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Peyer's Patch-Deficient Mice Demonstrate That *Mycobacterium avium* subsp. *paratuberculosis* Translocates across the Mucosal Barrier via both M Cells and Enterocytes but Has Inefficient Dissemination▽

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Mycobacterium avium* subsp. *paratuberculosis*, the agent of Johne's disease, infects ruminant hosts by translocation through the intestinal mucosa. A number of studies have suggested that *M. avium* subsp. *paratuberculosis* interacts with M cells in the Peyer's patches of the small intestine. The invasion of the intestinal mucosa by *M. avium* subsp. *paratuberculosis* and *Mycobacterium avium* subsp. *hominissuis*, a pathogen known to interact with intestinal cells, was compared. *M. avium* subsp. *paratuberculosis* was capable of invading the mucosa, but it was significantly less efficient at dissemination than *M. avium* subsp. *hominissuis*. B-cell knockout (KO) mice, which lack Peyer's patches, were used to demonstrate that *M. avium* subsp. *paratuberculosis* enters the intestinal mucosa through enterocytes in the absence of M cells. In addition, the results indicated that *M. avium* subsp. *paratuberculosis* had equal abilities to cross the mucosa in both Peyer's patch and non-Peyer's patch segments of normal mice. *M. avium* subsp. *paratuberculosis* was also shown to interact with epithelial cells by an $\alpha_5\beta_1$ integrin-independent pathway. Upon translocation, dendritic cells ingest *M. avium* subsp. *paratuberculosis*, but this process does not lead to efficient dissemination of the infection. In summary, *M. avium* subsp. *paratuberculosis* interacts with the intestinal mucosa by crossing both Peyer's patches and non-Peyer's patch areas but does not translocate or disseminate efficiently.

Mycobacterium avium subsp. *paratuberculosis* is the agent of Johne's disease, a debilitating condition of cattle and other ruminants that is associated with severe diarrhea and wasting (10, 32). Johne's disease causes significant economic loss, with a severe impact on the dairy industry (5, 37). Some studies have implicated *M. avium* subsp. *paratuberculosis* as one of the potential etiologic agents or opportunistic pathogens of Crohn's disease patients (5, 37).

M. avium subsp. *paratuberculosis* infects young calves and is usually transmitted by contaminated stools or milk (10, 29). In the first steps of pathogenesis, the bacterium crosses the intestinal mucosa of the infected host. Previous work has suggested that *M. avium* subsp. *paratuberculosis* crosses the intestinal mucosa by entering M cells in the Peyer's patches of calves (15). More recently, a goat kid animal model suggested that the ports of entry in the intestinal mucosa are not limited to M cells (24). In addition, work *in vitro* showed that *M. avium* subsp. *paratuberculosis* can invade bovine kidney epithelial (17) and murine intestinal epithelial (20) cells efficiently. *Mycobacterium avium* subsp. *hominissuis*, a subspecies related to *M. avium* subsp. *paratuberculosis* (26), invades the intestinal mucosa by interacting primarily with enterocytes (4, 21). AIDS patients acquire *M. avium* subsp. *hominissuis* infections mainly through the gastrointestinal tract (6), and studies of macaques have demonstrated that this microorganism is within enterocytes (L. E. Bermudez et al., unpublished observations). It should be noted that in earlier literature, this subspecies is referred to simply as *Mycobacterium avium* or *Mycobacterium avium* subsp. *avium* (5, 12, 31); the new taxonomic description (*M. avium* subsp. *hominissuis*) for microorganisms isolated from humans and swine is relatively recent (14) and was not immediately captured or accepted by the scientific community at large (31). Nonetheless, in spite of the close genetic relationship, *M. avium* subsp. *hominissuis* is likely the only truly environmental mycobacterium (33), often acquired by humans and pigs, but rarely isolated from birds or cattle (33). In contrast, *M. avium* subsp. *paratuberculosis* is the predominant, or possibly the only, mycobacterium isolated from ruminants with Johne's disease and associated with cases of Crohn's disease in humans, but rarely isolated from AIDS patients. Thus, these subspecies represent microorganisms associated with unique disease entities and epidemiological distributions.

The mouse model of *M. avium* subsp. *paratuberculosis* may not entirely reflect the disease in cattle, sheep, and goats; nevertheless, it has value for studying aspects of *M. avium* subsp. *paratuberculosis* pathogenesis in small laboratory animals (11, 16). *M. avium* subsp. *paratuberculosis* has been observed multiplying in the intestinal mucosa of athymic nude gnotobiotic mice (9), indicating that the bacterium can replicate efficiently in the intestinal mucosa in the absence of a competent immune system. The availability of recombinant

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mouse strains offers the opportunity to address the roles of M cells and enterocytes in the translocation of *M. avium* subsp. *paratuberculosis* through the intestinal wall. To study this process, we compared *M. avium* subsp. *paratuberculosis* dissemination in wild-type mice and B-cell knockout (KO) mice, which lack Peyer's patches and M cells. Our results indicate that *M. avium* subsp. *paratuberculosis* crosses the intestinal mucosa by invading both M cells and enterocytes and that it can translocate, although with less efficiency than *M. avium* subsp. *hominissuis*, across mucosal epithelial cells. Bacteria also infected dendritic cells after crossing the epithelial barrier.

