluorescent antibody stain.

Figure 7 shows the results of incubation of autoclaved *M. avium* serotype 2 with HIE cell monolayers versus results from monolayers inoculated with viable *M. avium* cultures. A remarkable decrease in uptake was evident compared to the control, but a small amount of endocytosis (3.08%) did occur. Comparison of the results of methanol fixed and unfixed preparations (Figure 8), indicates that minimal engulfment probably reflects extracellularly adhered bacilli.

Figure 9 shows the results of interactions of HIE cells with
Figure 7. Difference in Uptake of Autoclaved and Viable *M. avium* Serotype 2 by HIE Cells.
Figure 8. Staining Characteristics of Methanol-Fixed and Unfixed HIE Cells Infected with Autoclaved *M. avium* Serotype 2.
Figure 9. Difference in Uptake of Formalin-Killed and Viable *M. avium* Serotype 2 by HIE Cells.
formalin-killed and with viable *M. avium*. A significant inhibition 
(P = 0.05) in uptake of killed bacteria was found when compared to 
the control. Examination of methanol-fixed and unfixed preparations 
(Figure 10) indicated that the minimal level of endocytosis (3.79%) 
was more likely due to extracellular adhered bacteria than intra-
cellular bacteria, since the 2 preparations stain alike.

Suspensions of *M. avium* serotype 2 were exposed to ultraviolet 
irradiation for 0 to 60 seconds and their interaction with HIE cells 
was compared to that of nonirradiated bacteria. An exposure of 40 
seconds or more significantly reduced (P = 0.05) the infectivity of 
the organism (Figure 11). Results of methanol-fixed and unfixed prep-
arations were examined separately (Figure 12). After 40 seconds of 
irradiation preparations stained similarly indicating that the bacilli 
were not being taken into the host cell but were only adhered to the 
outside. After 50 to 60 seconds exposure the staining characteristics 
of methanol-fixed and unfixed preparations were identical.

Viability tests on the *M. avium* suspensions paralleled those 
shown for infectivity in Figure 11. After 8 weeks of incubation at 
37°C Middlebrook 7H10 Agar plates inoculated with suspensions exposed 
for 10, 20, or 30 seconds showed similar growth to plates inoculated 
with nonirradiated *M. avium*. Plates inoculated with bacterial cultures 
exposed for 40 seconds demonstrated a 65% reduction in growth compared 
to cultures containing nonirradiated bacilli. Plates inoculated with 
cultures exposed to 50 or 60 seconds of UV light exhibited no growth.

The effects of short-term irradiation (less than 60 seconds) 
on mycobacteria are thought to be confined to the nucleic acids.
Figure 10. Staining Characteristics of Methanol-Fixed and Unfixed HIE Cells Infected with Formalin-Killed M. avium Serotype 2.
Figure 11. Difference in Uptake of M. avium Serotype 2 Irradiated with Ultraviolet Light (260 nm) for 0 to 60 Seconds.
Figure 12. Staining Characteristics of Methanol-Fixed and Unfixed HIE Cells Infected with M. avium Serotype 2 Irradiated with Ultraviolet Light for 0 to 60 Seconds.
Therefore, it is likely that surface molecules would be unaltered in the suspensions irradiated in the present study. It may be inferred, from data obtained in these studies, that viability of the bacterium is an obligate requirement for endocytosis by HIE cells. The role of surface molecules in this process must be evaluated further.

Although immunity to mycobacterial infections is cell-mediated rather than antibody-mediated, the effect of local antibody on the infection process in swine tuberculosis is unknown. Porcine anti-M. avium IgA is not available and, therefore, anti-M. avium serotype 2 serum (rabbit origin) was employed to evaluate the interaction of this organism with HIE cells. Results are shown in Figure 13. Inhibition of infection/attachment was inversely proportional to the concentration of antiserum. This may have resulted from the masking of surface structures which are required for attachment. Cultures of the anti-sera treated suspensions revealed no reduction of growth, implying that viability is not the sole requirement for M. avium uptake.

Preliminary studies were conducted on the effect of milk and colostrum on uptake of M. avium by HIE cells. This was done to simulate conditions occurring in the gut of the neonate pig. Due to the extreme difficulty in obtaining milk and colostrum from sows, bovine milk and colostrum were substituted. Figure 14 indicates an increase in the number of internalized tubercle bacilli when M. avium was suspended in milk as compared to M. avium suspended in MEM. However, this increase was not statistically significant (P = 0.05). Figure 15 shows the results when epithelial cells were infected with M. avium
Figure 13. Interaction of *M. avium* Serotype 2 Suspended in Various Concentrations of Homologous Antiserum with HIE Cells.
Figure 14. Interaction of M. avium Serotype 2 Suspended in Bovine Milk or In MEM with HIE Cells.
Figure 15. Interaction of M. avium Serotype 2 Suspended in Bovine Colostrum or in MEM with HIE Cells.
suspended in colostrum. A statistically significant ($P = 0.05$) increase in the percent of bacterial uptake was found when bacteria were suspended in colostrum.

The difference in bacterial uptake between the 2 suspensions was possible due to their differences in lipid or immunoglobulin content. 22, 54, 70

If pigs are exposed to *M. avium* during the neonatal period, not only are they physiologically incapable of preventing bacterial invasion of the gut, but their diet at this time may also enhance susceptibility to infection. This may indicate that sanitation during the first few days after birth could be of some importance in preventing mycobacterial infection.
CHAPTER 4

CONCLUSIONS

An in vitro model using cultured Henle intestinal epithelial (HIE) cells was designed to study the early interaction between M. avium and the host cell in an attempt to better understand the pathogenic mechanism involved in swine tuberculosis. The results of this study indicate that:

1. Maximum infection of cultured HIE cells was achieved after 3 hours of incubation with M. avium serotype 2.

2. Differences in characteristics among different serotypes of M. avium and saprophytic or nonpathogenic mycobacteria may account for the observed differences in infection rate with these organisms.

3. Because the rate of infection of HIE cells was inhibited by reagents shown to inhibit particle uptake in known phagocytic cells, it was suggested that uptake of M. avium by cultured epithelial cells was endocytic in nature.

4. Studies involving infection of HIE cells with dead M. avium showed that viability of the tubercle bacilli is necessary for endocytosis to occur.

5. Surface molecules may play a role in attachment of the organism to the host cell, but metabolic activity on the part of the tubercle bacilli is also required for endocytosis.
LIST OF REFERENCES


95. Songer, J. G., and Bicknell, E. J.: Involvement of Wood Shavings Used as Bedding in the Epidemiology of Swine Tuberculosis. 59th Conference of Research Workers in Animal Disease, Abstract No. 70.


