INHIBITION OF PHAGOSOME-LYSOSOME FUSION IN MACROPHAGES BY CERTAIN MYCOBACTERIA CAN BE EXPLAINED BY INHIBITION OF LYSOSOMAL MOVEMENTS OBSERVED AFTER PHAGOCYTOSIS

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After phagocytosis of certain pathogenic microorganisms by cultured macrophages or monocytes, the characteristic fusion of phagosomes with primary or secondary lysosomes does not occur; direct contact of the organisms with lysosomal contents is evaded. The organisms include the human pathogens *Legionella pneumophila* (1), *Toxoplasma gondii* (2), and *Mycobacterium tuberculosis* (3), and the mouse pathogen *Mycobacterium microti* (vole tubercle bacillus) (4, 5). The mechanism of this effect (applying to viable organisms only) remains obscure. In the case of mycobacteria, we have previously reported two sets of observations that may be relevant: (*a*) various polyanions, of which at least two are present in tubercle bacilli, and also the weak base ammonia, which is produced abundantly in cultures, inhibit phagosome-lysosome (P-L)[1] fusion when yeasts are administered to normal macrophages (6–10); (*b*) certain of these lysosomotropic chemical inhibitors have been observed also to inhibit lysosomal saltatory movements, possibly by modifying microtubule control; we have suggested that such stasis prevents membrane contact and consequently reduces P-L fusion (11, 12). Further experiments have strengthened this interpretation by establishing an association between enhancement of fusion and acceleration of movements for many nitrogenous bases (13).

To clarify further the mechanism of mycobacterial inhibition of fusion, we have sought (*a*) to determine whether ingestion of live *M. microti* would be followed by stasis of periphagosomal lysosomes, as occurs throughout normal monolayers after treatment with the chemical inhibitors; (*b*) if so, to compare it with the lysosome movements after phagocytosis of a fusiogenic mycobacterium, such as *M. lepraemurium*; (*c*) to determine whether an *M. microti*–induced lysosome stasis could affect the fusion of lysosomes with separate phagosomes of a clearly distinguishable fusiogenic organism (i.e., *Saccharomyces cerevisiae*) ingested subsequently by the same cell.

[1] *Abbreviations used in this paper:* AO, acridine orange; EM, electron microscopy; FM, fluorescence microscopy; PCM, phase-contrast microscopy; P-L, phagosome-lysosome.

J. Exp. Med. © The Rockefeller University Press · 0022-1007/87/10/0933/14 $2.00 933
Volume 166 October 1987 933–946

Materials and Methods

Cell Cultures. Resident peritoneal macrophages from P-strain mice were established as monolayers on coverslips in Chang medium (containing 40% heat-inactivated horse serum) for 1–2 wk (3).

Microorganisms. M. microti (strain OV254, pathogenic for mice) was maintained by subculture in a modified Dubos-Middlebrook Tween 80–albumin liquid medium (14). (*a*) For the main experiments, centrifugates of 15–25-d cultures were washed well (to remove free ammonia); resuspended in 0.25% albumin/0.05% Tween in water; and lightly ultrasonicated to disperse further. The majority of the organisms were viable (fluorescein acetate assay), and the inoculum is referred to as live. For heat-killing, a portion of this suspension was spun, resuspended in Tween/water, heated in a water bath for 45 min at 100°C; washed again and finally resuspended in Tween/albumin water. Final suspensions contained single and small groups of bacteria and will be referred to as single units suspensions. (*b*) For some experiments, *M. microti* was subcultured on a Ratledge-Hall normal liquid medium (15) (but containing sucrose 3 g/liter instead of glycerol), giving large aggregates of bacteria that were reduced to a suitable size (~10–20 organisms) by glass-homogenization and ultrasonication (Tween was omitted from these procedures). Fresh (unless otherwise stated) commercial *Saccharomyces cerevisiae* were suspended in HBSS. *M. lepraemurium* (strain Douglas) was obtained by homogenizing spleens from infected P-strain mice (4).

Ingestion of M. microti and Yeasts. Monolayers, washed with HBSS, were overlaid for 2 h at 37°C with suspensions of live or heat-killed *M. microti*, diluted 1:5 in HBSS containing 5% heat-inactivated horse serum to give a ratio of bacteria to macrophages ranging from about 60:1 to 600:1. The monolayers were again washed and cultivated in Chang medium (usually incorporating 6.7 μg/ml streptomycin) for 18–24 h (unless otherwise stated), and then examined for lysosomal movements (omission of streptomycin did not appear to affect eventual results when compared with well-washed monolayers). Immediately thereafter, for experiments in which P-L fusion for yeasts was to be assessed, the monolayers were overlaid with the yeasts in BSS at 10^6–10^7 cells/ml, reincubated for 50 min, labeled with acridine orange (AO) (11), and scored for fusion by fluorescence microscopy (FM). Variations of this procedure, e.g., assessing at 1–3 h or at two or more days after infection are mentioned in the Discussion.