MATERIALS AND METHODS

Bacteria. *M. avium* subsp. *paratuberculosis* strain K-10 is a bovine isolate capable of causing disease in both cattle and mice (18). *M. avium* subsp. *paratuberculosis* was grown on Middlebrook 7H10 agar plates supplemented with 2 mg/liter of mycobactin J and oleic acid, albumin, dextrose, and catalase (OADC). *M. avium* subsp. *hominissuis* strain 101 is capable of infecting immunocompetent and immunosuppressed mice (2). *M. avium* subsp. *hominissuis* was cultured on 7H10 agar supplemented with OADC. Bacteria grown for 14 days (*M. avium* subsp. *hominissuis*) or 20 days (*M. avium* subsp. *paratuberculosis*) were used in the experiments. *Staphylococcus aureus* SA101 is a clinical isolate obtained from the purulent material of a cutaneous abscess. For the experiments, this bacterium was cultured on Mueller-Hilton agar plates for 48 h.

Mice. Eight- to 10-week-old female pathogen-free C57BL/6J black mice, weighing 25 g, were obtained from the Jackson Laboratory (Bar Harbor, ME) and were used after 2 weeks of quarantine. C57BL/6J B-cell-deficient mice (immunoglobulin H6 negative, 8 to 10 weeks old, weighing 25 g) were purchased from the Jackson Laboratory and were used after 2 weeks of quarantine. These animals have previously been shown to lack Peyer's patches and M cells (8). In some experiments, 10- to 12-week-old mice were used. All experiments were performed according to the guidelines of the institutional animal care and use committee.

Infection of mice. The first set of experiments compared the infection of C57BL/6J mice by *M. avium* subsp. *paratuberculosis* versus *M. avium* subsp. *hominissuis*. Bacteria were cultured on solid medium as described above, with the inoculum prepared at 2.4×10^7 CFU/ml (*M. avium* subsp. *hominissuis* 101) or 3.8×10^7 CFU/ml (*M. avium* subsp. *paratuberculosis* K-10). Mice were given 0.1 ml of the bacterial suspension *per os* and were followed for 1, 2, 4, 8, and 16 weeks postinfection (wpi). Ten mice were used for each time point in each of two sets of experiments. At each time point, mice were killed, and the spleens, livers, and terminal ilea were harvested, homogenized, serially diluted, and plated onto Middlebrook 7H10 agar plates supplemented with OADC, mycobactin J, and PACT (polymyxin B at 5 μg/ml, amphotericin B at 4.5 μg/ml, carbenicillin at 22 μg/ml, and trimethoprim at 2 μg/ml).

Intestinal loop. The intestinal-loop assay was carried out basically as described previously for *M. avium* subsp. *hominissuis* (21). Briefly, both C57BL/6 black mice and C57BL/6J B-cell-deficient mice (immunoglobulin H6 negative), 8 to 10 weeks old, weighing 25 g, were anesthetized by intraperitoneal administration of phenobarbital. Mice were maintained under profound anesthesia during the entire procedure. Following anesthesia, the abdominal cavity was carefully opened, and a segment of the small intestine that was approximately 3 cm long, proximal to the ileocecal area, was identified. A suture line was tied at both ends of the segment of intestine, tightly enough to close the intestinal lumen while not interfering with the blood flow. A suspension containing approximately 1×10^7 *M. avium* subsp. *hominissuis* or 2.5×10^7 *M. avium* subsp. *paratuberculosis* organisms in Hanks' balanced salt solution (HBSS) was injected into the proximal position of the isolated intestinal segment. Mice were kept alive for 1 and 3 h, after which they were killed, and the intestinal segment was removed, opened longitudinally, and rinsed with HBSS to remove unbound or weakly bound bacteria. The removed intestinal segment was placed in 5 ml of 7H9 broth with 30% glycerol and was homogenized. The suspension was then serially diluted in 7H9 broth before being plated onto 7H10 agar, supplemented with mycobactin J and PACT to inhibit intestinal biota, for quantification of viable organisms associated with the intestinal mucosa-submucosa. Plates were cultured for several days at 37°C, and the concentration of bacteria (expressed as CFU per gram of tissue) was calculated as (average CFU per plate × dilution factor × 5 ml)/(intestinal-segment weight).

Differential uptake by Peyer's patch and non-Peyer's patch segments. To determine whether *M. avium* subsp. *paratuberculosis* enters the intestinal mucosa preferentially by M cells or enterocytes, we performed an *in vivo* assay in which 10-week-old C57BL/6J mice were given *M. avium* subsp. *paratuberculosis* (3×10^8 bacteria) orally. At 4 h and 24 h, the mice (5 mice per time point per experimental group) were sacrificed. Their abdomens were opened, and for each mouse, 5 segments (length, 1 cm) comprising regions with Peyer's patches and 5 segments without Peyer's patches were obtained, opened longitudinally, washed, homogenized, and plated onto 7H10 agar with mycobactin J.

Histopathology. The intestinal ilea from four wild-type mice and four B-cell KO mice were obtained, fixed with 10% neutral buffered formalin, and stained with hematoxylin-eosin or an acid-fast stain as previously described (13).

Electron microscopy. Mice were infected with *M. avium* subsp. *paratuberculosis*, and 1 or 2 days after infection, the animals were harvested; the intestinal segment was cut and opened longitudinally, briefly washed in HBSS, and immersed in 4% paraformaldehyde and 2% glutaraldehyde for 12 h at 4°C. Then the material was placed in HBSS and stained with a 1% aqueous solution of osmium tetroxide for 1 h at 4°C, as previously described (17). Samples were dehydrated in ethanol at room temperature, embedded in resin, and polymerized at 52°C. Photographs were taken using a transmission electron microscope (EM) at a magnification of ×10,000.

