Effects of Anionic Inhibitors of Phagosome-Lysosome Fusion in Cultured Macrophages When the Ingested Organism is *Mycobacterium lepraemurium*

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The mouse pathogen *Mycobacterium lepraemurium* is readily phagocytosed by cultured mouse peritoneal macrophages. Ingestion is normally followed by fusion between phagosomes and lysosomes. The influence of some anionic compounds known to inhibit fusion in other systems was investigated by transmission electron microscopy after ingestion of *M. lepraemurium*. Fusion was markedly, although temporarily, inhibited by suramin and moderately inhibited by poly-D-glutamic acid. The effects are, however, not sufficient to permit these agents to be used to study the long-term effects of shutting off the secondary lysosome-phagosome fusion system in cultured macrophages infected with *M. lepraemurium*.

Ingestion of some pathogens, for example *Mycobacterium tuberculosis* and *Toxoplasma gondii*, by cultured mouse peritoneal macrophages is not followed by the fusion of lysosomes with phagosomes usually induced by foreign bodies (1, 9). This failure to fuse apparently involves primary as well as secondary lysosomes. Some lysosomotropic compounds also modify fusion of macrophage lysosomes with phagosomes containing suitable targets: polyanions, e.g., suramin and poly-D-glutamic acid (PGA), inhibit fusion, and lipophilic secondary or tertiary amines, e.g., chloroquine and tributylamine, enhance fusion (4, 7, 8, 8a; P. D'Arcy Hart and M. R. Young, *in* R. Van Furth (ed.), *Mononuclear Phagocytes—Functional Aspects*, in press). The site of action of these modifiers of fusion is probably the secondary lysosomes, at least in the case of the polyanion inhibitors, which enter the host cell by endocytosis.

In the studies carried out by Hart and co-workers (4, 7, 8, 8a), active compounds have been identified with dark-field vital fluorescence microscopy and transmission electron microscopy (EM). Lysosomes were prelabeled with suitable markers, and fresh living or lightly gamma-irradiated cells of bakers' yeast (*Saccharomyces cerevisiae*) were used as a fusion-promoting intraphagosomal target. This model offers a convenient means to study the kinetics of phagosome-lysosome fusion (P-LF) and to screen potential inhibitors and enhancers of this fusion.

However, primary lysosomes are probably unaffected by the agents so far identified, a fact that imposes limitations on the use of this model in studies on the role of the lysosome system in the fate of ingested microorganisms; such studies require all P-LF to be prevented. A further limitation is imposed by the target itself. Treating yeast cells drastically (e.g., heating at 100°C or exposing for 12 h to 1.5% glutaraldehyde) largely overcomes the inhibitory effect of the polyanions on P-LF. Hence, it might be expected that the fusion of secondary lysosomes with phagosomes containing fusion-inducing microorganisms might not be inhibited if the "fusion-promoting power" of these organisms was sufficiently high. The balance between promotion and inhibition of fusion can only be discovered in each case by trial, which must usually be by EM, since only large organisms (such as yeasts) can be adequately visualized by the vital fluorescence microscopy technique.

It seemed interesting, therefore, to test the effect of polyanion inhibitors of P-LF (recognized as such in the vital fluorescence microscopy or EM yeast system) after the ingestion of fusion-inducing pathogens. Using EM, Pesanti (11) observed *Listeria monocytogenes* ingested by mouse peritoneal macrophages and failed to find even transient inhibition of P-LF after pretreatment with suramin. We report here an EM investigation of the mouse pathogen *Mycobacterium lepraemurium* in macrophages pretreated with suramin or PGA. This organism, unlike *M. tuberculosis* but like *L. monocytogenes*, normally induces fusion of lysosomes with the great majority of phagosomes (5).

MATERIALS AND METHODS

Bacterial suspensions. Suspensions of *M. leprae-*

murium strain Douglas were isolated from livers of intravenously infected CBA mice, using procedures based on those described previously (3, 6).

