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Evidence for Inhibition of Fusion of Lysosomal and Prelysosomal Compartments with Phagosomes in Macrophages Infected with Pathogenic *Mycobacterium avium*

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Bone marrow-derived cultured macrophages were infected with the pathogenic organism *Mycobacterium avium*. Immediately after infection and at 1 to 28 days later, cells either were stained for acid phosphatase activity or given horseradish peroxidase, which served as a pinocytotic marker. With the former, fusions between phagosomes and lysosomes exclusively were assessed; with the latter, those between phagosomes and both pinosomes and lysosomes were determined. As a control, similar experiments were undertaken by infecting macrophages with gamma ray-killed *M. avium* and the nonpathogenic live organisms *Mycobacterium aurum* and *Bacillus subtilis*. After infection with live *M. avium*, fusions between phagosomes and acid phosphatase-positive vesicles (lysosomes) were inhibited. The same inhibition was observed whether phagosomes contained damaged or structurally intact (presumed to be live) bacteria, except for the early time points. This inhibition was, however, partial, suggesting that some of the live bacteria are resistant to the hydrolytic enzymes of the phagolysosomal environment. Fusions between horseradish peroxidase-positive vesicles (pinosomes and lysosomes) and phagosomes depended upon the morphological state of the bacteria. Damaged bacteria did not inhibit fusions, whereas with intact bacteria, a partial inhibition which increased with time was observed. The two types of experiment suggest that viable *M. avium* can impair phagosome-pinosome fusions.

It is now well established that *Mycobacterium avium* is an important human pathogen and that its intrinsic pathogenicity is strengthened by its resistance to antituberculous drugs (4, 26).

Like other pathogenic mycobacteria (7, 22), *M. avium* can circumvent the microbicidal activity of host defense cells (23), but the mechanisms responsible for this resistance property are not documented. Contrary to *Mycobacterium tuberculosis* H37Rv (20), *M. avium* does not escape from phagosomes and invade the cytoplasm of bone marrow-derived macrophages (23). It must therefore avoid or neutralize the host lysosomal attack by (i) decreasing the macrophage content in lysosomal enzymes (16), (ii) impairing the diffusion of lysosomal enzymes once lysosome-phagosome fusions have occurred (13), or (iii) inhibiting lysosome-phagosome fusions (1, 11–13, 17).

In addition, the survival mechanism of pathogenic bacteria inside host cell phagosomes is unknown, because the means of transfer of nutrients to phagosomes have not been investigated. Usually, macrophages interiorize substances from the extracellular medium in membrane-bound vesicles called pinosomes (24). The latter fuse with lysosomes, resulting in a thorough degradation of the vacuolar content. After phagocytosis of nonpathogenic bacteria, incoming pinosomes can also fuse directly with phagosomes (6). The presence of pathogenic bacteria might modify the latter pathway of pinocytic vesicles and thus perturb the transfer of nutrients or other substances, such as antibiotics, to phagosomes.

To gain insight into both the resistance and survival mechanisms of pathogenic *M. avium*, we infected bone marrow-derived macrophages with the live strain. At different times after infection, cells were stained for acid phosphatase (AcPase) activity to determine the extent of lysosome-phagosome fusions. In parallel, cells were given horseradish peroxidase (HRP), which served as a pinocytotic marker. Its fate was monitored under an electron microscope after cytochemical staining for the enzyme, and the occurrence of fusions between phagosomes and HRP-positive vesicles was analyzed. These vesicles correspond to lysosomes and also to vesicles of the prelysosomal compartment referred to variously as pinosomes, endosomes, intermediate vesicles, or receptosomes. By comparing the results obtained with both cytochemical methods, it was possible to deduce the extent of exchanges with the extracellular environment via pinosomes.

As a control, similar experiments were performed by infecting cells with (i) gamma ray-killed pathogenic *M. avium*, (ii) the nonpathogenic and slowly degraded organism *Mycobacterium aurum*, and (iii) the nonpathogenic and quickly degraded organism *Bacillus subtilis*.

MATERIALS AND METHODS

Cells and culture medium. Bone marrow macrophages were obtained by seeding 2×10^5 bone marrow cells isolated from 6- to 13-week-old C57BL/6 female mice per 35-mm tissue culture dish (Falcon, Becton Dickinson Labware, Oxnard, Calif.). Some dishes contained 12-mm-diameter cover slips. These macrophages were used because once differentiated, they can be maintained as stationary cultures for at least 3 months by simply changing the medium (23). This is of utmost importance for long-term experiments with slowly growing mycobacteria.

The culture medium consisted of Dulbecco modified Eagle medium with low glucose (1 g/liter) and high carbonate (3.7 g/liter) concentrations and enriched with 10% fetal calf

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serum heat inactivated for 40 min at 56°C, 10% L-cell conditioned medium, 2 mM L-glutamine, 100 μg of streptomycin per ml, and 100 U of penicillin per ml.

At 4 to 5 days after seeding, the attached cells were rinsed with Hanks balanced salt solution and refed with fresh medium devoid of antibiotics.

Bacteria and growth medium *M. avium* ATCC 15769, *M. aurum* CIPT 141210005, and *B. subtilis* SMY were used as exponentially growing cultures. Both *M. avium* and *M. aurum* were grown in RVB_{10} liquid medium (21) at 37°C, and *B. subtilis* was grown at 37°C in nutrient broth (Difco Laboratories, Detroit, Mich.).

M. avium was 90 to 95% viable, as determined by measuring the number of CFU in the bacterial suspensions on Lowenstein-Jensen medium, by measuring the turbidity at 650 nm with a Coleman Junior II spectrophotometer, and by making counts after Ziehl-Neelsen staining. For gamma ray-killed *M. avium* (4.5×10^6 rads administered in 10 h), bacterial counts on Lowenstein-Jensen medium showed that the preparations were completely sterilized ($<10^{-9}$ survivors). The nonpathogenic organisms *M. aurum* and *B. subtilis* were 95 to 100% viable.

Infection of macrophage monolayers. Macrophage cultures (7 to 10 days old) were overlaid with the appropriate bacterial suspension adjusted to yield a bacteria/macrophage ratio of 50 to 100:1. Cells were infected for 4 h with live or gamma ray-killed *M. avium*, for 4 h with *M. aurum*, or for 45 min with *B. subtilis*. Cells were then washed in two changes of phosphate-buffered saline to eliminate noningested bacteria and refed with fresh medium devoid of antibiotics. The medium was renewed every 5 days.