Translocation assay. Translocation assays were performed as previously described (17), using the Transwell 2-chamber culture system (Costar; Corning, NY) containing a 0.33-cm^2 porous membrane (pore size, 3.0 μm). Monolayers were established on the top of the membrane by seeding it with 1×10^6 Madin-Darby bovine kidney (MDBK) cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). The culture medium was changed every 2 days, and the integrity of the monolayer was monitored by the following methods: (i) microscopic observation, (ii) measurement of the transwell resistance using a Millipore (Bedford, MA) transwell device as reported elsewhere (17), and (iii) the trypan blue (0.25%) permeability assay (optical density at 580 nm), as described previously (3). Trypan blue (0.25%) was added to the monolayer, and 3 h later, the supernatant of the bottom chamber was obtained for a spectrophotometer reading. The control included the medium alone (baseline). The top chamber was infected either with 5×10^5 bacteria or with 2×10^5 bacteria that had previously been exposed to whole cow milk, as described previously (17). After 2, 6, or 24 h of infection, 500 μl of filtrate was collected from the bottom chamber, and the system was replenished with fresh culture medium. The translocation ability was calculated as the cumulative percentage of the initial inoculum that was recovered in the bottom chamber at each time point.

Bacterial uptake assays. Bacteria (10^5) were added to MDBK cells in medium with 10% FBS for 1 h. Extracellular bacteria were removed, and monolayers were washed three times with HBSS. Monolayers were then lysed and the number of intracellular bacteria quantified.

Bovine dendritic cells. Bovine dendritic cells were obtained by differentiation of bovine monocytes by treatment with human recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF; 10 μg/ml) and interleukin-4 (IL-4; 10 μg/ml) (Genzyme, Cambridge, MA). Mononuclear phagocytes from healthy cows at the Oregon State University (OSU) dairy farm, which are negative for Johne's disease, were isolated by using Percoll density gradient. Monocytes were then resuspended in RPMI 1640 supplemented with 10% FBS. The monocytes obtained were seeded at 2×10^5 per well and were treated with recombinant cytokines. Monocytes were matured for 7 days (35, 36) and were used when the morphology was indicative of dendritic cells. Dendritic cells were then maintained in RPMI 1640 supplemented with 10% FBS. To infect the dendritic cells (approximately 10^5 cells), we used 10^6 bacteria for 1 h. Afterward, monolayers were washed three times, and the lysate was plated onto 7H10 agar with mycobactin J. In some experiments, polarized monolayers of MDBK cells in a Transwell system were infected with 5×10^6 *M. avium* subsp. *paratuberculosis* bacteria for 2 h. Twenty-four hours after the infection, the translocated bacteria in the supernatant in the lower chamber were quantified. The cell culture supernatant was obtained and transferred to a 24-well plate with adherent dendritic cells. Then 1×10^5 dendritic cells were incubated with 1×10^5 translocated *M. avium* subsp. *paratuberculosis* bacteria for 1 h. As controls, bacteria in 7H9 medium supplemented with mycobactin J were used. Monolayers were repeatedly washed and lysed, and the internalized bacteria were quantified.

Statistical analysis. The comparisons among experimental groups and the control were evaluated for statistical significance by using Student's *t* test or the Mann-Whitney test.

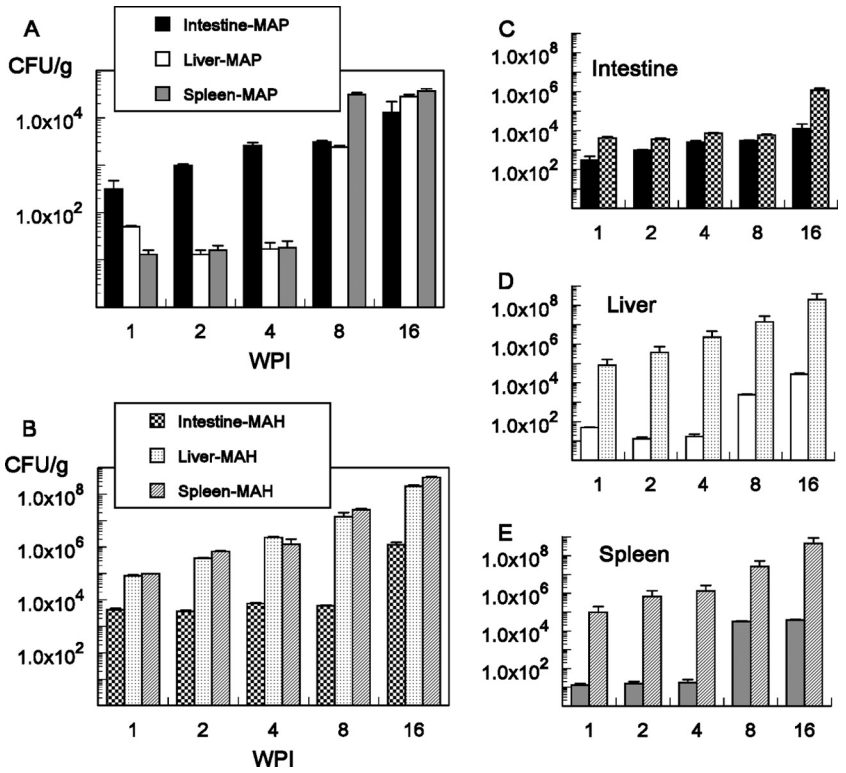

FIG. 1. Microbial burdens in organs of infected C57BL/6 black mice. (A and B) Mice were infected as described in Materials and Methods, and microbial burdens in animals infected with *M. avium* subsp. *paratuberculosis* (MAP) (A) or *M. avium* subsp. *hominissuis* (MAH) (B) were determined as a function of time. Note that different scales are used for the *y* axes in panels A and B. (C, D, and E) The same data are plotted by using the same *y* axis scale for all graphs, highlighting the comparison of organ burdens between the two *M. avium* subspecies. Mice were infected with 2.4×10^6 *M. avium* subsp. *hominissuis* organisms or with 3.8×10^6 *M. avium* subsp. *paratuberculosis* organisms. Each bar represents the mean data collected from 20 mice. Error bars, standard errors of the means. $P < 0.05$ for the comparison between *M. avium* subsp. *hominissuis* and *M. avium* subsp. *paratuberculosis* at the same time point for each of the organs.