Inhibitors of fusion in the yeast systems. Suramin (ICI Pharmaceuticals, Macclesfield, England) was sterilized in aqueous solution by filtration. Synthetic poly-α-D-glutamic acid (sodium salt; molecular weight, about 74,000) was obtained from Sigma Chemical Co.; it was dissolved in water, and the solution was neutralized and sterilized by filtration.

Culture and infection of cells. Unelicited resident mouse peritoneal macrophages from female albino P strain mice were established as monolayers in Y. T. Chang's long-term culture medium (containing 40% horse serum) on cover slips in Leighton tubes (1, 2). Cells were cultivated for 1 to 2 weeks to obtain mature cells capable of a lively lysosomal fusion response. The monolayers were exposed for 3 h to ferritin (Pentex; twice crystallized and "cadmium-free"; code 96-028, Miles Laboratories, Kankakee, Ill.) at 10 mg/ml in balanced salt solution (1, 2). On the next day, suramin (100 μg/ml) was added in fresh medium to some of the cultures, the remainder being left as untreated controls. The cultures were reincubated at 37°C for 6 days. After washing with balanced salt solution they were exposed for 1 h at 37°C to a heavy suspension (1×10^9 to 2×10^9 cells per ml) of *M. lepraemurium* in balanced salt solution. Sample monolayers were fixed for transmission EM at the end of this exposure; the remainder were washed, given fresh medium without suramin, reincubated, and fixed 23 h later.

The effect of PGA was tested similarly, after pretreatment of the macrophages with 200 μg of PGA per ml for 6 days at 37°C; in this case all cultures were exposed to bacteria for 1 h and fixed after a further 1 h in balanced salt solution.

EM. The method of fixation and subsequent procedures were as previously described (1). The cell profiles were systematically surveyed in thin sections, and P-LF was identified by the presence of ferritin label, derived from secondary lysosomes, within the bacterium-containing phagosomes (1, 2).

RESULTS

In thin sections of the control macrophages, ferritin was observed to mark both the cytoplasmic dense granules (secondary lysosomes) and nearly all of the bacterium-containing phagosomes (Fig. 1). In the suramin-treated cells many of the lysosomes were swollen, so that the ferritin appeared dilute and was often displaced to the periphery; the lysosomal contents were frequently of low density and reticulated. Autophagic vacuoles, a feature of suramin-treated cells (7), were evident. Ferritin was apparently absent from many of the bacterium-containing phagosomes in the suramin-treated macrophages fixed immediately after ingestion (Fig. 2). At 24 h, however, the appearances of phagosomes uniformly indicated fusion; the morphology of lysosomes and autophagic vacuoles was unchanged. The striking phenomenon of "loose" phagosomal membranes described after ingestion of yeasts by suramin-treated macrophages (8a) could not be identified with phagosomes containing *M. lepraemurium*, possibly because
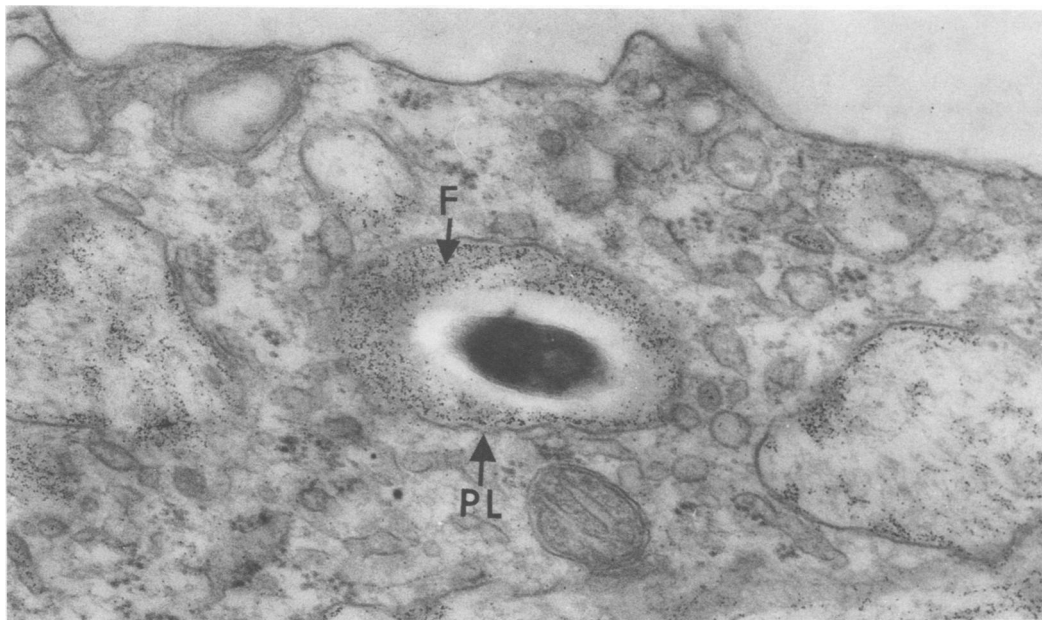