HRP uptake. At appropriate times after infection, cells were washed twice with Dulbecco modified Eagle medium containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)–saline to remove fetal calf serum from the medium (16a) and incubated for 60 min at 37°C with 1 mg of HRP per ml in Dulbecco modified Eagle medium–HEPES-saline. HRP uptake was arrested by washing the cells with ice-cold 10 mM HEPES–saline. Cells were then fixed for 15 min at room temperature with a mixture of 2.5% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 0.1 M sucrose. Cells were washed with the same buffer and fixed overnight at 4°C with 2.5% glutaraldehyde in the same buffer.

Cytochemical localization of HRP. The fixative was removed with two washes of buffer. Cells were incubated for 30 min at 22°C in the dark with diaminobenzidine-H_2O_2 by the technique of Graham and Karnovsky (10) as modified by Malmgren and Olsson (18). Cells were then washed twice in 0.1 M cacodylate buffer (pH 6.8) and prepared for electron microscopy as described below.

AcPase cytochemistry. Macrophage monolayers were fixed for 1 h at 4°C (in the culture dishes) with 1.25% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 0.1 M sucrose. They were washed overnight with the same buffer, rinsed once with 0.1 M acetate buffer (pH 5.0), and incubated for 30 min at 37°C in prewarmed Gomori reaction medium (9). Macrophages were rinsed twice with acetate buffer and once with cacodylate buffer. Cover slips were withdrawn from the dishes, rinsed with distilled water, and treated with 1% ammonium sulfide for the quantitation of AcPase-positive macrophages under a light microscope. Control experiments performed in the presence of 10 mM NaF were negative. The macrophages remaining in the dishes after withdrawal of the cover slips were prepared for electron microscopy as described below.

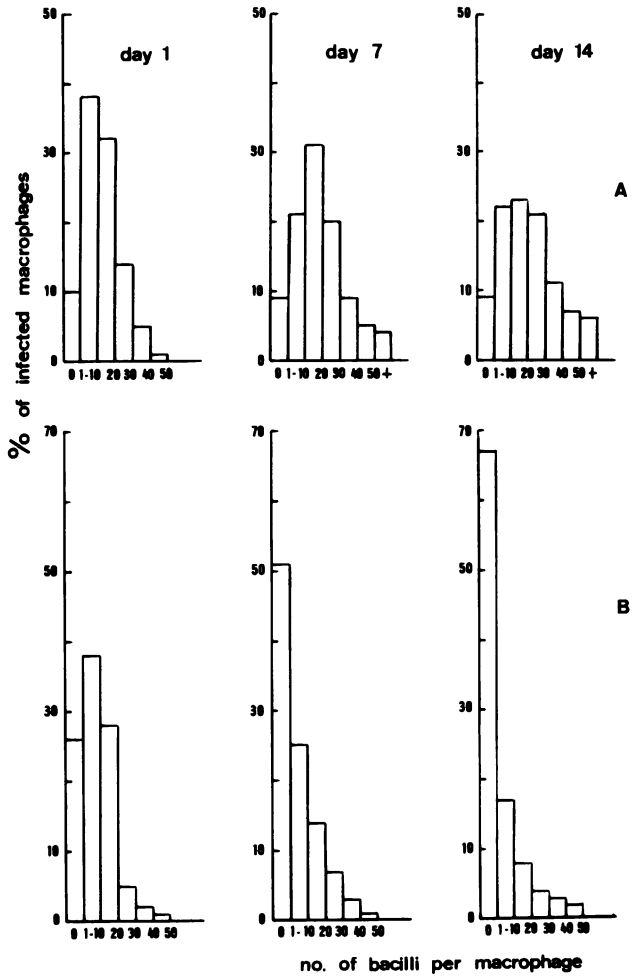

FIG. 1. Frequency distribution of intracellular Ziehl-Neelsen-positive bacteria after infection of macrophages with live (A) or gamma ray-killed (B) *M. avium*. Counts were made 1 to 14 days after infection on 100 to 200 different cells per sample. Results are those from a typical experiment. Whatever the initial number of bacteria per cell, the histogram distribution was similar in all experiments, i.e., a shift toward higher values for live bacilli (A) and toward zero for gamma ray-killed bacilli (B).

Subsequent processing for electron microscopy. Cells were fixed for 1 h at room temperature with 1% osmium tetroxide in 0.1 M cacodylate buffer. Cells were then scraped off the culture dishes with a rubber policeman, concentrated in agar, and treated for 1 h with 1% uranyl acetate in Veronal buffer at a final pH of 5.0. Samples were dehydrated in a graded series of acetone and embedded in Epon. Thin sections were stained with 2% uranyl acetate and lead citrate.

Assessment of intactness. The bacilli were considered to be intact only if they had maintained their rod shape, if their cytoplasm had preserved its ultrastructural organization and electron opacity, and if no breaks in the cell wall or cytoplasmic membrane were observed. Otherwise, the bacteria were considered to be damaged.

According to these criteria, 50 to 65% of the microorganisms in the live *M. avium* inoculum were intact, although 95% were viable, as described above. The criteria we have chosen to use are therefore more restrictive than those of