Assessment of Lysosomal Movements. Saltatory movements of secondary lysosomes were observed by direct visual phase-contrast microscopy (PCM). The method gives a qualitative assessment based on cells surveyed randomly over the monolayer area; its reliability for the present purpose has been previously supported by computer analyses (11).

Assessment of P-L Fusion. FM of the living cells, using AO as lysosomal marker and regarding the dye's presence within phagosomes as positive for fusion, has been previously described, accompanied by a critique of the method (11), from which it was concluded that, under the conditions normally used (9, 11) it is a satisfactory tool. This conclusion is supported by our recent (unpublished) tests of Lucifer Yellow (16), a nonpermeant lysosomotropic fluorescent probe, which revealed inhibition of P-L fusion both by a polyanion and by ammonium chloride, and enhancement by chloroquine, in each case similarly to AO. The AO method is semiquantitative, giving an overall evaluation. In the present case, when Tween/albumin cultures (containing single units of bacteria) were the source of the *M. microti*, the assay was confined to the estimation of fusion of phagosomes of the subsequently ingested yeasts with secondary lysosomes, since the transfer of AO to *M. microti*–phagosomes could not be visualized accurately. When, however, the alternative culture medium was used, larger aggregates of bacteria in the cells allowed fusion to be assessed by FM in their phagosomes too. Electron microscopy (EM), using ferritin as lysosomal label, and a system of scoring the cell profiles, have been previously described (3); the monolayers were fixed in situ on the coverslips and the cells then gently pushed off, i.e., still extended; after processing (3), the sections were cut in widely spaced interrupted series.

Results

Lysosomal Movements after Ingestion of M. microti

At 18–24 h after the ingestion of live or heat-killed *M. microti* obtained from Tween/albumin cultures (for shorter or longer periods see Discussion), the majority of macrophages in the monolayers appeared healthy. PCM showed the infection usually to be unevenly distributed. In extended cells infected with live cultures and showing many bacilli (≥20), the secondary lysosomes could be seen to move slowly or to be static (Table I); in cells with few or no visible bacilli, saltations appeared generally normal. Darkfield examination (DFM) indicated that this stasis applied also to the highly motile small vesicles not recognized by the PCM but considered to be also lysosomal (17). In cells containing comparable numbers of bacilli but in monolayers that had ingested heat-killed organisms, as well as in cells of normal (uninfected) controls, the lysosomes (by PCM) and the small vesicles (by DFM) moved actively throughout. To observe the effect of a mixed inoculum of killed and live *M. microti*, the monolayers were exposed to a suspension containing equal amounts of untreated organisms and of organisms intensely stained (and killed) by heating with carbolfuchsin, which enabled them to be distinguished from live organisms under PCM. Examples were then detected of areas that contained only untreated bacilli, around which lysosomes were static, while active movement was seen around stained bacilli in another area in the same cell.

Lysosomal Movements after Ingestion of M. lepraemurium

Like *M. microti*, *M. lepraemurium* is pathogenic for mice and multiplies in macrophage culture, but, unlike it, this species does not inhibit P-L fusion (as shown by EM assessment) (4). For a critical comparison, therefore, we examined lysosomal movements by PCM at different stages of a progressive infection in mouse macrophages. No evidence was observed of an association of the organisms with inhibition of lysosomal movements. Thus neither fusion nor movements are inhibited by this fusiogenic mycobacterium (see also Discussion).

P-L Fusion in M. microti-infected Macrophages

The inhibition of P-L fusion in macrophages by certain mycobacteria was originally established by EM. The small size and dispersed state of Tween/albumin–grown *M. microti* (single-unit suspensions) makes it impracticable to use FM to identify fusion by the passage of AO from labeled lysosomes to phagosomes. However, when the inoculum was derived from the alternative culture medium and included aggregates of the bacilli (very large aggregates tended to be extracellular), the occupied macrophages appeared healthy, and AO then could be detected in fused *M. microti*–phagosomes after the dye had penetrated an aggregate. By these means we were able to confirm the tendency of P-L fusion and nonfusion to be associated with ingested dead and live *M. microti*, respectively, using FM.

