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NOTES

Sequestration of *Mycobacterium tuberculosis* in Tight Vacuoles In Vivo in Lung Macrophages of Mice Infected by the Respiratory Route

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Following aerosol infection of mice with *Mycobacterium tuberculosis*, single mycobacteria or pairs of bacilli were observed within individual phagocytic vacuoles bound by tightly apposed vacuolar membranes. The virulent organism was not observed free in the cytoplasm of the parasitized cells or in the extracellular space of the lung granulomata. This study indicates that in vivo, virulent mycobacteria survive and probably replicate within a unique tight vacuole in the infected phagocyte within the lung.

Since *Mycobacterium tuberculosis* survives within macrophages, the host-parasite relationship is central to understanding the pathogenesis of tuberculosis. A number of in vitro infection models have been used to study the cellular interaction between the host phagocyte and the infecting mycobacterium. When either human or murine cells are infected in vitro, mycobacteria are rapidly phagocytosed and the organisms seem to reside in membrane-bound vacuoles within the cells (1, 2, 6, 9). Studies with these in vitro systems suggest that phagosome-lysosome fusion is altered following ingestion of the mycobacteria (1, 3, 4, 10). Recent data indicate that acidification of the mycobacterium-containing vacuole is prevented by exclusion of the proton-ATPase (10). It has also been suggested that virulent mycobacteria can escape the phagosomal vacuole into the cytoplasm of the cell, thus avoiding exposure to lysosomal enzymes and low pH (5). The size of the infectious inoculum and the viability of the organisms appear to be two of the important variables in determining the fate of the mycobacteria in the vacuole (1, 3). An important limitation of these intriguing experiments is that they have been carried out in vitro, usually with avirulent mycobacteria. Therefore, the results may not reflect the complexity of the host-parasite interaction of virulent organisms within the chronically infected host. In vivo, the localization of the mycobacteria at the ultrastructural level within the granulomatous infiltration has not been clearly documented.

In this study, we describe the host cell-mycobacterium interaction and the inflammatory response to virulent *M. tuberculosis* Erdman infection in vivo. Female B6D2/F1 (C57B/6 × DBA2) mice (BCG^{rs} genotype and BCG^{r} phenotype), 7 to 9 weeks old, obtained from the Trudeau animal breeding facility (Saranac Lake, N.Y.) were inoculated via the respiratory route with *M. tuberculosis* Erdman (Trudeau Institute mycobacterium culture collection) by exposure to aerosolized mycobacteria with a Middlebrook Airborne Infection Apparatus (TRJ-R Instruments, Rockville, N.Y.). The aerosol was generated by a nebulizer loaded with 10 ml of a saline suspension containing approximately 5×10^6 viable organisms per ml. Mice were exposed to the aerosol for 30 min, resulting in implantation of approximately 200 organisms into the lungs of each mouse, as confirmed by plating lung homogenates 2 h after infection. The alveolar route of infection was utilized because of its importance as the natural mode of transmission for human tuberculosis. Also, this route of infection has been reported to lead to an aggressive disease in the lungs of mice, with a faster mycobacterial generation time compared to the more traditionally used intravenous model of infection (7, 8). Alternatively, mice were infected by intravenous injection of 5×10^5 organisms per mouse in a lateral tail vein. The progression of the infection was evaluated weekly by light and electron microscopy. The bacterial loads in the lungs, livers, and spleens of infected mice were evaluated as described previously (7).

The high levels of mycobacterial multiplication achieved following aerosol infection of mice afforded us the opportunity to study the ultrastructural detail of the host-parasite relationship in an in vivo model that closely mimics the pattern of human tuberculosis infection. The cellular composition of the infiltrate and the localization of mycobacteria in the cells of the granulomata within the infected lungs were examined by light and electron microscopy. Lungs of infected mice were fixed in 10% formalin for 24 h. Paraffin-embedded sections were stained with hematoxylin-eosin and with Ziehl-Neelsen stain for determination of acid-fast bacilli (AFB) by light microscopy. Morphometry of the lesions was performed with Microcomp, a computer-based image analysis system. A calibration micrometer slide was used to determine the area evaluated. The electron microscopy study was carried out as previously described (9).