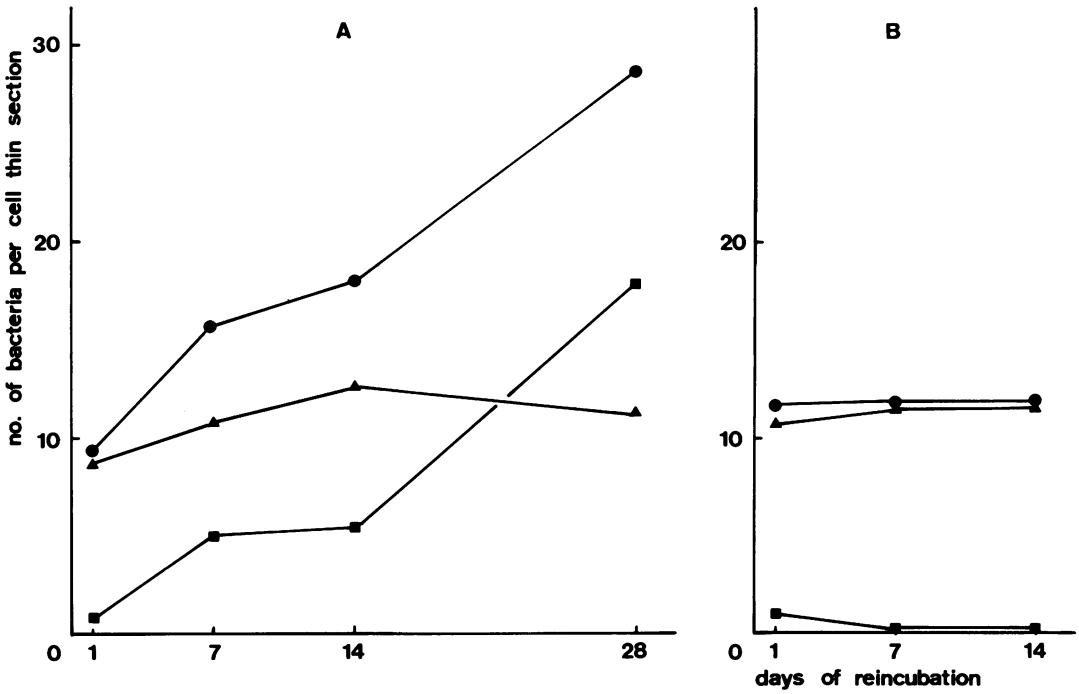

FIG. 2. Mean number of damaged (▲), structurally intact (■), and total (●) mycobacteria per cell thin section of macrophages infected with live (A) or gamma ray-killed (B) *M. avium*. Counts were made 1 to 28 days after infection on 50 to 100 different cell profiles per sample. Results are those from a typical experiment. Whatever the initial number of bacilli per cell thin section after infection with live bacilli (A), the total number of bacilli increased with time and the number of structurally intact bacilli either increased or reached a plateau but never decreased.

viability, which strongly suggests that the bulk of intact bacilli are viable.

Assessment of fusion. The presence of electron-dense material within phagosomes after AcPase cytochemistry identified fusions with lysosomes, whereas the dense material observed after staining for HRP activity revealed fusions with lysosomes or pinosomes.

To statistically assess phagosome-lysosome or phagosome-pinosome fusions, we examined 40 to 80 macrophages per sample. This allowed us to examine 300 to 1,500 phagosomes per time point. In all experiments, care was taken to avoid serial sections, and only profiles exhibiting a nucleus were examined.

TABLE 1. Percentages of bacterium-containing phagosomes that showed fusion with AcPase-positive vesicles at different times after infection with live *M. avium*

Length of reincubation after infection	Appearance of bacilli in phagosomes	No. of phagosomes encountered	% Fusion with AcPase-positive vesicles	
			Positive	Negative
0 min	Intact	203	20.8[a]	79.2[a]
	Damaged	423	37.5[a]	62.5[a]
1 day	Intact	318	45.0[a]	55.0[a]
	Damaged	1,087	52.9[a]	47.1[a]
7 days	Intact	178	16.3	83.7
	Damaged	826	15.7	84.3
14 days	Intact	93	16.1	83.9
	Damaged	576	12.1	87.9

[a] The differences within the pairs were significant ($P < 0.01$; χ^2 test).

Morphometry methods. The initial requirements for the random sampling of the cell population were easily met with macrophages, since a random sample of the population was included in each block. Thirty profiles per time point were analyzed with a multipurpose test grid by the method of Weibel et al. (25) on micrographs enlarged to a final magnification of 28,000×. These micrographs were used to determine the volume of the HRP-positive vesicle compartment with respect to the total cell volume by counting the number of test points that fell in the positive vesicles and in the entire cell.

Chemicals. HEPES, HRP type II, β-glycerophosphate, and glutaraldehyde grade I were purchased from Sigma Chemical Co., St. Louis, Mo.; 3,3'-diaminobenzidine tetrachlorohydrate and H_2O_2 were purchased from Prolabo, Paris, France; paraformaldehyde was purchased from Serva, Heidelberg, Federal Republic of Germany; fetal calf serum and antibiotics were purchased from GIBCO Europe, Paris France; and Dulbecco modified Eagle medium and glutamine were purchased from Biopro, Mulhouse, France.

RESULTS

Growth of *M. avium* in macrophages. *M. avium* multiplication was determined by evaluating the number of bacteria per macrophage at different times after infection (0 to 14 days) either on Ziehl-Neelsen-stained whole cells under a light microscope or on thin-sectioned cells under an electron microscope. As a control, the same evaluation was carried out with gamma ray-killed bacteria.

Observations of Ziehl-Neelsen-stained macrophages showed that the number of bacteria per cell increased with