P-L Fusion of S. cerevisiae Present in the Same Macrophages as M. microti

FM Assessment. The inhibition of lysosomal movements by live *M. microti*, in correlation with its inhibition of P-L fusion, raised the question whether the

fusion behavior of a normally fusiogenic organism, e.g., *S. cerevisiae*, would be affected if surrounded by static lysosomes induced by *M. microti*. In a considerable proportion of such *M. microti*–infected cells, *S. cerevisiae* ingested 18–24 h after *M. microti* could be seen to be present in addition. After ingestion of aggregates of live but not of heated *M. microti*, failure to fuse was indeed observed not only for the mycobacteria but also for many of the accompanying yeasts. As expected, yeast phagosomes, when apparently alone in cells, induced normal P-L fusion (with the occasional exception of cells adjoining those cells that showed inhibition associated with the larger aggregates). An overall assessment of the yeast P-L fusion in *M. microti*–infected monolayers (mostly in those that had ingested inocula from Tween/albumin cultures) confirmed these appearances of individual yeast phagosomes (Table I). The scored level of inhibition was modest, possibly because of the proportion of cells that contained yeasts without *M. microti*, which would have induced normal fusion.

EM Assessment. The FM-AO technique used for scoring P-L fusion (as in Table I) enables a large number of cells to be examined, but it is semiquantitative, giving an overall evaluation. The EM survey–scoring method assesses a limited number of cells in greater detail, and is more quantitative (18). The P-L fusion behavior of both *M. microti* and *S. cerevisiae* was evaluated by EM in macrophages prelabeled with ferritin before an 18-h *M. microti* infection. The latter was then followed (at 18 h) by ingestion of fresh yeasts; incubation for 50 min; and fixation. In a proportion of cell profiles neither organism was visibly present, but cells containing one or the other or both (in variable numbers) were sufficient for recording purposes. The two organisms were always in separate phagosomes. Of the *M. microti*, some 70–80% were seen in crosssection in the cell profiles, the remainder being diagonal or (rarely) longitudinal. The appearances of fusion and nonfusion are illustrated in Fig. 1, and the scores of P-L fusion for each in an experiment based on a Tween/albumin culture (single units) of *M. microti*, together with a note on procedures, is given in Table II (Table III, showing distributions of the two organisms in the profiles, is analyzed in the Discussion).

TABLE I

Lysosome Movements in Macrophages after Ingestion of M. microti, and Fusion of Yeast Phagosomes with AO-labeled Lysosomes after Subsequent Ingestion of S. cerevisiae (Direct Visual Observations)

Pretreatment of *M. microti*	Change of movements from normal*	Fusion of yeast phagosomes‡	
		Mean of 17 experiments	Range
		%	
Untreated (live)	−1 to −2	40	30–50
Heat-killed	0	70	60–80
No bacteria	0	70	55–80

* Score key: −2, no movement; −1, slow movement; 0, normal movement (see Materials and Methods). In the monolayers infected with live organisms, the score varied with the numbers of bacilli in a cell.

‡ Scored as proportion of yeast-containing phagosomes showing intraphagosomal fluorescent rims or colored yeasts (see Materials and Methods).

TABLE II

Phagosome Fusion with Ferritin-prelabeled Lysosomes in Profiles of Macrophages after Ingestion of M. microti and, later, of S. cerevisiae (EM Assessment)

Pretreatment of *M. microti*	After ingestion of *M. microti*			After ingestion of *S. cerevisiae*			
				M. microti–positive		*M. microti*–negative (yeasts alone)	
	Bacilli encountered	Phagosomes encountered	P-L fusion	Yeast phagosomes encountered	P-L fusion	Phagosomes encountered	P-L fusion
			%		%		%
Heat-killed	1,256	497	74*	74	57‡	59	63
Untreated (live)	856	483	15*	85	18‡,§,‖	104	55§
No bacteria						169	44‖

Yeasts were ingested by the monolayers 18 h after ingestion of the *M. microti*, many of them entering cells already containing *M. microti* (*M. microti*–positive) and others entering cells without *M. microti* (*M. microti*–negative); the yeasts were never seen in phagosomes already containing *M. microti*. Sampled cell profiles were systematically surveyed, and the contents of all with visible *M. microti* or *S. cerevisiae* organisms, or both, were recorded (cells without visible organisms being disregarded). Phagosomes, and bacilli within them, varied in number from cell to cell; yeast phagosomes contained only single organisms. Mean number of bacilli per *M. microti*–positive cell profile was 17 for heat-treated and 14 for untreated inocula; for accompanying yeasts the numbers were 1.0 and 1.3, respectively. If P-L fusion of phagosomes of companion yeasts is restricted to those seen in cell profiles containing 1–9 *M. microti*, the inhibition is still significant; thus, in 31% of such cells one or more yeast phagosomes showed fusion when the inoculum was of untreated (live) *M. microti*, compared with 70% for heat-treated inoculum and 85% when the cell profiles contained yeasts but no visible bacilli ($p = 0.05$ and <0.01, respectively.

*‡§‖ The differences within the pairs of percentages are significant ($p < 0.01$) (see text).

