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Pathology

Prevention of phagosome–lysosome fusion in cultured macrophages by sulfatides of *Mycobacterium tuberculosis*

(electron microscopy/endocytosis/membranes/polyanions)

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ABSTRACT **Intracellular parasites (e.g., *Mycobacterium tuberculosis, Toxoplasma gondii,* and some *Chlamydiae*) may promote their survival within the host by acting from within phagosomes to prevent phagolysosome formation, thus avoiding exposure to the lysosomal hydrolases. The present studies demonstrate that when sulfatides of *M. tuberculosis* (anionic trehalose glycolipids largely responsible for the neutral red reactivity of virulent strains) are administered to cultured mouse peritoneal macrophages, they accumulate in the secondary lysosomes, which are rendered incompetent for fusion with phagosomes containing suitable target particles such as viable yeasts. This antifusion effect is also exhibited when small amounts of sulfatide are introduced directly into phagosomes by attachment to the target yeasts prior to their ingestion. The sulfatides evidently exert a selective inhibitory influence on membrane fusion, analogous to what occurs typically when macrophage cultures are infected with tubercle bacilli. This effect may be due to ionic interaction between the polyanionic micelles of bacterial sulfatide and organelle membranes, modifying the latter and inducing dysfunction.**

The sulfatides of *Mycobacterium tuberculosis* (1) have been characterized as several closely related glycolipids—more specifically, multiacylated trehalose 2-sulfates (2). Because a significant correlation has been established between production of strongly acidic lipids, including sulfatides (SL), and order of "infectivity" (3) or virulence (4) for some 40 strains of *M. tuberculosis*, it was inferred that these substances might be a factor in the virulence of these organisms.

We have previously speculated that in parasitized host cells the glycolipids might influence phagosomal or lysosomal membranes, so as to interfere with their capability to fuse and form phagolysosomes (4). Such interference could underlie a survival mechanism for some intracellular parasites, as exhibited by viable virulent tubercle bacilli during infection of cultured macrophages (5, 6). A similar failure of lysosomes to fuse with phagocytic vacuoles containing viable *Chlamydiae* (7) or *Toxoplasma gondii* (8) has also been established.

Our present studies with purified sulfatides from *M. tuberculosis* strain H37Rv show that they are potent inhibitors of phagolysosome formation in cultured mouse peritoneal macrophages. The inhibition may involve dysfunction in either phagosomal or lysosomal membranes—or both. We present a preliminary report of these investigations.

MATERIALS AND METHODS

Tissue Culture and Related Procedures. Unelicited peritoneal macrophages of normal female mice of the albino P strain were maintained as coverslip monolayers in Chang medium (9) in Leighton tubes as described previously (5, 10). The monolayers were used after about 10 days in culture.

Sulfatides of *M. tuberculosis*. Thirty-day surface cultures of strain H37Rv were harvested and the principal sulfatide (SL-I), a 2,3,6,6′-tetraacyl trehalose 2′-sulfate (molecular weight about 2400), was recovered and purified as previously described (11). [*1*-^{14}C]propionate, which is readily incorporated into unique methyl-branched substituents in SL (2), was included in the culture medium to effect a light radiolabeling.

Sulfatide Emulsions. Very fine aqueous dispersions of SL of prolonged stability were prepared (without emulsifying agents) by modifications of a method developed by M. Kato for preparing aqueous emulsions of cord factor (6,6′-dimycoloyl trehalose) (12).

Attachment of Sulfatides to Yeast Cells. Fresh yeasts were suspended in 0.3 M glucose to give 10^8 cells per ml; 0.5 ml of suspension was mixed with 0.1 ml of aqueous emulsion containing 400 μg of SL-I. After 10–15 min, 20 μl of 5% aqueous postassium alum (about 2 μmol of Al^{3+}) in the tip of a pasteur pipette were inserted below the surface of the suspension and the mixture was rapidly aspirated back and forth. After standing for 15 min, the suspension was made up to 1.5 ml with 0.3 M glucose.

Radioassay. Uptake of radioactive SL by macrophage monolayers was determined by scintillation counting. Fixing the washed monolayer in 5 ml of absolute ethanol for several hours extracted the sulfatide quantitatively for recovery and counting in toluene–Triton X-100 scintillation fluid.