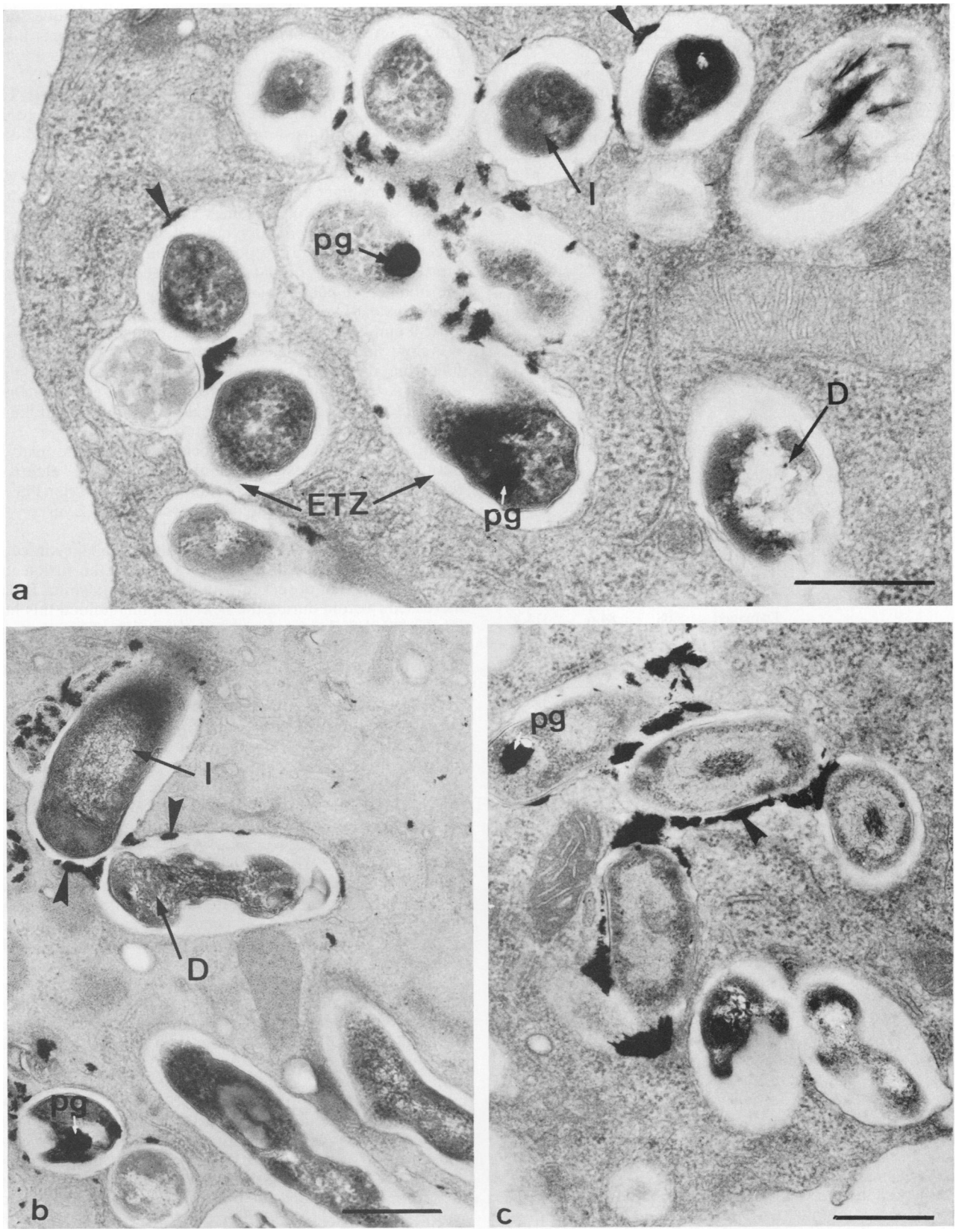

FIG. 3. Macrophages stained for AcPase 1 day (a) or 14 days (b) after infection with live *M. avium*. Discrete patches of deposit (arrowheads) were located between the ETZ and the phagosome membrane. (c) Cells stained 1 day after infection with gamm ray-killed *M. avium*. The phagosomes were heavily stained, and the deposits (arrowhead) entirely filled the space between the phagosome membrane and the outer layer of the cell wall of bacteria devoid of ETZ. I, Intact bacteria; D, damaged bacteria; pg, bacterial phosphate granule inclusions. Bars, 0.5 μm.

TABLE 2. Percentages of bacterium-containing phagosomes that showed fusion with AcPase-positive vesicles at different times after infection with control organisms

Organism	Length of reincubation after infection	Appearance of bacilli in phagosomes	No. of phagosomes encountered	% Fusion with AcPase-positive vesicles	
				Positive	Negative
Gamma ray-killed *M. avium*	0 min	Intact	110	32.4	67.6
		Damaged	505	39.1	60.9
	1 day	Intact	114	54.1	45.9
		Damaged	804	66.1	33.9
	7 days	Intact	28	57.9	42.1
		Damaged	637	65.6	34.4
	14 days	Intact	7	ND[a]	ND
		Damaged	612	35.9	64.1
M. aurum	0 min	Intact	186	62.9	37.1
		Damaged	389	67.8	32.2
	1 day	Intact	270	65.0	35.0
		Damaged	690	74.0	26.0
B. subtilis	0 min	Intact	224	77.6	22.4
		Damaged	298	89.0	11.0
	30 min	Intact	38	78.9	21.1
		Damaged	508	84.6	15.4
	60 min	Intact	13	ND	ND
		Damaged	660	84.8	15.2

[a] ND, Not determined.

time (Fig. 1A) and that, starting from day 7, 5 to 10% of the cells had more than 50 bacilli each. Throughout the 14-day period, only 10% of the macrophages were free of mycobacteria. In contrast, after infection with gamma ray-killed *M. avium*, the percentage of macrophages devoid of bacteria increased wih time (Fig. 1B).

Under the electron microscope, damaged and structurally intact bacilli could be distinguished from each other. Whatever the percentage of intact bacteria in the inoculum (50 to 65%), the number of intact bacilli per macrophage was low during the first 24 h after infection (10 to 20%), indicating a degradation of bacteria. During the next 7 days, the number of intact bacilli started to increase. This was not due to a reinvasion by bacteria that had not been eliminated by the washings. Their number was too low to account for such an increase, and they could not multiply in the medium (23). Afterward, the number of intact bacilli either continued to increase (Fig. 2A) or reached a plateau, but it never decreased. After infection with gamma ray-killed *M. avium*, the number of damaged bacilli remained constant, whereas the number of intact bacilli decreased after day 1 (Fig. 2B) in all experiments.

These data, added to the fact that the bulk of intact bacilli are viable (23), show that *M. avium* actually multiplies within macrophages, as does *Mycobacterium intracellulare* (8). They also indicate that *M. avium* is slowly degraded, since damaged bacilli persisted for at least 15 days within phagosomes.

It is worth noting that the Ziehl-Neelsen technique stains only intact and partially damaged bacteria, whereas under the electron microscope, strongly damaged bacteria are also assessed.

Fusion of AcPase-positive vesicles with phagosomes. (i) Live *M. avium*. Macrophages were stained for AcPase at the onset of reincubation in fresh medium (0 min) and at 1 to 14 days later.

For each time point, the number of phagosomes with damaged or intact bacilli was tabulated, and the percentage of AcPase-positive phagosomes was determined (Table 1). Each phagosome generally contained a single bacterium, except at the 0-min time point, at which phagosomes contained several bacteria, intact or damaged. In the latter case, the number of bacteria surrounded by AcPase was determined instead.

At 0 min, the percentage of AcPase-positive phagosomes was low and increased to 45 to 50% on day 1. In the following days, the percentage of AcPase-positive phagosomes decreased strongly. No differences were detected between phagosomes containing intact and damaged bacteria, except for the early time points.

The reaction product was located in discrete patches between the phagosome membrane and the electron-transparent zone (ETZ) surrounding most bacteria (Fig. 3a and b). It never penetrated this translucent layer or the bacterial cytoplasm.

(ii) Gamma ray-killed *M. avium*. In macrophages infected with gamma ray-killed *M. avium* (Table 2), the fusion rate was similar to that observed with live bacilli at 0 min. It then increased twofold during the next 24 h, maintained this high level until day 7, and decreased thereafter. Except at the 0-min time point, this percentage was higher than after infection with live *M. avium*. The differences were small on day 1 and increased strongly in the following days.

Bacteria were usually devoid of an ETZ, and the deposit entirely filled the space between the phagosome membrane and the outer layer of the bacterial cell wall (Fig. 3c). The intensity and size of the deposits were stronger than after infection with live *M. avium* (Fig. 3c). AcPase was sometimes detected in the cytoplasm of strongly degraded bacteria; this was never the case with live *M. avium*.

(iii) Nonpathogenic bacteria. After *M. aurum* infection (Table 2), 60 to 75% of the phagosomes containing damaged or intact bacteria were AcPase positive at 0 min and on day 1. Experiments were not extended to later time points because the few uningested bacteria remaining in the medium after the washings multiplied rapidly and reinvaded the cells.