Table II shows that the incidence of P-L fusion after the ingestion of live *M. microti* (15%) was much (and significantly) less than that in comparable macrophages inoculated with dead organisms (74%), thus confirming previous reports (4, 5). The new observation is that the yeast phagosomes reflected these differences: those in the same cell profiles as *M. microti* phagosomes from the untreated (live) *M. microti* inoculum showed a fusion score of 18%, significantly lower than the 57% when the *M. microti* inoculum was of dead organisms. When restricted to cell profiles with 10 or more *M. microti*, the difference was even greater (7% against 57%). Two figures serve as controls for the 18% score. One is the significantly higher incidence of fusion of yeast phagosomes when alone in cell profiles of the same *M. microti*–infected monolayers (55%, probably underrated because of the likelihood of some bacilli being in fact present below or above the profiles). The other is the significantly higher score for yeast phagosomes in different monolayers entirely free of *M. microti* (44%). Finally, there is no evidence of inhibition (or enhancement) of fusion by yeast phagosomes in the dead *M. microti*–inoculated macrophages, since the score for those in profiles containing the bacilli as well (57%) was similar to the score for those in profiles containing yeasts alone (63%). These observations by EM thus confirm and extend those by FM. An experiment based on a culture containing aggregated bacilli gave essentially similar results to those described here for a single unit culture.