Phagolysosome Formation: Observation by Fluorescence Microscopy. A recent application of dark-field vital fluorescence microscopy (13) was used. Macrophage secondary lysosomes are prelabeled with acridine orange (AO) and thus acquire a bright orange-red fluorescence due to concentration of the dye (14). The phagocytes are then permitted to ingest live baker's yeast cells (*Saccharomyces cerevisiae*). The progress of lysosome–phagosome fusion, giving rise to phagolysosomes, is recognized from passage of the fluorescent marker into the yeast-containing phagosomes. The latter exhibit vivid orange confluent "rims" of fluorescence at early stages (e.g., after 45 min at 37°); this is followed by progressive coloration of the yeast cells (at first green and later red) as increasing levels of AO and mixed lysosomal contents accumulate in the phagolysosomes. However, in the absence of fusion, the phagosomes appear as dark spaces against a background of fluorescent lysosomes. Results derived from fluorescence microscopy were confirmed at the ultrastructural level, using ferritin as the lysosomal marker.

Electron Microscopy and Ferritin Prelabeling of Lysosomes. Basic procedures, applied to experimental and control cultures, were as described previously (5, 13, 15); and, to achieve optimal visualization of intracellular membranes, included

Abbreviations: SL, sulfatide; SL-I, principal sulfatide of *M. tuberculosis*, strain H37Rv; AO, acridine orange.

‡ On sabbatical leave at Mill Hill, July–December, 1974.