In the positive phagosomes, large patches of lead precipitate were observed between the bacterial cell wall and the phagosome membrane (Fig. 4a). In some of the strongly damaged bacteria, the precipitate was also localized in the cytoplasm (Fig. 4b).

In the case of *B. subtilis* infection, intact bacteria accounted for 40% of the ingested bacilli at the outset of reincubation; at 1 h later, virtually all the bacteria were degraded. Cells were therefore stained for AcPase after short reincubation times (0 to 60 min). Quantitation of AcPase-positive phagosomes indicated that the majority of those containing damaged bacteria had undergone fusion with lysosomes (Table 2). For phagosomes with intact bacilli, the extent of fusion was not significantly lower ($0.5 < P < 0.750$).

In *B. subtilis*-containing phagosomes, the lead precipitate was first circumscribed to the peribacillar space between the

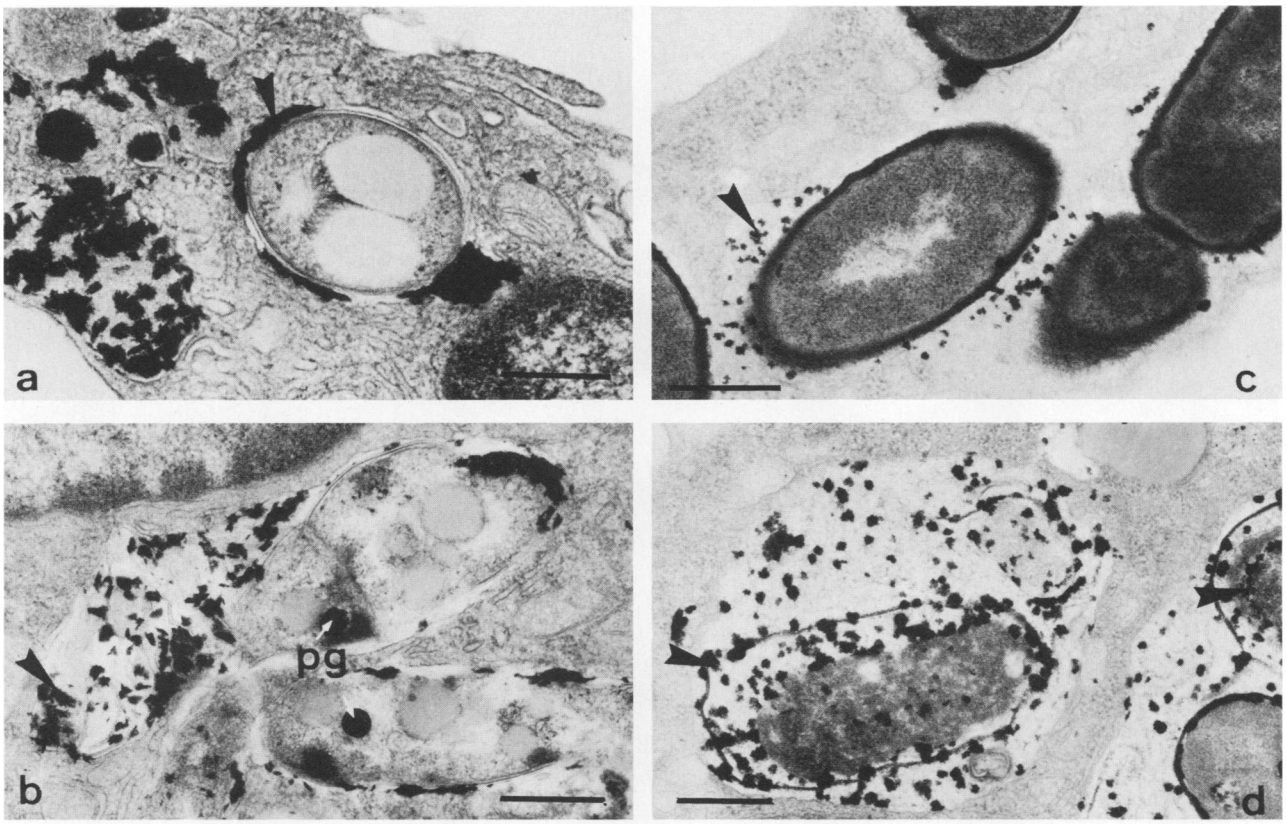

FIG. 4. Macrophages infected with *M. aurum* (a and b) or *B. subtilis* (c and d) and stained for AcPase activity. The cytochemical product (arrowheads) surrounded the cell wall of intact bacteria within phagosomes (a and c) and even invaded the cytoplasm of strongly damaged bacilli (b and d). pg, Bacterial phosphate granule inclusions. Bars, 0.5 μm.

cell wall and the phagosome membrane. It then invaded the cell wall and finally the cytoplasm of damaged bacteria. (Fig. 4c and d).

AcPase activity of infected macrophages. To rule out the possibility that infection with live *M. avium* resulted in an inhibition of the macrophage AcPase activity, we assessed the percentages of AcPase-positive cells after infection with the four strains. Uninfected cells served as a control (Table 3).

After infection with *B. subtilis*, the percentage of AcPase-positive macrophages was already higher than in uninfected (control) populations at the onset of reincubation. This was probably related to the rapid degradation of this organism.

With live or gamma ray-killed *M. avium* or *M. aurum*, the percentages of AcPase-positive macrophages were similar to that observed in control cells at the onset of reincubation. On day 1, however, these percentages increased strongly and reached the high value of *B. subtilis*-infected cells. Afterward, the percentage remained high in cells infected with gamma ray-killed *M. avium*. In contrast, it decreased in cells infected with live *M. avium* but was never lower than that in uninfected cells (Table 3).

Fusion of HRP-positive vesicles with phagosomes. (i) Live *M. avium*. The fusion of *M. avium*-containing phagosomes with lysosomes or pinosomes was examined by exposing macrophages to HRP fo 60 min at the onset of reincubation

TABLE 3. Percentages of AcPase-positive macrophages infected with live or gamma ray-killed *M. avium*, *M. aurum*, and *B. subtilis*[a]

Length of reincubation after infection	% of AcPase uninfected macrophages	% of AcPase-positive macrophages infected with:			
		Live *M. avium*	Gamma ray-killed *M. avium*	*M. aurum*	*B. subtilis*
0 min	47.6 ± 5.2	49.3 ± 2.1	54.1 ± 5.3	48.3 ± 7.7	78.8 ± 4.1
30 min	ND	ND	ND	ND	73.5 ± 2.9
1 day	48.0 ± 5.3	73.5 ± 3.4	75.0 ± 7.3	78.1 ± 6.2	ND
7 days	44.1 ± 8.1	49.5 ± 7.8	80.0 ± 6.1	ND	ND
14 days	41.0 ± 7.1	44.1 ± 3.4	63.0 ± 2.5	ND	ND

[a] Uninfected macrophages served as a control. Counts were made 0 to 14 days after reincubation in fresh medium. Data represent the mean ± standard error of the mean for three experiments. For each sample, 100 to 200 cells were examined. ND, Not determined.