***M. tuberculosis*-induced pathology in the lungs and localization of AFB by light microscopy.** Mice infected by the respiratory route with an aerosol dose which resulted in the seeding of 200 *M. tuberculosis* bacilli/mouse (both lungs) showed a progressive and rapid granulomatous response in the lungs. At 7 days after infection, histologic examination revealed normal-appearing lungs, with the exception of a few mononuclear

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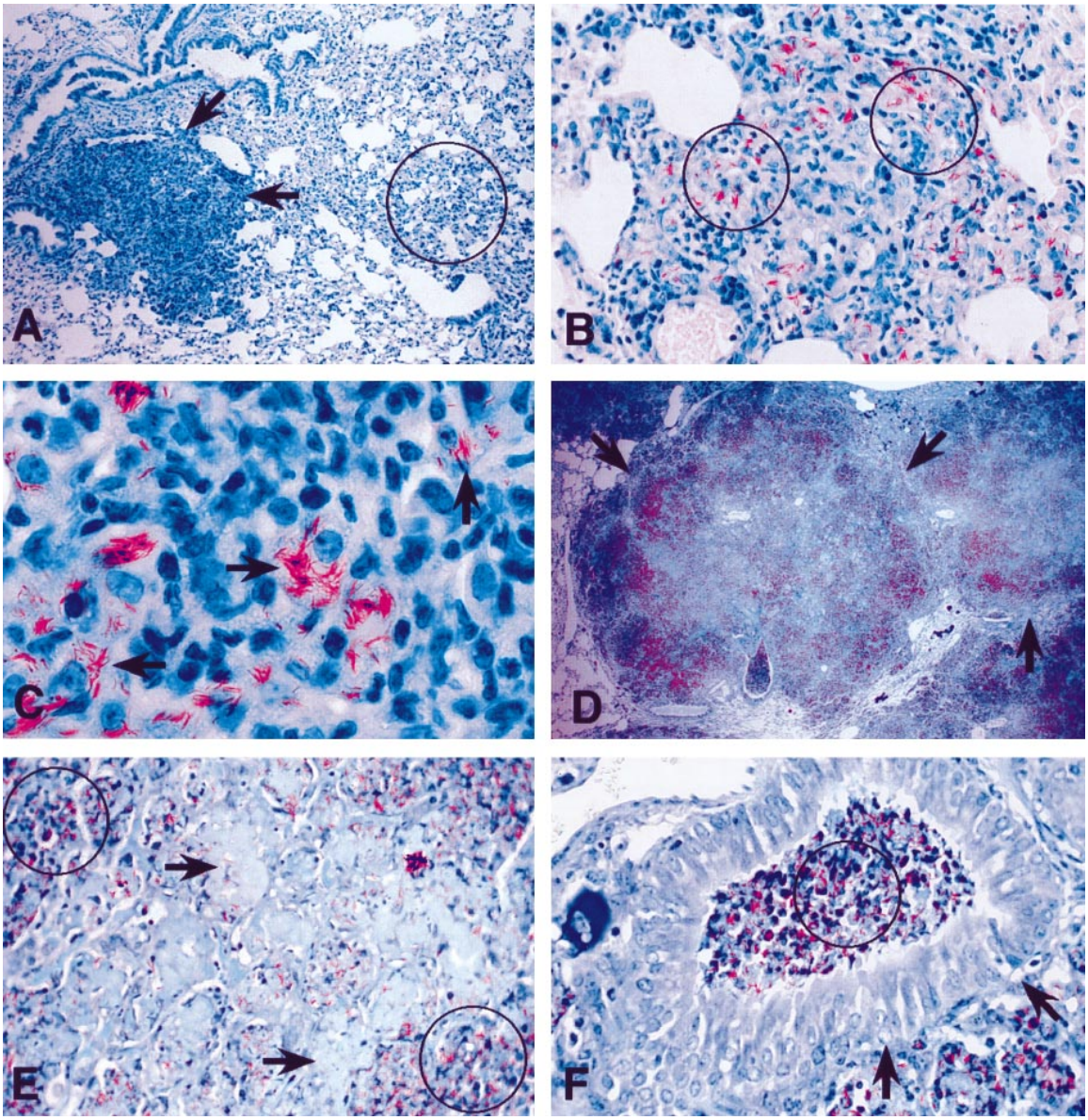