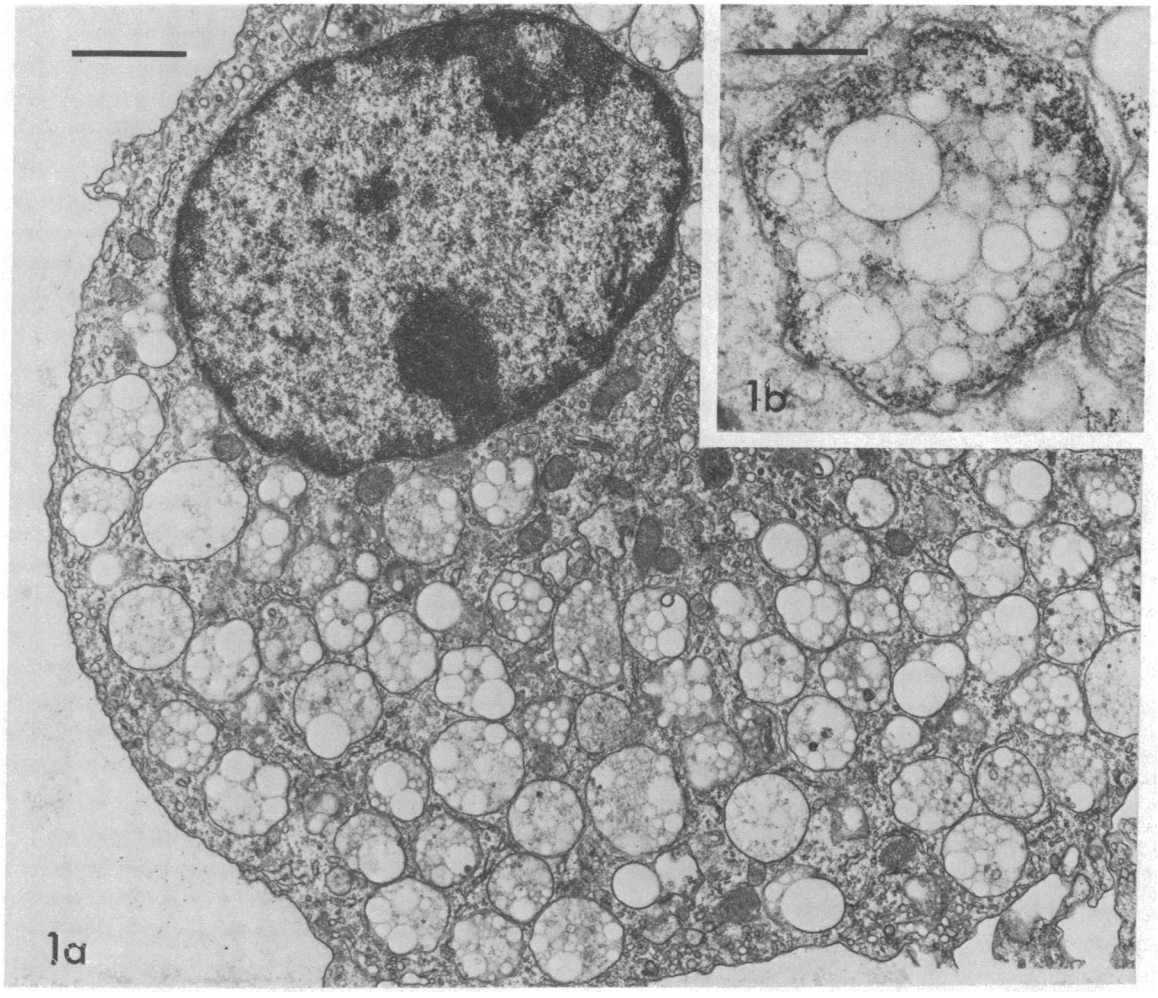

FIG. 1. (a) Thin section of a swollen macrophage from a culture incubated 72 hr with 300 μg of SL-I/ml, revealing massive accumulation of lucent "sulfatide droplets" within membrane-bound cytoplasmic vesicles. (Electron micrograph: bar represents 1 μm.) (b) Higher magnification from a similar sulfatide-treated (20 hr) macrophage prelabeled with ferritin. Ferritin particles and the "sulfatide droplets" are seen together in the same vesicle identified as a secondary lysosome. (Bar represents 250 nm.)

routine fixation by the Hirsch-Fedorko technique (16). To assess the extent of phagosome-lysosome fusion, monolayers were first pulse-labeled with ferritin; on the next day half of the cultures were exposed for 20 hr at 37° to SL-I, the remainder serving as controls. After the monolayers were washed, live yeasts were added to all cultures, which were reincubated for 50 min prior to fixation.

RESULTS

Uptake and lysosomotropism of sulfatide

In cultures of macrophages treated with relatively high concentrations (200–400 μg/ml) of SL-I in the medium for 24 hr at 37° the cells became enlarged and visibly foamy. More prolonged exposure, up to 72 hr, usually provoked further swelling, rounding, generalized vacuolation, and a tendency to aggregate. These morphological changes were less marked, or absent, after exposure doses of 100 μg/ml or less for 20 hr. Assessment of the phagocytic capability towards yeasts of SL-treated macrophages (100 μg/ml for 20 hr) showed no appreciable difference from that of untreated controls. Sulfatide is probably adsorbed at least in part at the macrophage surface: thus, normal macrophage monolayers showed little or no affinity, after formalin fixation, for neutral red, but cells exposed to SL were almost uniformly stained by this cationic dyestuff, resembling the complexing of the dye which is shown by virulent tubercle bacilli themselves, and which is believed to be associated with peripherally located sulfatide (1, 2, 4).

Electron micrographs of sectioned macrophages after exposure to SL (at 100–300 μg/ml) provided evidence of lysosomotropism. Typical thin sections (Fig. 1a) revealed that at high dose levels the normal population of lysosomal dense granules had been replaced by a profusion of membrane-bound vesicular structures throughout the cytoplasm, containing both granular material and large numbers of electron-lucent droplets, each with an opaque margin or interface; the droplets varied in size up to around 350 nm in diameter. In experiments in which the lysosomes had been labeled with ferritin before SL treatment (Fig. 1b), ferritin particles and these droplets were located within the same vesicular profiles, the ferritin being usually displaced to the periphery. Droplets were also recognized regularly within small pinocytic vacuoles, some profiles of which were evidently fusing with lysosomes; but droplets were never found free in the cytoplasm, nor in the nucleus or mitochondria. From these ultrastructural features it is inferred that after entering the macrophages by endocytosis, the sulfatide (or some derivative thereof) becomes concentrated selectively, in droplet form, within distended secondary lysosomes; this alone could account for the cellular swelling described above.