TABLE 4. Morphometric measurement of the HRP-positive vesicle compartment with respect to cell volume at different times after infection with live *M. avium*

Length of reincubation after infection	Pv^+/Pc[a] (mean ± SEM)
None (uninfected cells)	0.147 ± 0.012
0 min	0.122 ± 0.010
1 day	0.131 ± 0.009
7 days	0.141 ± 0.009
14 days	0.141 ± 0.019

[a] *P*, Number of test points that fell in a given compartment. V^+, HRP-positive vesicle compartment; *c*, entire cell.

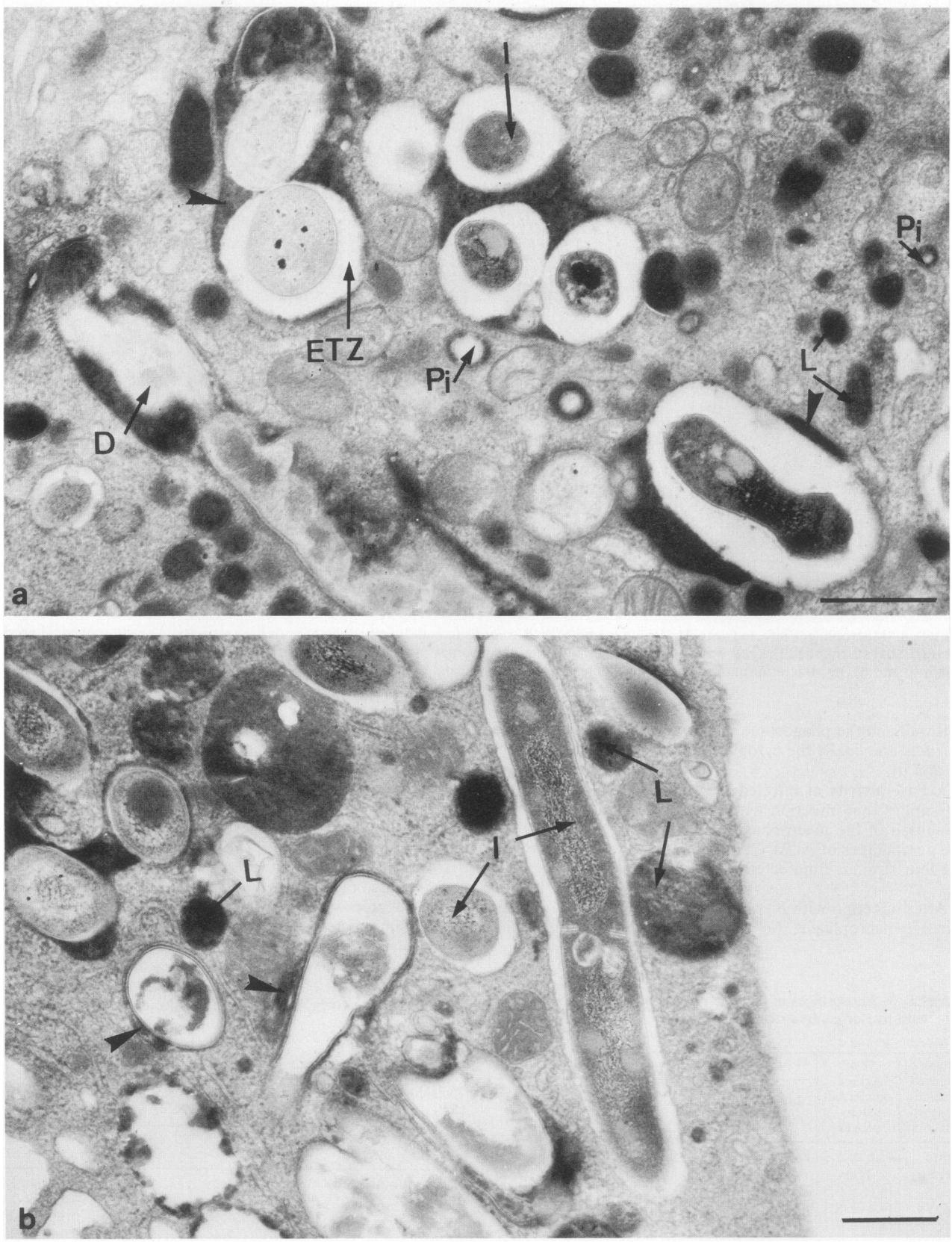

FIG. 5. Localization of HRP in macrophages infected with live *M. avium*. Cells were exposed for 1 h to HRP 1 day (a) or 14 days (b) after infection. On day 1, large dense deposits (arrowheads) were observed between the ETZ and the phagosome membrane whether bacteria were intact (I) or damaged (D); on day 14, the deposits (arrowheads) were smaller. HRP-negative phagosomes containing intact bacilli were encountered and were surrounded by HRP-positive vesicles. HRP-positive vesicles with a rim of reaction product corresponded to pinosomes (Pi), and those filled with a dense reaction product corresponded to lysosomes (L). Bars, 0.5 μm.

in fresh medium (0 min) and at 1 to 28 days later. As a control, uninfected macrophages were given HRP in parallel.

The presence of bacteria in macrophages did not impair the subsequent uptake of HRP even after multiplication of the pathogenic strain. At least 95% of the macrophages displayed HRP-positive vesicles under the light microscope at all times points. Moreover, morphometric measurements (Table 4) showed that at 1 to 14 days after infection, the size of the HRP-positive vesicle compartment with respect to total cell volume was similar to that observed in uninfected cells. A slight decrease in size was observed only at the 0-min time point, probably because phagocytosis and transfer to fresh medium momentarily inhibited pinocytosis. Finally, the usual types of HRP-positive vesicles were encountered (Fig. 5a) (16a); they appeared at the same time as in control cells, and their relative amounts were similar.

The extent of fusions was assessed (Table 5) as described above for AcPase experiments. At the 0-min time point, HRP was detected in 80% of the phagosomes. No differences were observed between phagosomes containing intact or damaged bacilli. Afterward (days 1 to 28), more than 90% of the phagosomes with damaged bacilli stained for HRP. In contrast, the percentage of HRP-positive phagosomes with intact bacilli was already significantly lower ($P < 0.01$) on day 1 and continued to decrease afterward.