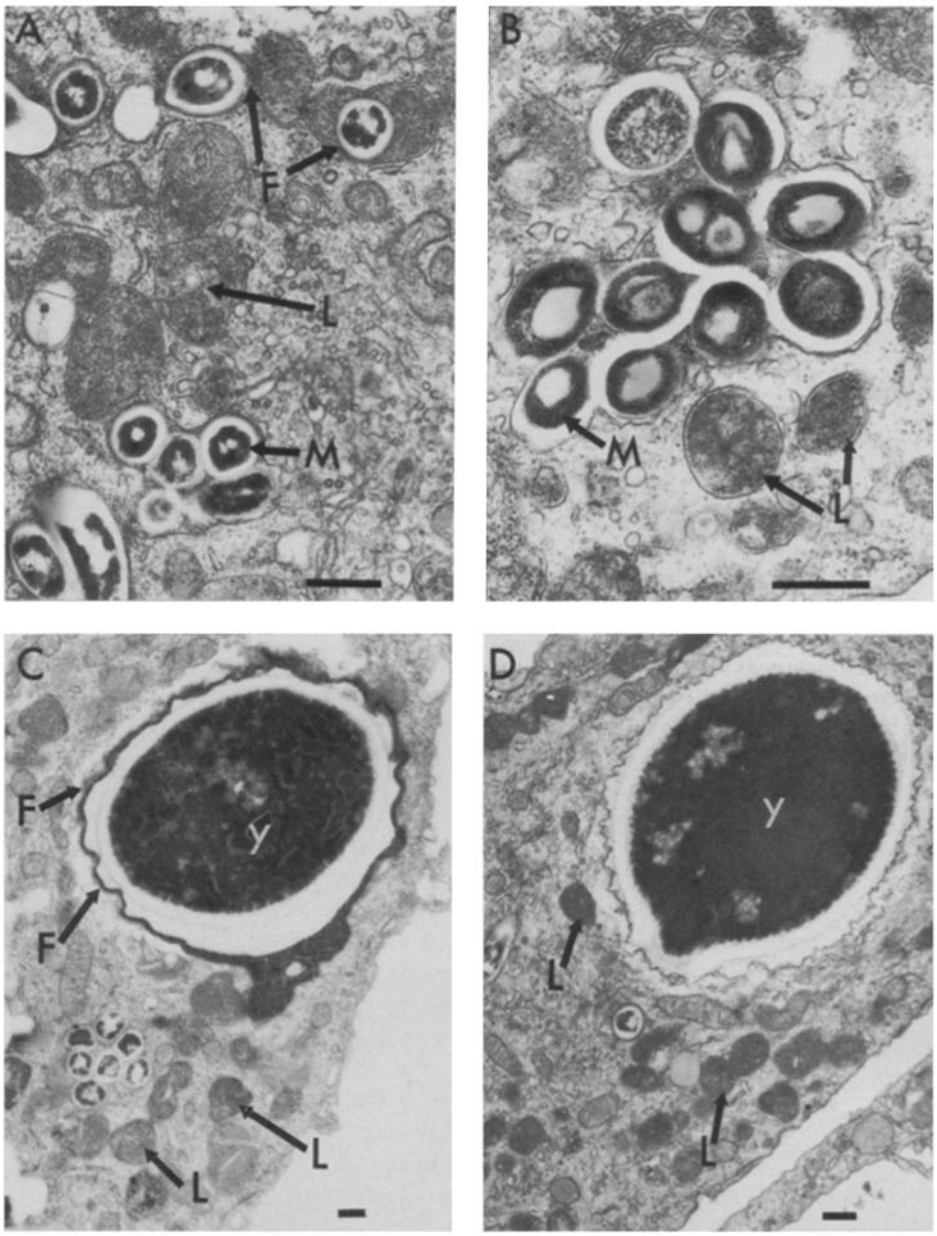

FIGURE 1. Ferritin-labeled cultured macrophages that had ingested, first, *M. microti* and, later, *S. cerevisiae*. Electronmicrographs illustrate the appearances of P-L fusion and nonfusion for each microorganism (Bars = 0.5 μm). (*A*) Heat-killed *M. microti* (*M*) in phagosomes, with labeled secondary lysosomes (*L*) in the cytoplasm, and label (*F*) within the phagosomes, indicating fusion. (*B*) Unheated (presumed live) *M. microti* (*M*), with labeled lysosomes (*L*) in cytoplasm but no label seen in phagosomes; no fusion. (*C*) *S. cerevisiae* (*Y*) in phagosome, with labeled lysosomes (*L*) in cytoplasm, and label (*F*) within the phagosome surrounding the yeast; indicates fusion. (*D*) *S. cerevisiae* (*Y*), with labeled lysosomes (*L*) in cytoplasm but no label seen in phagosome; no fusion.

Discussion

Specificity of the Mycobacterium-induced Effects: Appraisal of the Evidence. In the original studies (3, 4) of the responses of cultured macrophages to *M. tuberculosis* and *M. microti*, small intracellular bacterial populations were adequate to reveal P-L nonfusion by EM. On the other hand, to observe mycobacterially induced changes in lysosomal saltations in living macrophages by PCM, and changes in the fusion pattern of companion secondary organisms by FM or EM, larger bacterial populations have been considered advantageous, on the expectation that these changes are related to the strength of the stimulus. However, against this advantage is a risk of overloading and damaging the host cells, with consequent nonspecific effects, which might include the observed lysosomal stasis.

We believe that we have minimized this risk: (*a*) by using a mouse-passaged strain of *M. microti* that had been subsequently subcultivated continually for many months, a procedure that reduces the level of virulence and slows the progress of infection in the macrophage cultures but does not affect the capacity to inhibit P-L fusion; (*b*) by adjusting the inoculum of this strain to give an infection that does not appear initially to disturb the general health of the host cells to an important extent, as judged by the maintained extension of the cells on the coverslips, the absence of morphological deterioration detected by PCM or FM, and most important, an ability to phagocytize yeasts equal to that by control monolayers. Some 50 or more organisms were tolerated initially in infected cells, though the average was lower (see later); (*c*) by selecting mainly 18–24 h, but sometimes several days, of cultivation after ingestion of the bacteria as suitable and stable times for the lysosomal assessments (following the previously reported mycobacterial assessments of fusion at 1, 2, or 4 d (3, 4). However, P-L fusion is a relatively rapid event that takes place normally within 30 min of ingestion, and we therefore made assessments on infected macrophages also at 1–3 h. The notable contrast between the lysosomal stasis after a live inoculum (from single units, Tween/albumin cultures) and the continued normal movements after a heat-killed inoculum was already apparent; but the occasionally observed occurrence of stasis in the latter case too suggested temporary shock at this early postinfection period. This shock was transient, and cell recovery appeared to have taken place some hours later, the contrast in lysosomal movements being regularly evident at 18–24 h and persisting for a week or more, depending on the progress of the live infection and how long the monolayer remained intact. The postinfection period was thus divisible into a phase of stable and apparently healthy cells, lasting several days, and a later phase when the progression of the infection had become manifest.

Using and monitoring continually these arrangements of *M. microti* strain, inoculum, and times of assessment, we have gathered evidence that *M. microti*–induced lysosomal stasis, and the associated change in fusion pattern of subsequent companion yeasts, is not a nonspecific event secondary to a general depression of host cell activity. This evidence is discussed below.

Taking the inoculation and other conditions of the central EM experiment of Tables II and III as typical, the bacillary content of similar macrophages 1 d after infection was estimated in two ways: (*a*) by counts of intracellular acid-fast

TABLE III

Distribution of M. microti and S. cerevisiae in Macrophage Profiles (Supplementary Data from EM Experiment)

Pretreatment of *M. microti* inoculated	Cells encountered	After ingestion of *M. microti*			After ingestion of *S. cerevisiae*			
		Bacilli encountered	Bacilli per cell profile (mean)	Bacilli per *M. microti*–positive profile (mean)	Yeasts encountered	Yeasts per cell profile (mean)	Yeasts per *M. microti*–positive profile (mean)	Yeasts per *M. microti*–negative profile (mean)
Heat-killed	116	1,131	10	14	100	0.9	0.9	0.8
Untreated (live)	110	733	7	11	89	0.8	0.9	0.6
No bacteria	236	—	—	—	216	0.9	—	0.9