FIG. 1. Histologic sections of lungs of B6D2F1 mice infected by aerosol with *M. tuberculosis* Erdman, stained for AFB (Ziehl-Neelsen stain). Micrographs are representative of the observations gathered from five mice per time point. (A) Micrograph of the lung of a mouse at 14 days postinfection, showing the formation of a granuloma (arrows) and significant inflammatory infiltration of the lung parenchyma between the granulomata (circle). Magnification, ×50. (B) Micrograph of granuloma in the lung of a mouse at 21 days postinfection, showing leukocytes parasitized by AFB (circle). Magnification, ×200. (C) Micrograph of large parasitized macrophages (arrows) in a lung granuloma at 21 days postinfection. Magnification, ×500. (D) Micrograph of the lung of a mouse at 28 days postinfection. The granulomata (arrows) are larger than those at days 14 and 21 and are almost confluent. More than 80% of the lung parenchyma is occupied by infiltrating mononuclear cells and edema. Magnification, ×20. (E) Micrograph showing necrosis in the center of a granuloma at 28 days postinfection. Mycobacteria are associated with cells (circle) but not with the fully necrotic areas (arrows). Magnification, ×200. (F) Micrograph showing the obstruction of a bronchus (arrows) with *M. tuberculosis*-infected leukocytes (circle) at 28 days postinfection. Magnification, ×200.

leukocytes that accumulated in small foci in the alveoli. By 14 days postinfection the focal inflammatory aggregates in the alveolar space were larger and contained numerous mononuclear leukocytes (Fig. 1A). This is in contrast to the relative paucity of mycobacteria in the lung at 14 days following an intravenous infection. Large macrophages parasitized by AFB were also evident at 21 days postinfection (Fig. 1B and C). At 28 days after alveolar infection, the granulomata were extensive and often coalescent, occupying most of the alveolar space (Fig. 1D). Necrosis was prominent in the center of the granulomata (Fig. 1D and E), which were surrounded by heavily parasitized macrophages. Mononuclear phagocytes containing AFB were also observed inside the lumen of the bronchi, suggesting erosion or invasion of terminal bronchi (Fig. 1F). Although the size of the granulomata increased with time, the number of granulomata per 10 mm^2 of lung tissue did not change significantly (Table 1).

In these experiments, the generation time for *M. tuberculosis* in the lungs during the first 14 days after aerosol infection was 0.96 day (Table 1), in contrast to 1.69 days for the intravenous infection (not shown). On subsequent days (14 to 35 days postinfection), *M. tuberculosis* continued to grow faster in the