At the early time points, the HRP reaction product filled the space between the phagosome membrane and the ETZ surrounding most bacilli (Fig. 5a). Starting from day 7, phagosomes contained less reaction product, which appeared as small and discrete patches (Fig. 5b). It is worth noting that the HRP-negative phagosomes were surrounded by HRP-containing vesicles (Fig. 5b).

(ii) Controls. After infection with gamma ray-killed *M. avium* or *M. aurum*, more than 90% of the phagosomes stained for HRP at all time points, whether they contained intact or damaged bacilli (Table 6). In the case of *B. subtilis* (Table 6), bacterial degradation was so rapid that more than 95% of the bacilli were damaged during the first hour of HRP uptake after infection. These phagosomes virtually all stained for HRP. It was not possible to determine the percentage of HRP-positive, intact bacillus-containing phagosomes because their number was too low for an adequate quantitation.

With the three organisms, the reaction product was found in close apposition with the bacterial cell wall and even completely invaded the cytoplasm upon bacterial degradation, especially for *B. subtilis* (Fig. 6a to c).

TABLE 5. Percentages of bacterium-containing phagosomes that showed fusion with HRP-positive vesicles at different times after infection with live *M. avium*

Length of reincubation after infection[a]	Appearance of bacilli in phagosomes	No. of phagosomes encountered	% Fusion with HRP-positive vesicles	
			Positive	Negative
0 min	Intact	207	78.0	22.0
	Damaged	670	81.9	18.1
1 day	Intact	294	79.9[b]	20.1[b]
	Damaged	1,124	93.5[b]	6.5[b]
7 days	Intact	524	79.1[b]	20.9[b]
	Damaged	1,427	93.3[b]	6.7[b]
14 days	Intact	306	68.9[b]	31.1[b]
	Damaged	488	91.2[b]	8.8[b]
28 days	Intact	280	67.1[b]	32.9[b]
	Damaged	175	92.6[b]	7.4[b]

[a] Cells were exposed to HRP for 60 min at the indicated reincubation times.
[b] The differences within the pairs were significant ($P < 0.01$; χ^2 test).

TABLE 6. Percentages of bacterium-containing phagosomes that showed fusion with HRP-positive vesicles at different times after infection with control organisms

Organism	Length of reincubation after infection[a]	Appearance of bacilli in phagosomes	No. of phagosomes encountered	% Fusion with HRP-positive vesicles	
				Positive	Negative
Gamma ray-killed *M. avium*	0 min	Intact	57	87.7	12.3
		Damaged	428	92.5	7.5
	1 day	Intact	40	95.0	5.0
		Damaged	399	97.5	2.5
	7 days	Intact	2	ND[b]	ND
		Damaged	307	92.9	7.1
	14 days	Intact	2	ND	ND
		Damaged	332	91.7	8.3
M. aurum	0 min	Intact	360	97.0	3.0
		Damaged	262	99.0	1.0
	1 day	Intact	285	91.2	8.8
		Damaged	273	94.8	5.2
B. subtilis	0 min	Intact	3	ND	ND
		Damaged	225	98.0	2.0

[a] Cells were exposed to HRP for 60 min at the indicated reincubation times.
[b] ND, Not determined.

DISCUSSION

In the present work, phagosome-lysosome fusions were assessed by the AcPase technique. In bone marrow-derived macrophages infected with the pathogenic organism *M. avium*, the cytochemical labeling was lighter and the percentage of AcPase-positive phagosomes was always lower than in those infected with the nonpathogenic organism *B. subtilis* and *M. aurum*. Therefore, *M. avium* shares with other pathogenic bacteria (5, 14), including mycobacteria (7, 11, 13), the ability to inhibit phagosome-lysosome fusions.

At the outset of reincubation (0-min time point), the extent of fusions was similar whether macrophages had been infected with live or gamma ray-killed *M. avium*. Afterward, the percentage of AcPase-positive phagosomes was always lower with live bacilli than with gamma-killed bacilli. The differences were very small on day 1, but they were quite large on the following days. Because *M. avium* is very slowly degraded (Fig. 2B), it is reasonable to think that surface or cell wall constituents of gamma-killed bacteria or both were still unmodified or barely modified at the outset of reincubation and then progressively degraded with time. Consequently, they could counteract fusions to the same extent as live bacteria do at early time points but not at later ones. Our results suggest that *M. avium* surface or cell wall