This table is based on further sections sampled from the same blocks as for Table II. All cell profiles encountered were recorded, i.e., including those with no visible organisms of either species (unlike Table II [see its footnote], where the record was of all phagosomes, but not of all cell profiles, encountered).

bacilli in the monolayers (stained by Ziehl-Neelsen method); (*b*) by acid-fast counts of bacilli in ultrasonicates of the infected monolayers (after dislodging the cells manually from the coverslips). Estimates made by extrapolating bacillary numbers per cell profile (Tables II and III) to numbers per whole cell involve assumptions that render that approach less reliable than actual counts of bacillary content. The results for method *a* were 9–30 (mean 18) per cell and 18–42 (mean 27) per infected cell (eight experiments); for method *b*, 7–36 (mean 24) per cell (six experiments). As stated above, such numbers derived from the strain of *M. microti* used do not appear to destabilize significantly the P-strain monolayer macrophages.

The heat-killed bacillary content of the macrophages in our experiments was closely similar to that after a live inoculum (judged qualitatively by PCM, by acid-fast stain, and by EM; see Tables II and III). Because lysosomal movements were actively maintained in cells containing comparable numbers of bacilli to those associated with stasis after a live infection, this stasis is unlikely to be due to numbers per se e.g., by lack of available cell space.

M. microti was compared with another mouse pathogen, *M. lepraemurium* (virulent Douglas strain, freshly mouse-passaged), which also multiplied in the macrophages (generation time 7–12 d, compared with ~1 d for *M. microti*). The characteristically very large bacterium/cell ratio required for ingestion of organisms of *M. lepraemurium* made contemporaneous comparisons difficult; however, (*a*) macrophages infected with this organism 4 d previously had a similar small uptake to that of *M. microti*, in cells with a much reduced inoculum of the latter, assessed on day 1; (*b*) after 2 mo of infection by *M. lepraemurium* (monolayers still intact), the number of bacilli per cell was of a similar considerable amount to those after 1 d of infection by *M. microti*, when receiving its usual inoculum. Under these conditions of comparability, we observed that, in contrast to the *M. microti*, there was at no time an association of the *M. lepraemurium* either with lysosomal stasis or (after ingestion of yeasts) with inhibition of P-L fusion. The failure of this mycobacterium, of at least equal virulence to *M. microti*, and with comparable intracellular numbers of live bacilli, to induce stasis supports the view that the latter is a specific result of the *M. microti* infection.

Further evidence that *M. microti*–induced lysosomal stasis and the associated nonfusion of yeast phagosomes is not due to damage by an excessive bacillary

load is provided by two sets of observations: (*a*) the graded effect of reducing the inoculum to give a much lighter infection, with fewer than five bacilli per infected cell. Instead of cells showing either slowing or normal lysosomal movements, we observed in some of them a group of bacilli with focal (presumably periphagosomal) slowing of lysosomal movements around it, while the lysosomes were moving normally in other areas of the same cell where bacilli were not seen. This evidence is supported by the focal distribution of inhibition of movements around live bacilli, and normal movements around identifiable Ziehl-Neelsen–killed bacilli, in the same cell (p); (*b*) by the additional analysis of the data in Table II that indicates that even when the bacillary content of live *M. microti*–positive cell profiles was <10 (mean, 6.5 compared with the general mean of 14), inhibition of fusion was still significantly evident in associated yeasts (judged by comparison with killed *M. microti*–positive or *M. microti*–negative profiles).

It still remains a possibility that even at the *M. microti* population figures given above for whole cells and for cell profiles, and in spite of the morphologically healthy appearances, infection with live *M. microti* renders the host cells functionally unhealthy, causing nonspecific loss of fusion capacity in associated yeasts compared with unaffected cells or cells containing heat-killed bacilli. This matter was further examined by using the observation that monolayers with cells reversibly or irreversibly depressed manifest this effect by ingesting fresh yeasts poorly or not at all; should some unhealthy cells be present in an otherwise healthy monolayer, they too should be detected. Although the many observations by FM did not indicate differences in uptake of fresh yeasts by individual cells that could be related to the different numbers of organisms therein, more quantitative data are available from the monolayers of the day 1 sample in Tables II and III. Table II shows that, on average, yeasts were seen as frequently in *M. microti*–positive cell profiles containing live bacilli as in those containing dead bacilli (1.3 and 1.0 per cell profile, respectively). The more inclusive population data of Table III also show that, under the conditions of the experiment, the cells containing live *M. microti* were not at a disadvantage, judged by their response when exposed to yeasts; thus the number of yeasts seen was no less in *M. microti*–positive than in *M. microti*–negative cell profiles, whether the inoculum was of live or dead bacteria, and the figure was much the same when no bacteria were inoculated.