RESULTS

Infection of C57BL/6J with *M. avium* subsp. *hominissuis* or *M. avium* subsp. *paratuberculosis*. To evaluate whether *M. avium* subsp. *paratuberculosis* invades the intestinal mucosae of mice, and if it does so differently from *M. avium* subsp. *hominissuis*, mice were infected orally, and the numbers of bacteria in the terminal ileum, liver, and spleen were monitored over time. In C57BL/6 black mice, the intestinal bacillary counts for *M. avium* subsp. *paratuberculosis* increased monotonically during the 16-week period. In contrast, *M. avium* subsp. *paratuberculosis* did not grow to significant numbers in the liver or spleen at 4 wpi, but moderate dissemination to the liver and the spleen was observed at 8 and 16 wpi (Fig. 1A). Intestines remained the organ with the greatest microbial burden during this period. Infection with *M. avium* subsp. *hominissuis* followed a different course, with bacillary counts increasing monotonically in the liver and the spleen, while intestinal counts remained approximately constant, increasing only at 16 wpi (Fig. 1B). Comparison on a per-organ basis led to the observation that *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *hominissuis* reached statistically significantly different levels in the intestines of infected animals, but the difference was less than 100-fold for all cases (Fig. 1C). Dramatic differences, with extremely high counts for *M. avium* subsp. *hominissuis*-infected animals, were observed in the liver (Fig. 1D) and the spleen (Fig. 1E). In all cases, *M. avium* subsp. *hominissuis* burdens 500-fold greater than *M. avium* subsp. *paratuberculosis* burdens were observed. In summary, *M. avium* subsp. *paratuberculosis* is less efficient at dissemination in the host than *M. avium* subsp. *hominissuis*. These results further confirmed that the mouse can be used as a model for investigating the interaction between *M. avium* subsp. *paratuberculosis* and the intestinal mucosa.

Intestinal loop. To determine whether *M. avium* subsp. *paratuberculosis* needs Peyer's patches in order to cross the intestinal mucosa, we used an intestinal-loop model with C57BL/6 wild-type and B-cell knockout mice, which lack Peyer's patches. As seen in Table 1, both *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *hominissuis* (used as a control) (21) are able to enter the intestinal mucosa equally well in the presence or absence of Peyer's patches. That finding, though, does not necessarily indicate that *M. avium* subsp. *paratuberculosis* invades the intestinal mucosa through the Peyer's patches and Peyer's patch-deficient segments in the wild-type mouse.

Preferential site of invasion. To determine the site of entry of *M. avium* subsp. *paratuberculosis* into the intestinal tract, mice were infected orally, and three segments (1 cm each) containing Peyer's patches and three segments containing non-Peyer's patch regions were homogenized and plated. As shown

TABLE 1. Efficacy of invasion of the intestinal mucosae of C57BL/6 mice and B-cell-deficient mice (lacking Peyer's patches) by *M. avium* subsp. *paratuberculosis* using an intestinal-loop model[a]

Bacterium	% of inoculum inside the mucosa[b]			
	C57BL/6J mice		B-cell-deficient mice	
	1 h	3 h	1 h	3 h
M. avium subsp. *paratuberculosis*	5.1 ± 0.3	36 ± 10	4.3 ± 0.5	30 ± 6
M. avium subsp. *hominissuis*	7.6 ± 0.4	54 ± 9	6.8 ± 0.3	52 ± 4

[a] Bacteria (1.7×10^7 *M. avium* subsp. *hominissuis* organisms or 2.5×10^7 *M. avium* subsp. *paratuberculosis* organisms) were injected into the intestinal loop.
[b] The experiment was repeated twice, with a total of 10 mice per time point. *P* was >0.05 for comparison of the percentages of invasion of C57BL/6J versus B-cell-deficient mice.

in Table 2, *M. avium* subsp. *paratuberculosis* enters equally well into the mucosal area of Peyer's patches and into surrounding areas that do not contain M cells. Moreover, *M. avium* subsp. *paratuberculosis* bacilli were visualized in association with enterocytes by transmission electron microscopy (Fig. 2).

To determine whether the enterocyte route was important for infection, we carried out an *in vivo* experiment in which wild-type C57BL/6J mice were infected orally with *M. avium* subsp. *paratuberculosis*, and 4 h and 24 h later, the number of bacteria in the intestinal wall was determined. As shown in Table 2, comparison between the number of bacteria in Peyer's patch and non-Peyer's patch areas identified a slight preference for the bacteria to enter the non-Peyer's patch segments, though the differences were not statistically significant ($P > 0.05$). Histopathological observations indicated that wild-type mice infected with *M. avium* subsp. *paratuberculosis* displayed approximately the same number of bacteria in the submucosa as that in corresponding tissues from B-cell KO mice (data not shown).