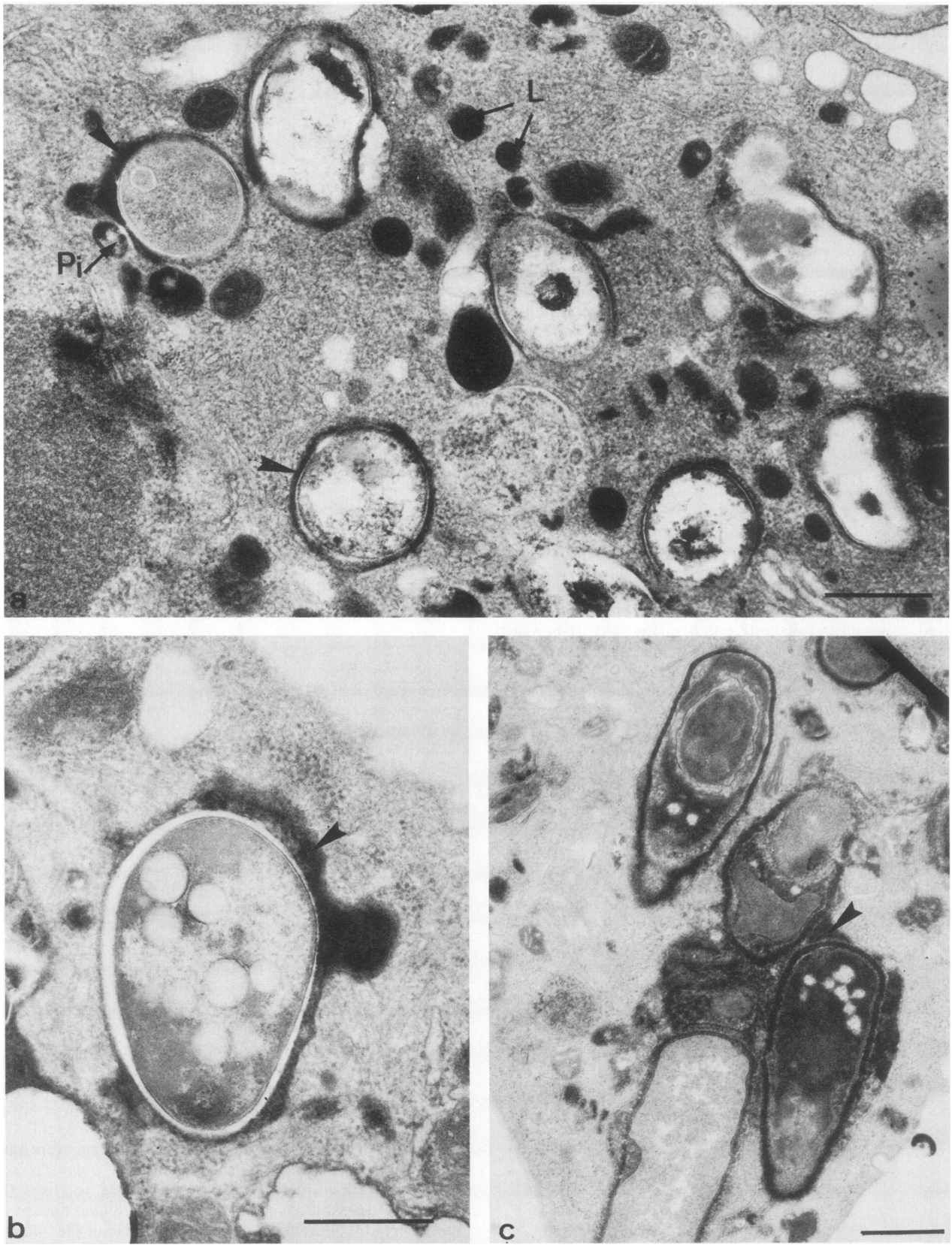

FIG. 6. Localization of HRP in macrophages infected with gamma ray-killed *M. avium* (a), *M. aurum* (b), or *B. subtilis* (c). The cytochemical product (arrowheads) surrounded the cell wall of bacteria within phagosomes and even invaded the cytoplasm of strongly damaged bacilli. Pi, Pinosomes; L, lysosomes. Bars, 0.5 μm.

constituents or both could be responsible for the impairment of phagosome-lysosome fusions, as proposed for *Nocardia asteroides* (5).

Previous reports on pathogenic bacteria (1, 5, 13) indicated that only intact bacilli inhibited phagosome-lysosome fusions. In contrast, with *M. avium*, both damaged and intact bacilli inhibited fusions throughout our experiments.

With *M. avium*, the inhibition of phagosome-lysosome fusions was, however, partial, especially on day 1, when 50% of the phagosomes with intact bacilli stained for AcPase. Therefore, *M. avium* is also able to survive in the hydrolytic phagolysosomal environment, as suggested for *Mycobacterium lepraemurium* (13) and *N. asteroides* (5). This was not the case for *Legionella pneumophila* (15). The ETZ surrounding the bacteria could be responsible for this resistance property by acting as a barrier to the diffusion of lysosomal enzymes. This is suggested by the fact that lead deposits always remained outside the ETZ.

After infection with *Mycobacterium leprae* (16) or *N. asteroides* (2), a reduction in macrophage AcPase activity was observed. This does not seem to be the case for *M. avium*, because the percentage of AcPase-positive macrophages was never lower than that in uninfected populations. On day 1, the percentage was even higher than that in uninfected cells, which seems to indicate a transient activation of macrophages. With gamma ray-killed bacteria, this activation was maintained throughout the infection, as observed in *M. tuberculosis*-, *Mycobacterium bovis* BCG-, and *Mycobacterium microti*-infected cells (16).

In all previous reports concerning bacterial survival in macrophages, only phagosome-lysosome fusions were investigated either by AcPase cytochemistry or by loading lysosomes with HRP, ferritin, or thorium (1, 5, 12–14). In our experimentals, HRP-loaded vesicles corresponded not only to lysosomes but also to vesicles of the prelysosomal compartment (pinosomes). By comparing the percentages of AcPase-positive and HRP-positive phagosomes over time and with respect to the morphological state of the bacilli, it was possible to obtain new insight on phagosome-pinosome fusions.

HRP-positive vesicles behaved differently from AcPase-positive lysosomes in their fusion with *M. avium*-containing phagosomes: (i) Just after infection, the percentage of HRP-positive phagosomes was high (80%), irrespective of the morphological state of the bacilli. In contrast, that of AcPase-positive phagosomes was low. (ii) In the following days, the percentage of HRP-positive phagosomes with damaged bacilli remained as high as in the controls (>90%), whereas with intact and, presumably, live bacilli, this percentage decreased with time. In contrast, the percentage of AcPase-positive phagosomes decreased abruptly after day 1, whether the bacilli were damaged or intact. All these results indicate that another type of vesicle, namely, pinosomes, is implicated in the fusion process and suggest that the presence of intact bacilli inhibits not only phagosome-lysosome fusions but also phagosome-pinosome fusions.

We mentioned above that just after infection, gamma ray-killed *M. avium* was as effective as live *M. avium* in inhibiting fusions of AcPase-positive vesicles with phagosomes. In contrast, fusions of HRP-positive vesicles with phagosomes were not inhibited just after infection with live or gamma ray-killed bacteria. These results suggest that the surface components present at the outset of the reincubation period are not implicated in the inhibition of phagosome-pinosome fusions. Rather, *M. avium* would progressively acquire new or enhanced properties that would prevent these fusions. The mechanism by which pathogenic *M. avium* inhibits phagosome-pinosome fusions would therefore differ from that concerned with phagosome-lysosome inhibition. To examine this hypothesis, studies will be performed after modification of the bacterial cell wall by various treatments.

In conclusion, inhibition of phagosome-lysosome fusions and resistance to the phagolysosomal environment are two of the means used by *M. avium* to counteract macrophage microbicidal activity. In addition, live bacilli seem to modify the interactions of the host cell with the extracellular environment by inhibiting phagosome-pinosome fusions. This modification does not seem to impede the transport of nutrients, because bacteria survive and multiply. The nutrients could be transferred to phagosomes by diffusion of metabolites or could result from the breakdown of extracellular material by lysosomal enzymes (3). The transfer of antibiotics to phagosomes is only partially documented (19). Certain lipophilic antibiotics, such as rifampin, diffuse through the membrane, but others probably need to be pinocytosed. In the latter case, the inhibition of phagosome-pinosome fusion would prevent the transfer of drugs that could no longer exert their bactericidal effects. Resistance to antibiotics observed in cultures of *M. avium* (7) would then be exacerbated when bacteria are enclosed in the host phagosomes.

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