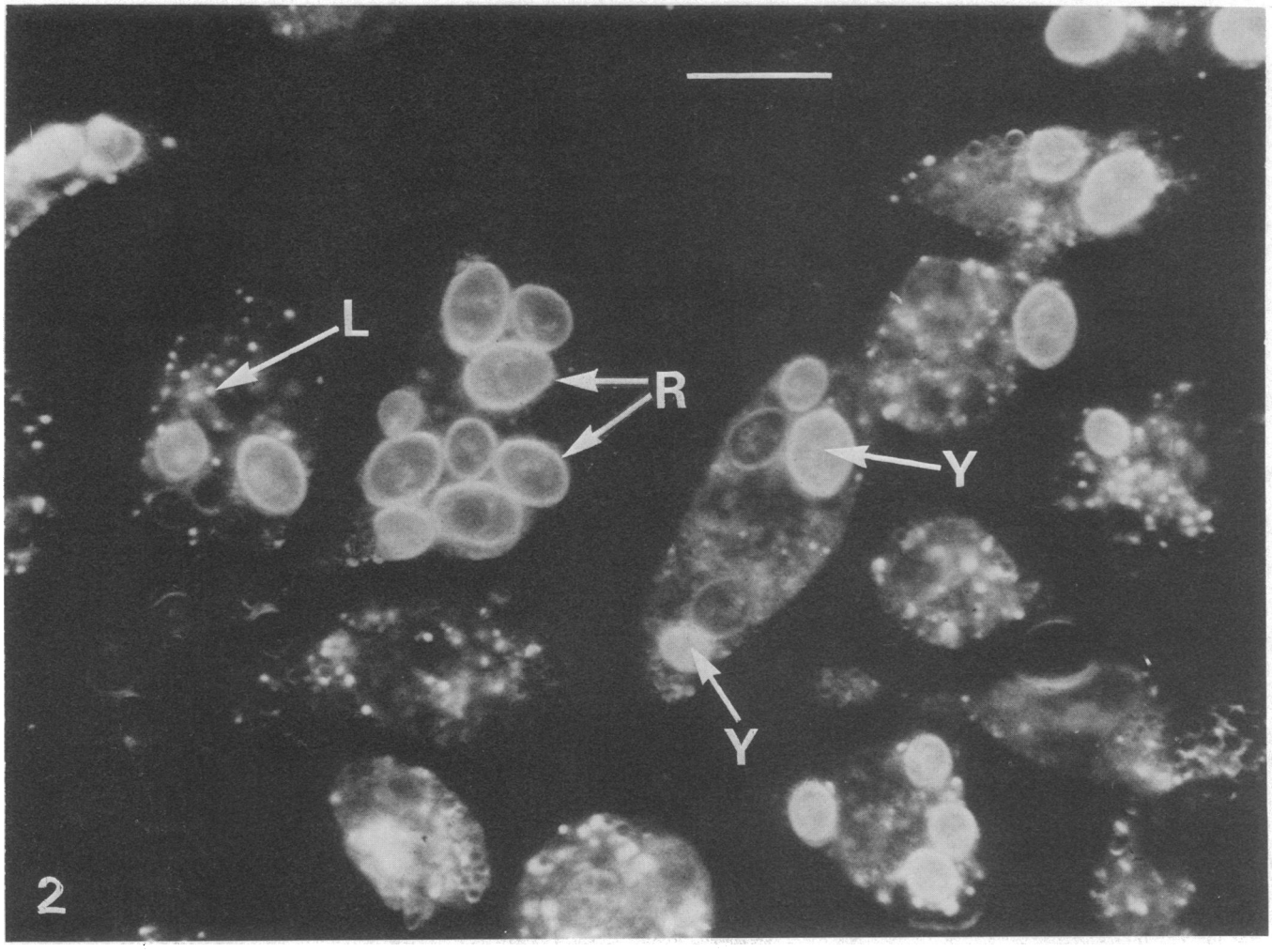

FIG. 2. Living macrophages with acridine-stained lysosomes (L) after incubation with live yeasts at 37° for 45 min. Some yeasts were surrounded by bright intraphagosomal fluorescent "rims" (R), and others showed an intense overall fluorescence (Y), indicating diffusion of the lysosome-marker dye into the yeast cell. This is the normal pattern of phagosome–lysosome fusion response. (From a color micrograph: bar represents 10 μm.)