Interactions with a polarized monolayer. *M. avium* subsp. *paratuberculosis* incubated with polarized MDBK epithelial cells crossed the monolayer from the apical to the basolateral surface. As shown in Table 3, significant crossing occurs after 2 h. Crossing of the monolayer by *M. avium* subsp. *paratuberculosis* did not alter the transmembrane resistance. When bacteria were incubated in the presence of whole milk, a significantly greater number of organisms translocated MDBK cells at early time points (Table 3).

Role of $\alpha_5\beta_1$ integrin in *M. avium* subsp. *paratuberculosis* uptake by MDBK cells. Past work had suggested the possible role of fibronectin in the translocation of the bacterium through the intestinal tract. To investigate if *M. avium* subsp. *paratuberculosis* invades MDBK cells using the fibronectin receptor, we treated MDBK monolayers with a mouse anti-$\alpha_5\beta_1$ IgG antibody or with an irrelevant anti-*Escherichia coli* lipopolysaccharide (LPS) IgG2a antibody at 30 μg/ml. Because we did not know whether the anti-human integrin would bind to the bovine antigen, we used a *Staphylococcus aureus* clinical isolate as a control. Incubation of *S. aureus* in the presence of the anti-$\alpha_5\beta_1$ antibody led to an 85% ± 5% reduction in uptake in 1 h. In contrast, the entry of *M. avium* subsp. *paratuberculosis* was reduced by 37.3%. In the initial inoculum, most of the *M. avium* subsp. *paratuberculosis*, which was internalized by MDBK cells, entered by a different pathway than the fibronectin receptor. As shown in Table 4, the control antibody results support the absence of nonspecific effects.

TABLE 2. Preferential site of *M. avium* subsp. *paratuberculosis* invasion of the intestinal mucosae of C57BL/6J mice

Time	Concn of bacteria (CFU/g of intestinal tissue)[a]	
	Peyer's patch segments	Non-Peyer's patch segments
4 h	3.6 ± 0.4	4.1 ± 0.8
24 h	8 ± 2	9.3 ± 1.7

[a] Number of colonies in 1 cm of terminal ileum. Each value is the average for five mice (three segments per mouse). $P > 0.05$ for all comparisons.

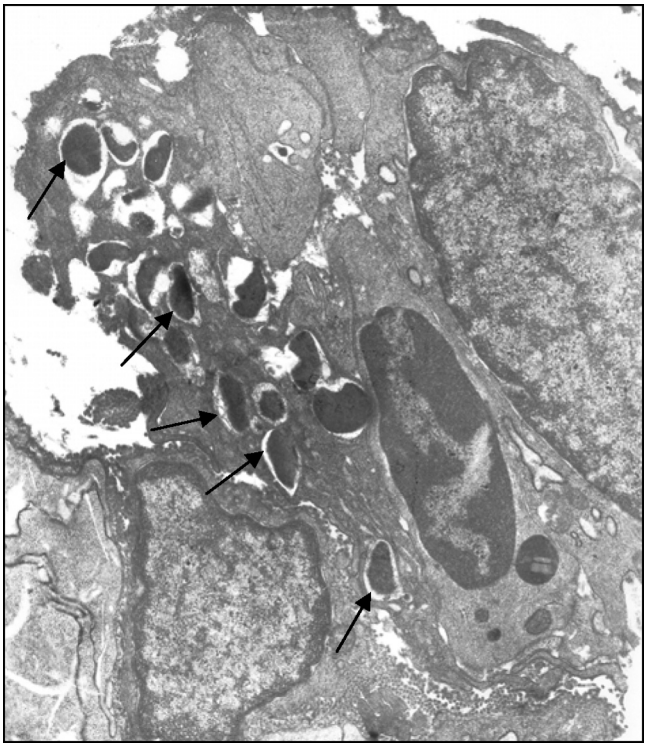

FIG. 2. Transmission electron micrograph of a sample from a mouse orally infected with *M. avium* subsp. *paratuberculosis*. The intestine was harvested at 2 days postinfection. *M. avium* subsp. *paratuberculosis* can be observed within enterocytes (arrows). Magnification, ×10,000.

Uptake by dendritic cells. *M. avium* subsp. *paratuberculosis* was placed in the presence of dendritic cells, and the percentage of bacteria that were internalized was determined. After 15 min, $(3 \pm 0.4) \times 10^1$ *M. avium* subsp. *paratuberculosis* bacteria were observed inside dendritic cells (0.003% of the inoculum), while at 30 min and 1 h, $(6 \pm 0.6) \times 10^2$ (0.01% of the inoculum) and $(1.5 \pm 0.3) \times 10^4$ (1.8% of the inoculum) bacteria were ingested by dendritic cells, respectively (Table 5). Since *M. avium* subsp. *paratuberculosis* is likely to encounter dendritic cells upon translocation of the mucosal epithelium, we infected polarized MDBK cells and used the translocated bacteria to infect dendritic cells. As shown in Fig. 3, translocated bacteria were significantly more efficiently internalized by dendritic cells than bacteria grown on plates. Observation of

TABLE 3. Translocation of *M. avium* subsp. *paratuberculosis* across an epithelial cell monolayer

Condition	2 h		6 h		24 h	
	% of inoculum that translocated	Transmembrane resistance (Ω)[a]	% of inoculum that translocated	Transmembrane resistance (Ω)	% of inoculum that translocated	Transmembrane resistance (Ω)
No exposure to milk (5×10^5 bacteria)	0.2 ± 0.05	320 ± 10	0.4 ± 0.04[c]	310 ± 30	0.6 ± 0.05	330 ± 24
Preincubation with milk (2×10^5 bacteria)[b]	0.4 ± 0.06	320 ± 26	0.7 ± 0.04[c,d]	328 ± 24	1.5 ± 0.06[c,d]	325 ± 39

[a] Before the addition of bacteria, the transmembrane resistance was 320 Ω.
[b] *M. avium* subsp. *paratuberculosis* was incubated in the presence of whole milk for 2 h, washed, and used in the experiments.
[c] $P < 0.05$ for comparison with the previous time point.
[d] $P < 0.05$ for comparison with *M. avium* subsp. *paratuberculosis* incubated without milk.

monolayers for as long as 10 days showed a stable number of intracellular bacteria without significant growth.