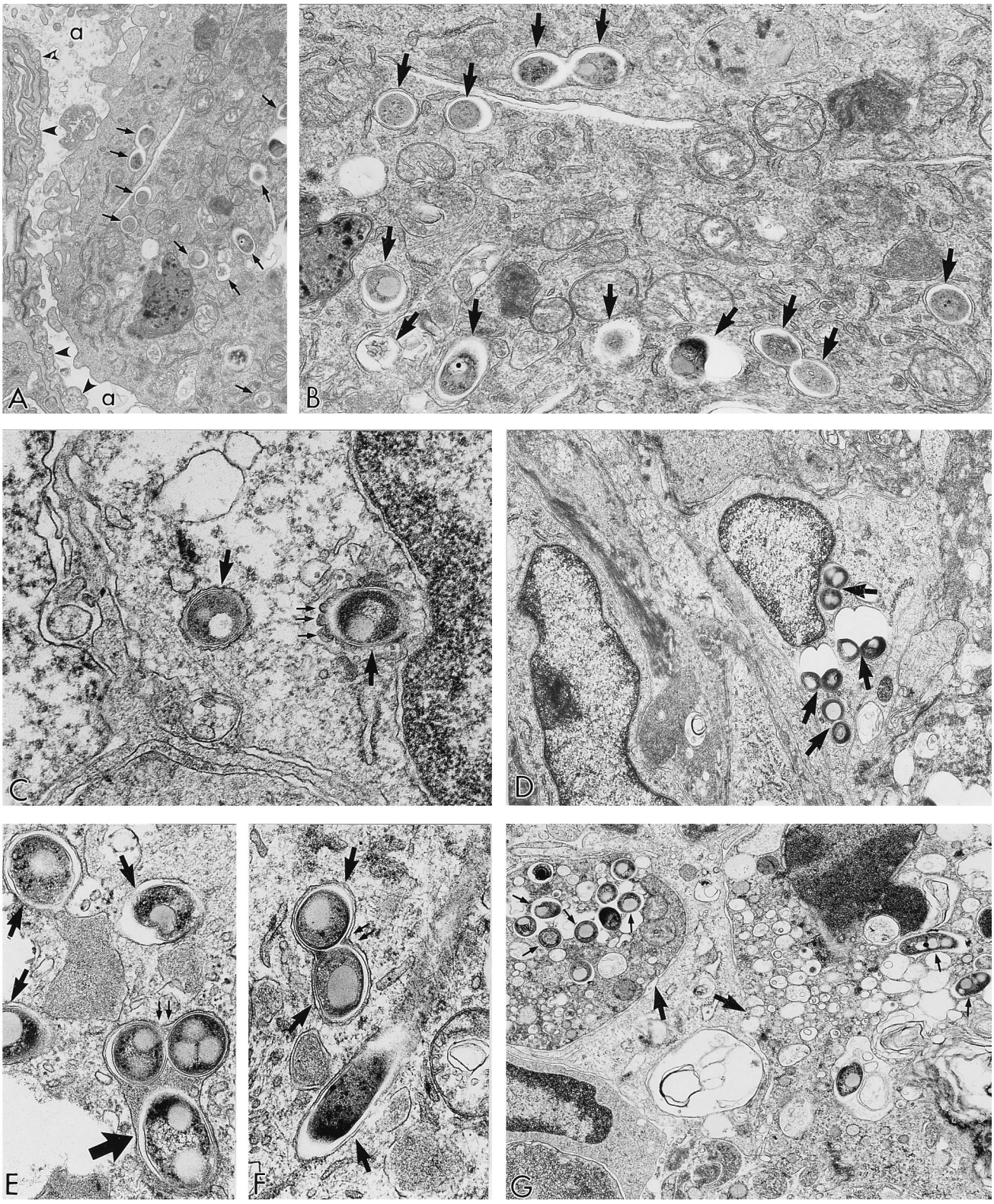

FIG. 2. Electron micrographs of the lungs of mice infected by aerosol with *M. tuberculosis* Erdman. (A) Micrograph of the lung of a mouse at 14 days postinfection, showing single and pairs of mycobacteria inside tight vacuoles (arrows) in the cytoplasm of a parasitized macrophage. Arrowheads point to the alveolar endothelial lining. The macrophage is localized to the alveolar space (a). Magnification, ×7,900. (B) Higher magnification of panel A, showing single and pairs of mycobacteria in tight phagocytic vacuoles. Magnification, ×18,000. (C) Micrograph showing single mycobacteria inside a phagocytic cell at 21 days postinfection. Note the membrane vesiculation associated with the phagocytic vacuole (small arrows). Magnification, ×28,500. (D) Micrograph of pairs of organisms, presumably dividing (arrows), inside vacuoles within a parasitized macrophage at 21 days postinfection. Magnification, ×9,500. (E and F) Micrographs of single and pairs of organism inside membrane-bound vacuoles (large arrow). Small arrows indicate vacuoles containing pairs of bacteria at 21 days postinfection. Magnification, ×26,500. (G) Micrograph showing phagocytosis of cell debris (large arrows) with multiple mycobacteria inside vacuoles (small arrows) at 28 days postinfection. Magnification, ×10,000.