Effects of sulfatide on phagolysosome formation

SL-loaded macrophage monolayers examined by the AO technique after ingestion of yeast cells showed a virtually total failure of the lysosomes to fuse with yeast-containing phagosomes during the period of observation. Control AO-labeled monolayers, examined 45 min after addition of the yeasts, showed numerous phagolysosomes at various stages of fusion (Fig. 2). In striking contrast (Fig. 3), as late as 2 hr after the ingestion of the yeasts by the SL-treated macrophages (240 μg/ml for 18 hr), the phagocytic vacuoles appeared as black nonfluorescing spaces amidst a brightly fluorescing background of lysosomal granules. In subsequent experiments, exposure of monolayers to smaller doses of SL (e.g., 100 μg/ml for 20 hr, or 40–100 μg/ml for 30 min followed at once by offer of the yeasts) resulted in a similar inhibitory effect on phagosome–lysosome fusion. It may be added that after 100 μg/ml for 30 min the uptake of SL, judged by radioassay, is of the order of 1 μg per monolayer; and none of the gross morphological changes mentioned earlier were apparent.

Interrupted serial thin sections of yeast-containing ferritin-prelabeled macrophages, with and without SL-I pretreatment (100 μg for 20 hr), were surveyed systematically by electron microscopy, and the evidence of lysosome–phagosome fusion was compared quantitatively (5). In the absence of SL (i.e., in control cultures) ferritin marker was found in abundance within 80% of the 175 yeast-containing phagosomes that were scored (see Fig. 4), and profiles of lysosomes in the process of fusion were common, as described elsewhere (5, 13). In cultures pretreated with SL, however, any evidence that lysosomal fusion had occurred was restricted to 20% of the yeast-containing phagosomes (260 scored). In the population of phagosomes that remained unfused following SL treatment (i.e., 80% of the total) the phagosomal membrane was in most cases applied closely to the contained yeasts (Fig. 5), as in the control macrophages where fusion was the rule; but about one in five exhibited a much wider, clear space around the contained yeasts, so resembling the "loose" phagosomes reported previously in the case of suramin-induced nonfusion (13). It was also evident (see Fig. 5) that after the prior exposure to moderate doses of SL the lysosomal accumulation of the droplets was markedly reduced in comparison with larger dose levels as illustrated in Fig. 1.

Ingestion of yeast cells coated with sulfatide

In further experiments normal macrophages, prelabeled with AO or ferritin, were permitted to ingest yeast cells to which sulfatide droplets had been attached through the intervention of small amounts of Al^{3+}. Introduced thus, directly into phagosomes with the target yeasts, sulfatide again induced al-