The lysosomal stasis induced by live *M. microti* can be reversed: (*a*) by substituting heat-killed *S. cerevisiae* (100°C in Ringer solution for 1 h) for the fresh yeasts, to be ingested at 1 or 4 d after an infection by live *M. microti*. Heated yeasts are much more fusiogenic in macrophages than fresh yeasts, normally causing accelerated lysosomal movements, rapid P-L fusion, and degranulation. When ingested by a previously *M. microti*–infected cell and examined by PCM 10–30 min later, restoration of lysosomal movements could be observed around the (presumed intraphagosomal) heated yeasts, while in other areas of the same cell the *M. microti*–associated stasis still prevailed; moreover, AO showed enhanced P-L fusion of these yeast phagosomes to have occurred. This behavior is the converse of the sequence after ingestion by fresh yeasts after *M. microti* infection, where both focal and general stasis was unaffected and the yeast

phagosomes showed inhibited P-L fusion; (*b*) by infecting the monolayers with live *M. microti* and, 1 d later when lysosomal stasis was evident, adding drugs to the culture medium to kill the bacteria in situ. Because we have shown that chloroquine in the medium for 7 d would reduce the viability of *M. tuberculosis* in macrophages by over 90% (19) we used this amine (at 20 μM), plus a high dose (130 μg/ml) of streptomycin in the expectation that *M. microti* too would be killed. Chloroquine is also an accelerator of lysosomal movements and of P-L fusion (11), and these properties might assist in inducing a reversal. Movements were restored in most infected cells after 7 d of treatment at 37°C, and the former inhibition of fusion of the phagosomes of then-added fresh yeasts was also reversed. Controls of untreated monolayers showed continued stasis and inhibited fusion of yeast phagosomes, while uninfected monolayers treated or untreated showed normal movements and yeast P-L fusion.

These reversals of lysosomal stasis show that the latter was not due to a permanent inactivation resulting from the mycobacterial infection. The continued presence (at least in the case of the heat-killed yeasts) of the live *M. microti* population in cells showing reversal supports the evidence from heat-killed *M. microti* (see above) that (apart from an exceptionally heavily loaded cell) the original stasis was not due simply to restricted available host cell space impeding organelle movement. Moreover, the reversals were induced by agents (heat-killed yeasts, *M. microti* killed within their phagosomes, and chloroquine itself) that have known associations with lysosomal movement, rather than by agents that act by generalized stimulation of cell activity. This suggests that the original stasis is specific rather than caused by host cell functional damage, e.g., from the toxicity of the infecting bacilli.

From the foregoing evidence we infer that lysosomal stasis after infection of macrophages with live *M. microti* is as true and specific a phenomenon as the previously reported P-L nonfusion for this mycobacterium, for *M. tuberculosis*, and for certain other species of microorganisms. We interpret our present observations accordingly.

Interpretation of the Observations. The phagosomes of live, but not of dead, *M. microti*, like those of *M. tuberculosis*, fail to fuse with lysosomes in macrophages (4, 5). The two main observations now reported throw some light on how this comes about. One is the associated inhibition or paralysis of the saltatory movements of periphagosomal lysosomes after the ingestion of live, but not of dead, *M. microti*, in contrast to their continued movements in response to the fusiogenic *M. lepraemurium*. This observation suggests an explanation for the inhibited P-L fusion, for if the lysosomes are slowed or stationary, contact between them and phagosomes is likely to be much reduced, since the movements of phagosomes are relatively very slow. This concept is strengthened by the second main observation, that the nonfusion induced by the *M. microti* infection is "transmitted" to the phagosomes of a different and unrelated organism, *S. cerevisiae*, when it is ingested into the same cell; its normal stimulation of fusion is in most cases converted to inhibition. The slowing or stasis of the lysosomes (induced by the *M. microti*) seems to provide a simple and compelling explanation for the nonfusion acquired by the yeasts in the same cell, and thus also for at least a main part of the nonfusion shown by the mycobacterium-containing phagosomes themselves. The alternative view, that inhibition of the fusion of the

mycobacteria-containing phagosomes is caused differently from that of the yeast phagosomes, seems very unlikely.