DISCUSSION

In this study, we developed a novel low-dose (ca 2.5×10^7-CFU) oral-infection model of mice in order to compare the pathogenesis of *M. avium* subsp. *hominissuis* and *M. avium* subsp. *paratuberculosis*. In contrast, other studies using oral infection of mice have used a significantly higher dose (1.0×10^{10} to 1.0×10^{11} CFU). High-dose oral experimental infection with *M. avium* subsp. *paratuberculosis* resembles the intraperitoneal infection model, resulting in higher microbial burdens in the livers and spleens of infected animals than in the intestines (11). Our model reflects more closely the natural infection of ruminants, in that there is a higher level of colonization of the intestinal mucosa by *M. avium* subsp. *paratuberculosis* early in the infection (up to 4 wpi). However, at a similar infection dose, *M. avium* subsp. *hominissuis* is significantly more effective at disseminating in the host, reaching high colonization levels in the liver and spleen. Thus, this model reveals significant differences in the pathogenesis of these subspecies, as reflected in natural infections. In this context, *M. avium* subsp. *paratuberculosis* infects calves, and most of the bacteria remain in the intestinal wall, leading to lesions characteristic of Johne's diseases in the adult animal (25). A small percentage of the bacteria disseminate, with some spreading to distant tissues, such as the mammary gland (27) and local lymph nodes (28). *M. avium* subsp. *hominissuis*, in contrast, crosses the intestinal mucosa by entering enterocytes (21), where it suppresses chemokine production (22) and, therefore, inflammatory cell migration (13). *M. avium* subsp. *hominissuis* disseminates to mesenteric lymph nodes (19) and, in immunosuppressed individuals, to distant sites such as spleen and bone marrow (12).

M. avium subsp. *paratuberculosis* infects young calves through the gastrointestinal tract (9, 12, 27). It has been hypothesized that this microorganism crosses the intestinal mucosa by the Peyer's patches, reaching the submucosa (12, 19). However, a recent study with goat kids suggested that *M. avium* subsp. *paratuberculosis* enters the intestinal mucosa, translocating through enterocytes (20). By using B-cell knockout mice deficient in Peyer's patches, it was observed that *M. avium* subsp. *paratuberculosis* can still enter the intestinal wall. *M. avium* subsp. *paratuberculosis* can infect the mucosa similarly in C57BL/6J wild-type mice and in B-cell knockout animals, and when given orally, the bacterium can be found both in regions with Peyer's patches and in regions in which there are no Peyer's patches. The histopathological alterations at the initial stage of infection appear to be the same, suggesting that the two subspecies cross the mucosa similarly, but the kinetics of dissemination differ significantly. The molecular basis for this difference between these closely related organisms is unknown. Nonetheless, comparison of the two genomes unveiled the occurrence of genome segments specific for either *M. avium* subsp. *paratuberculosis* or *M. avium* subsp. *hominissuis* (38). The study of these genomic regions might help to explain these phenotypic differences. Alternatively, differential gene expression of key virulence determinants in these two subspecies may account for these observations.

By using a mouse strain lacking Peyer's patches (B-cell KO mice) and comparing the level of infection with that of the

TABLE 4. Impact of the β_1 integrin receptor on the entry of *M. avium* subsp. *paratuberculosis* into MDBK cells

Experimental group	*S. aureus*		*M. avium* subsp. *paratuberculosis*	
	No. of intracellular bacteria[a]	% Δ[b]	No. of intracellular bacteria[a]	% Δ
Control (no antibody)	$(3.4 \pm 0.5) \times 10^4$		$(2.6 \pm 0.4) \times 10^3$	
Anti-$\alpha_5\beta_1$	$(5.1 \pm 0.3) \times 10^3$	85[c]	$(9.7 \pm 0.2) \times 10^2$	37.3[d]
Anti-LPS	$(3.2 \pm 0.4) \times 10^4$	−5[d]	$(3.0 \pm 0.3) \times 10^3$	15[d]

[a] The inoculum was 1.8×10^5 organisms for *S. aureus* and 1.1×10^5 organisms for *M. avium* subsp. *paratuberculosis*.
[b] Δ, reduction.
[c] $P < 0.05$ for comparison with the no-antibody control.
[d] $P > 0.05$ for comparison with the no-antibody control.

TABLE 5. Infection of bovine dendritic cells[a] by *M. avium* subsp. *paratuberculosis*

Time	No. of intracellular bacteria	% of inoculum
Uptake		
15 min	$(3 \pm 0.4) \times 10^1$	0.003
30 min	$(6 \pm 0.6) \times 10^2$	0.01
Intracellular growth		
1 h	$(1.5 \pm 0.3) \times 10^4$	
4 days	$(3.8 \pm 0.3) \times 10^4$	1.8

[a] A total of 1×10^6 monocyte-derived macrophages were incubated with human GM-CSF and IL-4 for maturation into dendritic cells.