FIG. 1. *Ferritin-labeled control macrophage immediately after ingestion of M. lepraemurium. Profile of intact bacillus surrounded by ferritin (F) within phagolysosome (PL), showing that lysosomal fusion has occurred. ×56,000.*
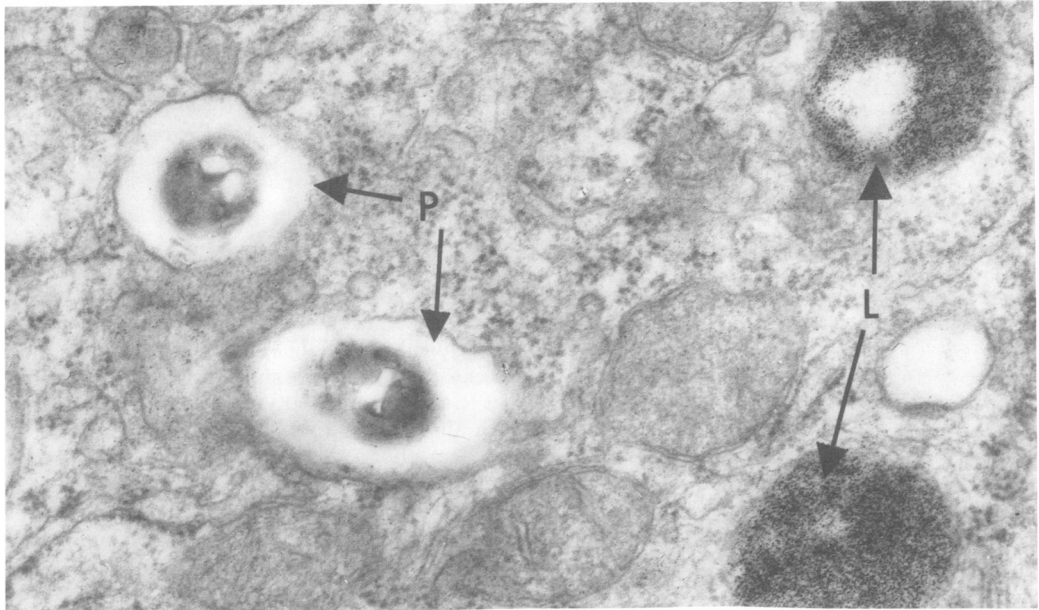
FIG. 2. *Conditions as in Fig. 1, but macrophages pretreated with suramin. Lysosomes (L) are labeled, but ferritin is absent from phagosomes (P). Nonfusion.* ×56,000.

of the smaller size of the latter. The appearances of the secondary lysosomes in PGA-treated cells (not shown) were similar to those after suramin treatment, but autophagic vacuoles were rare.