TABLE 1. Granuloma formation in the lungs of B6D2F1 mice after aerosol infection with *M. tuberculosis* Erdman[a]

Day postinfection	Granuloma formation		*M. tuberculosis*	
	No. per 10 mm^{2b}	Size $(mm^2)^b$	CFU $(\log_{10})^b$	Generation time (days)
14	4.9 ± 0.8	0.03 ± 0.004	6.67 ± 2.0	0.96
28	6.4 ± 1.2	2.73 ± 0.5	9.4 ± 1.0	1.53

[a] Morphometry of the lung lesions was performed with Microcomp, a computer-based image analysis system. A calibration micrometer slide was used to determine the area evaluated. The generation time was calculated as previously described (11) for between days 0 and 14 postinfection and between days 15 and 28 postinfection.

[b] Means ± standard errors of the means for five mice per time point.

lungs of mice infected by the aerosol route, with a doubling time of 1.53 days, while in intravenously infected mice, growth was steady but slower, with a doubling time of 8 days. These observations are in agreement with a previous report demonstrating a more aggressive lung disease in mice infected by aerosol than in mice infected intravenously (7).

Mycobacterial localization in the macrophages of the granulomata. In the lungs of mice infected by aerosol, there was a progressive increase in the bacillary load as revealed by electron microscopy from day 14 to 35. By 21 days postinfection, parasitized phagocytes were observed in every histologic section from infected lungs. Electron-dense mycobacteria were observed singly or in pairs inside tight membrane-bound cytoplasmic vacuoles within macrophages (Fig. 2). In some cases small membrane vesicles appeared to be either fusing with or pinching off the phagocytic vacuole (Fig. 2C, small arrows). Two or more bacilli were often seen within tight membrane-bound vacuoles. On occasion, invagination of the membrane between two organisms in a single vacuole was suggestive of bipartition of the vacuoles (Fig. 2A, B, and D to F). These observations together with the increasing colony counts from the lungs at different time intervals are indicative of mycobacterial multiplication. Similar observations have been made for macrophages infected in vitro with either *Mycobacterium smegmatis* (2), BCG (6), or *M. tuberculosis* H37Rv or H37Ra (9) at a low multiplicity of infection. In contrast to a previous report (5), bacilli were not observed free in the cytoplasm or in the extracellular space in the lungs of mice following infection by the alveolar route with this virulent strain of *M. tuberculosis.*

Mycobacteria were rarely observed in the acellular necrotic center of the granulomata (Fig. 1E) but were always localized within cells in vacuoles containing organisms alone or organisms and cell debris (Fig. 2G). Thus, it appears that damaged macrophages containing mycobacteria may be engulfed by other phagocytic cells (Fig. 2G) at the inflammatory site. Indeed, it was only in these circumstances that several mycobacteria were seen within the same vacuole. Polymorphonuclear leukocytes often containing a few phagocytosed mycobacteria were also found in and around the necrotic centers of the granulomata. Dead or damaged polymorphonuclear leukocytes were also observed within phagocytic vacuoles of macrophages.

Our data indicate clearly that in vivo, in mice infected with virulent *M. tuberculosis* by aerosol, the bacilli are localized within tight membrane-bound vacuoles in the phagocytic cells of the lung. The nature of this special vacuole and its role in the maintenance of the host-parasite relationship are currently under investigation.

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