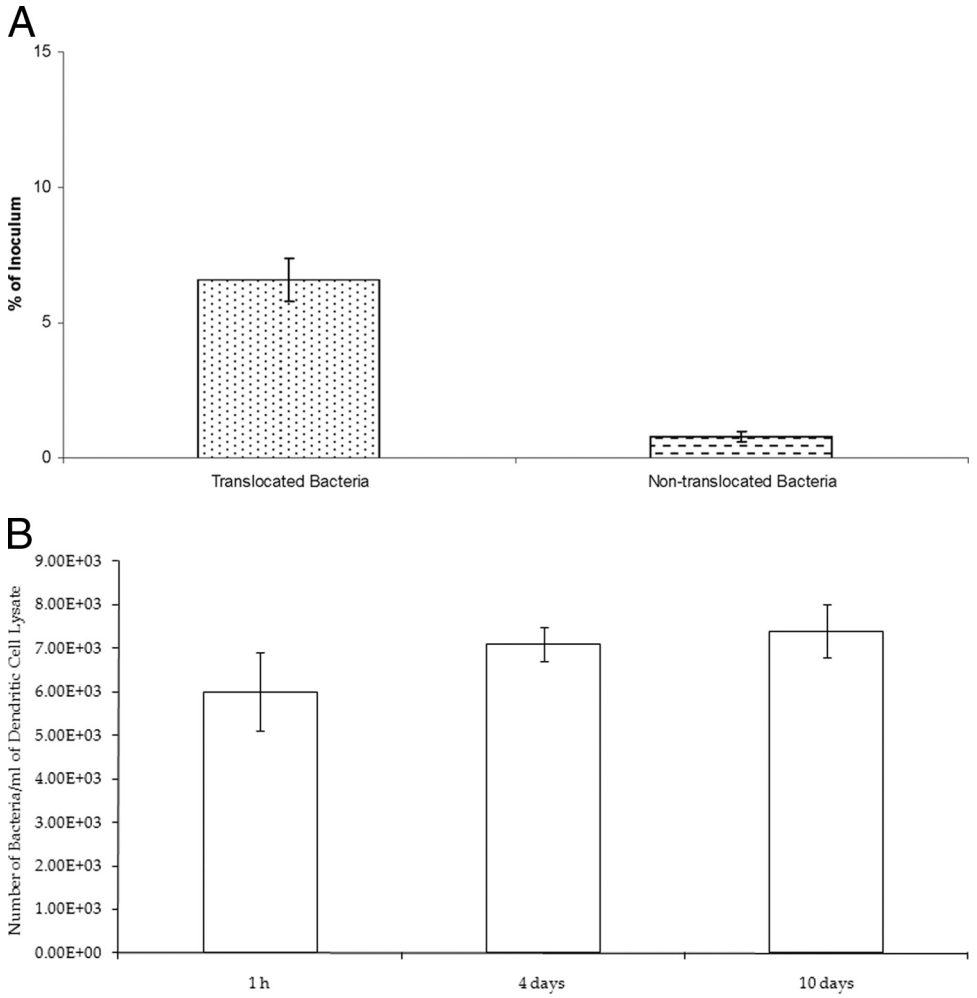

FIG. 3. Uptake and survival of *M. avium* subsp. *paratuberculosis* in dendritic cells. (A) Uptake of *M. avium* subsp. *paratuberculosis* by bovine dendritic cells following translocation across a polarized monolayer of MDBK cells. Uptake by dendritic cells was allowed for 1 h. Bacteria obtained from 7H9 broth medium supplemented with mycobactin J were used as a control. (B) Number of *M. avium* subsp. *paratuberculosis* bacteria in dendritic cells 10 days after infection (no significant differences in growth were observed).

wild-type mouse, it became evident that the levels of tissue infection were similar, independently of the existence of Peyer's patches. In the presence of Peyer's patches, the bacterium used the M cells to cross the mucosa, but in the absence of the patches, *M. avium* subsp. *paratuberculosis* was capable of interacting with enterocytes (Fig. 2). Additional experiments confirmed that bacteria delivered orally infected both Peyer's patches and non-Peyer's patch regions (Fig. 4 depicts a schematic model). Whether the mechanisms of interaction are the same is currently unknown. Bacterial genes required for the invasion of bovine epithelial cells have been identified (1), but all the evidence so far supports the hypothesis that uptake by M cells follows binding to the β_1 integrin receptor (15, 23, 24). Our study confirmed that *in vitro*, invasion of MDBK cells by *M. avium* subsp. *paratuberculosis* does not depend on the β_1 integrin receptor as a primary mechanism of uptake.

What would be the evolutionary advantage for *M. avium* subsp. *paratuberculosis* of crossing the intestinal mucosa by two different pathways? We hypothesize that the function of the M-cell pathway is to recognize and contain the infection, while the enterocyte pathway represents the pathogenic route leading to dissemination. This hypothesis is consistent with the use of the enterocyte entry pathway by *M. avium* subsp. *hominissuis*, the intrinsically more virulent subspecies. The other plausible hypothesis is that each mechanism of entry represents a different outcome, both of which are important for the disease. Interaction with the Peyer's patches would result in rapid interaction with phagocytic cells, local spread, and an inflammatory response. In contrast, the infection of enterocytes would lead to a slow inflammatory response, translocation, and dissemination to distant sites, including the mammary glands. In the case of *M. avium* subsp. *hominissuis*, infection of enterocytes results in slow translocation followed by faster systemic dissemination (21). Alternatively, cells of the dendritic cell lineage might play a role in the dissemination of *M. avium* subsp. *paratuberculosis*. In fact, *M. avium* subsp. *paratuberculosis* infection of dendritic cells is inefficient. However, if the bacterium translocates across the epithelial barrier, uptake into dendritic cells increases 3- to 6-fold. This finding suggests that once bacteria cross the intestinal mucosa, they are readily