If it is accepted that inhibition of P-L fusion is largely caused by an earlier event, i.e., lysosomal stasis, it then becomes necessary to explain how the presence of phagosomes containing live mycobacteria can bring about this earlier event (which may itself involve the microtubule system). One possibility is an entirely membrane effect, initiated by contact of the bacterium with plasma or phagosome membrane. Another is a cytoplasmic soluble factor; such a factor would have to become available almost immediately after phagocytosis. Possible candidates are one or more of the nitrogenous bases that can modulate lysosomal movements as well as P-L fusion in normal macrophages (10, 11, 13). Thus ammonium chloride, applied to monolayers, leads rapidly to inhibition both of fusion (yeast target, AO lysosomal label [FM]; and yeast target, ferritin label [EM]) and of movements (10, 11). Many mycobacteria produce ammonia copiously in culture media (10) (though so do other organisms). Macrophages themselves also have ammonia-producing capacity (e.g., from glutamine and glutaminase [20]). A model of intraphagosomally produced ammonia in macrophages, consisting of urease-linked carboxylated latex beads (3.0 μm-diam) followed, after phagocytosis, by urea, mimicked *M. microti* by inducing stasis of periphagosomal lysosomes (the effect sometimes extending to adjoining cells); moreover, P-L fusion of the phagosomes of yeasts ingested subsequently into the same cell was inhibited (our unpublished observations). However, we have no direct evidence at present for a role for ammonia in the mycobacterium-induced inhibitions.

Based on the present information, we draw the limited conclusion that, as with the lysosomotropic chemical inhibitors of P-L fusion previously described (11), inhibition of lysosomal movements is largely responsible for the mycobacterium-induced inhibition of fusion. We have yet to determine how this stasis is produced by the infection, i.e., whether through a soluble factor or by other means. Also, the mediatory role of microtubules requires clarification. A preliminary experiment using a monolayer in which lysosome stasis was induced chemically, and an immunofluorescence technique (antitubulin), did not show appreciable morphological differences from controls, but clearly further study is indicated.

A previous study by FM and EM (21) sought to determine if infection of macrophages by the P-L fusion–inhibitor *T. gondii* would interfere with the ability of the lysosomes of the same cell to fuse with *Trypanosoma cruzi*, a fusiogenic organism. The results were negative. An explanation of this difference from our findings may be that, perhaps because the two infections were simultaneous or within 1 h of each other, compared with our 18 h, very few of the large number of macrophages that became infected contained both parasites (3–7%, compared with our 60–80%) so that (presumably) the comparisons were mainly of the two organisms in different cells; from our observations, the inhibition of the yeast-induced fusion was usually not evident outside the cell containing the inhibitory *M. microti*. Another explanation might be simply that P-L fusion after ingestion of fresh yeasts is more easily converted to nonfusion than in the case of *T. cruzi*.

Summary

We have investigated the mechanism of the inhibition of phagosome-lysosome

(P-L) fusion in macrophages known to occur after infection by *Mycobacterium tuberculosis* (3) and by the mouse pathogen *Mycobacterium microti* (4, 5). We have used an *M. microti* infection and have studied, first, the saltatory movements of periphagosomal secondary lysosomes by means of visual phase-contrast microscopy (a similar use of the method having been previously supported by computer analyses [11, 12]). The movements became slow or static after ingestion of live but not of heat-killed *M. microti*. They were unaffected by a fusiogenic mycobacterium *M. lepraemurium*.

Second, we studied the behavior of a normally fusiogenic unrelated organism, *Saccharomyces cerevisiae*, after its phagocytosis by cells already containing live *M. microti* ingested 18 h previously. We observed, using a fluorescent assay of fusion (6, 11, 18), that many of these yeast phagosomes now also failed to fuse with the lysosomes; in contrast, when the host *M. microti* had been heat killed the yeast phagosomes fused normally. These observations were extended by ultrastructural quantitative analyses (3) of P-L fusion, which confirmed the nonfusion of phagosomes of live *M. microti* and, more particularly, the change to nonfusion from the normal fusion behavior of the separate phagosomes of accompanying yeasts.

Third, we have assembled evidence against the likelihood that these *M. microti*–induced phenomena are nonspecific, i.e., secondary to a general depression of activity of heavily infected host cells. The evidence includes the feasibility of adjusting the degree of infection so as to facilitate visual assessment of organelle movements without the presence of detectable damage to the cells studied; the absence of lysosomal stasis after comparable infection with another mycobacterium of comparable virulence (*M. lepraemurium*); and the reversibility of the stasis. We conclude that inhibition of lysosome saltatory movements (and consequently its secondary effect on the associated yeasts) is a significant, specifically induced phenomenon.

From these observations and considerations, therefore, in conjunction with the analogous inhibition of lysosomal movements in normal macrophages by some chemical inhibitors of P-L fusion, and our suggestion that this association is causally related (11), we now suggest that *M. microti*–induced focal lysosomal stasis is also the main means by which the inhibition of P-L fusion is brought about by this organism. This concept is strengthened by the observations on *S. cerevisiae*, which provide strong evidence that stasis can cause suppression of fusion.

We thank Mrs. I. Mandavia for preparing macrophage cultures and for the intracellular mycobacterial counts.

Received for publication 3 February 1987 and in revised form 27 May 1987.

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