The extent of P-LF after ingestion of *M. lepraemurium* is shown in Table 1. In the untreated (control) macrophages the great majority of phagosomes containing mycobacteria showed evidence of fusion with ferritin-labeled lysosomes, thus confirming previous observations (5). When fixed immediately after ingestion of the bacteria, the suramin-treated macrophages showed a pronounced and significant contrast (28% phagosomes fused compared with 90% in the controls; $P < 0.01$, assessed by a graphic method [10] derived from Fisher's formula for calculating exact probabilities). This contrast had disappeared by 24 h, P-LF being then universal in both groups. PGA-treated macrophages showed a moderate and significant inhibition of fusion when fixed 1 h after the ingestion of the mycobacteria (40% phagosomes fused compared with 83% in the controls; $P < 0.01$).

In the type of preparation used, EM shows most of the *M. lepraemurium* to be intact; it has been inferred that they have high viability (5).

DISCUSSION

These results emphasize that the target chosen for ingestion by cultured mouse peritoneal macrophages in experiments to test for inhibi-

TABLE 1. *Proportions of phagosomes showing fusion with ferritin-prelabeled lysosomes in macrophages treated with polyanions and then infected with M. lepraemurium[a]*

Pretreatment of macro- phages	Time after start of ingestion of bacteria (h)	No. of phagosomes encoun- tered	% of phago- somes fused[b]
None	1	120	90[c]
Suramin	1	177	28[c]
None	24	100	100
Suramin	24	100	98
None	2	90	83[d]
PGA	2	100	40[d]

[a] Lysosomes of mouse peritoneal macrophage monolayers were labeled with ferritin, and some of the cultures were treated with suramin (100 µg/ml) or PGA (200 µg/ml) at 37°C for 6 days, the remainder being left untreated; then all were exposed to *M. lepraemurium* for 60 min and were fixed for EM at stated times thereafter.

[b] i.e., containing visible ferritin label.

[c] Difference significant at $P < 0.01$, assessed by the method referred to in the text.

[d] Difference significant at $P < 0.01$, assessed by the method referred to in the text.

tion of P-LF by chemical compounds is of critical importance. Our initial selection of fresh living yeast cells was perhaps fortunate, since they normally induce fusion (judged from vital fluorescence microscopy) in slow and visibly distin-

guishable stages, and this fusion can be readily and consistently suppressed or inhibited by various anionic compounds, such as suramin and PGA. The yeast model has been used, for example, in a study of phagosome ultrastructure (8a) and to assess the altered fusion behavior of macrophages from a virus-infected host (G. J. Jakab, G. A. Warr, and P. L. Sannes, RES J. Reticuloendothel. Soc. **23**: 27i, Abstr. no. 51, 1978).

We had noted, however, that heat-killed yeasts accelerated the fusion process, which was then only marginally inhibited by suramin. Other targets, such as latex spheres and zymosan, also gave less satisfactory or variable results in our hands. Judged from EM, the fusion response after ingestion of *L. monocytogenes* (a fusion promoter) is not inhibited by suramin (11). We have now shown that intracellular *M. lepraemurium* (also a fusion-promoting pathogen) is associated with a marked and significant, but transient, inhibition by suramin and with only moderate (although also significant) inhibition by PGA. These particular agents cannot, therefore, be used to examine the long-term effects of shutting off the secondary lysosome-phagosome fusion system upon cellular and subcellular behavior in infections of cultured macrophages with *M. lepraemurium*. However, the activity of agents effective in the yeast system may be of basic significance in some pathogenic infection systems. For example, all of the anionic compounds that have been shown to inhibit P-LF in the yeast system accelerate the progress of an experimental infection with the fusion inhibitor *M. tuberculosis* in mouse peritoneal macrophage cultures. Conversely, all of the amines that enhance fusion in the yeast system, and all that have been tested, suppress or inhibit the progress of the tuberculous infection (8; Hart and Young, *in Mononuclear Phagocytes—Functional Aspects*, in press). The mechanism of this contrasting behavior is at present unknown.

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