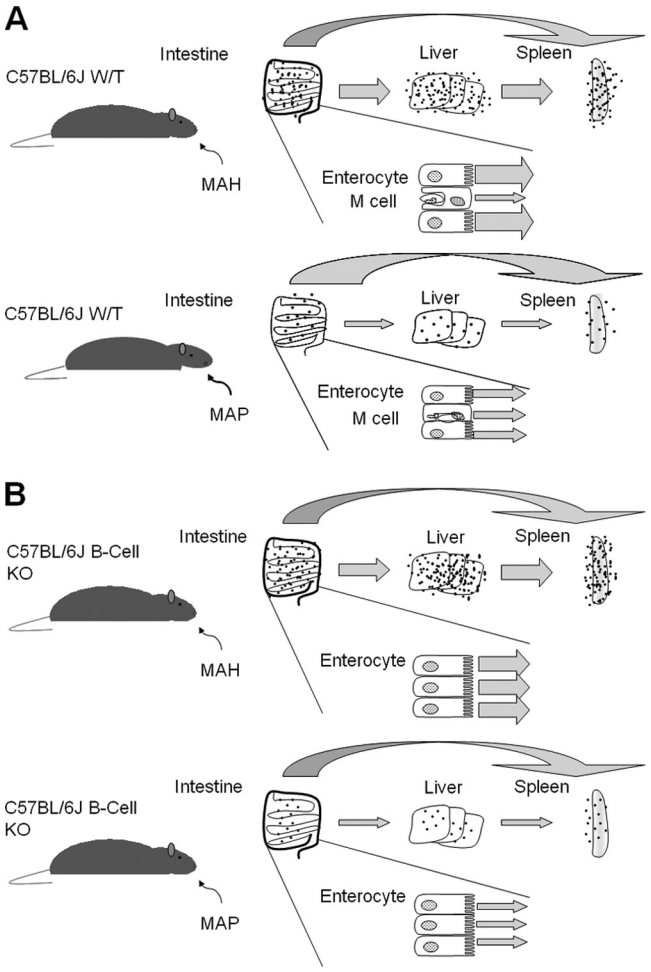

FIG. 4. Mouse model of *M. avium* infection. Shown are predicted outcomes of oral infection of wild-type (C57BL/6J WT) (A) and B-cell-deficient (C57BL/6J B-cell KO) (B) mice with *M. avium* subsp. *hominissuis* (MAH) or *M. avium* subsp. *paratuberculosis* (MAP). Dots indicate bacillary burdens, and arrow widths are proportional to the numbers of translocated bacilli. In the model depicted, *M. avium* subsp. *hominissuis* is translocated mostly via enterocytes in WT mice.

ingested by dendritic cells. Once within dendritic cells, *M. avium* subsp. *paratuberculosis* was able to persist for 10 days, despite the lack of growth. Thus, dendritic cells may be responsible for the rapid spread of *M. avium* subsp. *paratuberculosis* to mesenteric lymph nodes, as observed by Wu and colleagues (39).

The finding that *M. avium* subsp. *paratuberculosis* translocates through enterocytes in our mouse model may also have implications for the potential colonization of the human epithelium. Recently, Golan et al. developed an *M. avium* subsp. *paratuberculosis* infection model using SCID mice transplanted with fetal human intestinal xenografts (7). Mice were infected by intraluminal challenge using a relatively low dose (approximately 5×10^7 CFU). The results indicated that goblet cells were predominantly infected compared to enterocytes. However, there are significant differences in the SCID model, including the use of human xenografts, the inoculation route, the complete absence of both B and T cells, and a complex interplay of both mouse and human innate immune cells. These differences may explain the predilection of *M. avium* subsp. *paratuberculosis* to invade either enterocytes or goblet cells. In this regard, intracellular pathogens, such as *Salmonella* spp., have been shown to invade both M cells and enterocytes, and, to a lesser extent, goblet cells, in mouse models, as reviewed by van Asten (34).

Our *in vitro* studies using polarized monolayers suggest that binding and internalization of *M. avium* subsp. *paratuberculosis* do not cause a decrease in transmembrane resistance up to 24 h of infection. In addition, the bacterium translocated through the epithelial monolayer, a finding that is in agreement with recent observations by Wu and colleagues that *M. avium* subsp. *paratuberculosis* is found in the mesenteric lymph nodes of cows just 2 h after infection (39). Although surgery and intestinal clumping can be associated with increased permeability and can explain, in part, the results of Wu and colleagues, the observation appears to suggest that *M. avium* subsp. *paratuberculosis* crosses the mucosal barrier. However, the percentage of bacteria that translocates is very small. Histopathological observations of cattle infected with *M. avium* subsp. *hominissuis* and *M. avium* subsp. *paratuberculosis* indicate that while *M. avium* subsp. *hominissuis* spreads quickly from the intestinal mucosa, *M. avium* subsp. *paratuberculosis* disseminates very slowly (30), which, in fact, was observed in our system.

In conclusion, this study demonstrates that *M. avium* subsp. *paratuberculosis* uses both Peyer's patches and enterocytes to cross the intestinal mucosa, a mechanism slightly different from that of *M. avium* subsp. *hominissuis*, which enters the mucosa preferentially by enterocytes (21). *M. avium* subsp. *paratuberculosis* also differs from *M. avium* subsp. *hominissuis* in the efficiency of dissemination. Further studies should focus on the understanding of those differences at the molecular level.

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