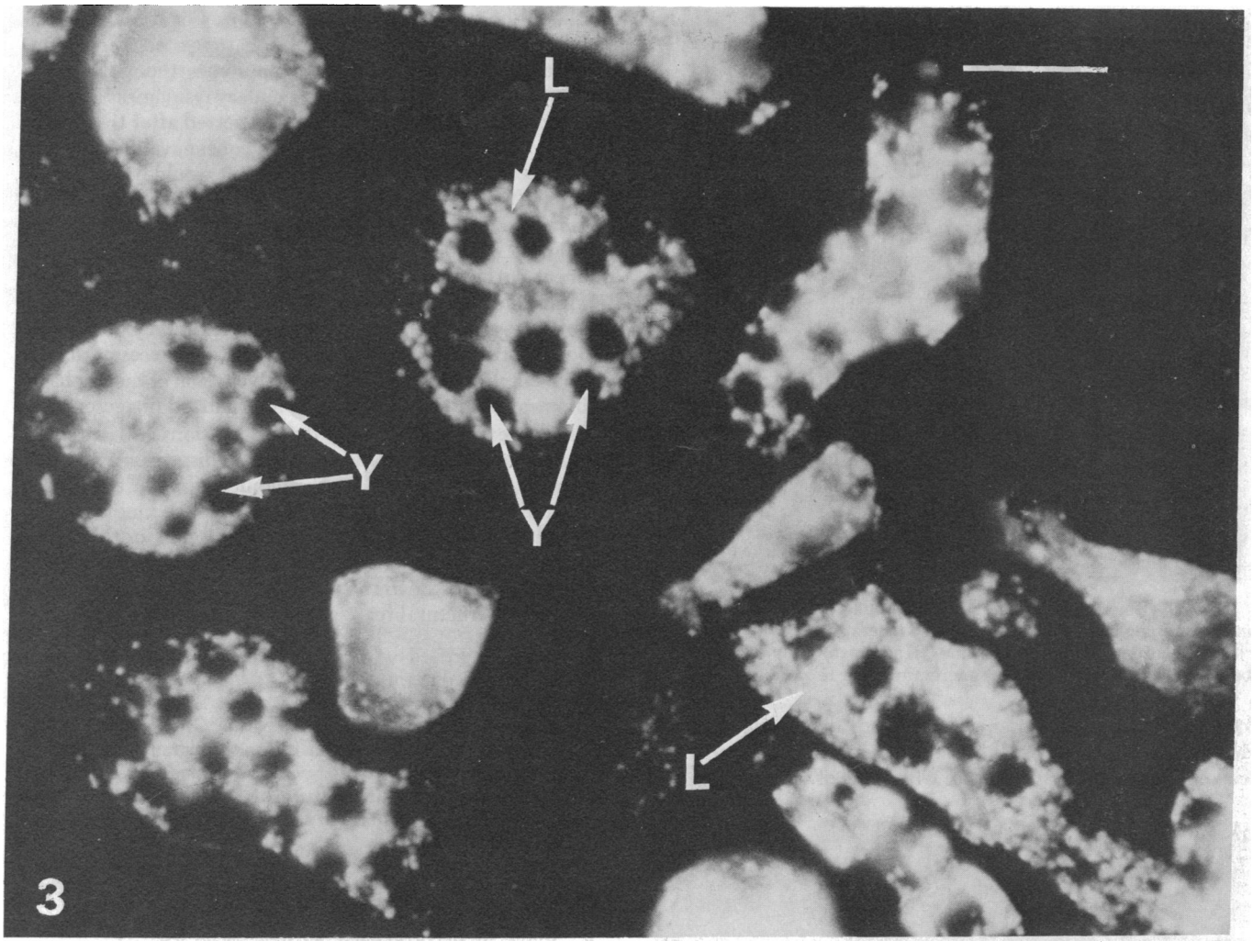

FIG. 3. Living macrophages pretreated with sulfatide [SL-I, 240 μg/ml (0.1 mM) for 18 hr at 37°] before the acridine staining, and then incubated with live yeasts for 2 hr. The macrophages appeared swollen, with densely packed lysosomes (L) surrounding dark spaces (Y) which were unstained yeasts in nonfused phagosomes. This is the nonfusion pattern of response. (From a color micrograph: bar represents 10 μm.)

most total suppression of phagolysosome formation as judged by fluorescence microscopy. Electron microscopy revealed that about one third of the yeast-containing phagosomes had failed altogether to fuse with the ferritin-labeled lysosomes, and that in most others the fusion was minimal. In contrast, abundant fusion was observed in almost 100% of yeast-containing phagosomes in control macrophages that had ingested yeasts pretreated with alum alone. Close inspection revealed, in addition, that in these experiments with "coated" yeasts a few lipid-like droplets (identical to those described above after SL-loading) were present inside some of the secondary lysosomes, more particularly those in close proximity to yeast-containing phagosomes. The source of such lysosomal droplets is uncertain; however, perhaps surprisingly, profiles strongly suggesting the occurrence of endocytic transportation of droplets from phagosome to lysosome were occasionally also identified.

DISCUSSION

Our findings indicate that the sulfated trehalose glycolipids from a typical virulent strain of *M. tuberculosis* are lysosomotropic; further, that after mouse peritoneal macrophages are exposed to even very small amounts of SL these products of the tubercle bacillus somehow prevent or delay phagosome–lysosome fusion, as shown towards ingested yeast cells which normally promote a massive fusion response. The evidence suggests the possibility that the fusion-inhibiting property of the sulfatide may contribute to the similar dysfunction observed (5, 6) during tuberculous infection of cultured macrophages, and so possibly assist the pathogen to survive and multiply within the host cell. This suggestion is strengthened by the observation that fusion inhibition occurs not only when the macrophages have sequestered SL in the secondary lysosomes before ingestion of the target yeasts, but also when the glycolipid is introduced via the phagosomes, attached to the yeasts. However, it is not yet clear how far the latter necessarily indicates a direct effect of SL on the phagosomal membrane, for, as we have noted, sulfatide-like droplets were sometimes to be seen simultaneously within the nearby secondary lysosomes. Hence caution is necessary in interpreting sulfatide activity even in the yeast-coating experiments in terms of the mycobacterial model, though the parallels seem impressive; also, use of ferritin as the indicator of lysosomal fusion, though precise and very reliable (5), necessarily confines observations at this time to the population of secondary lysosomes.

Since the inhibitory influence of SL on phagolysosome formation is regularly associated with lysosomal accumulation of droplets, it is reasonable at this stage to consider the lysosomes,

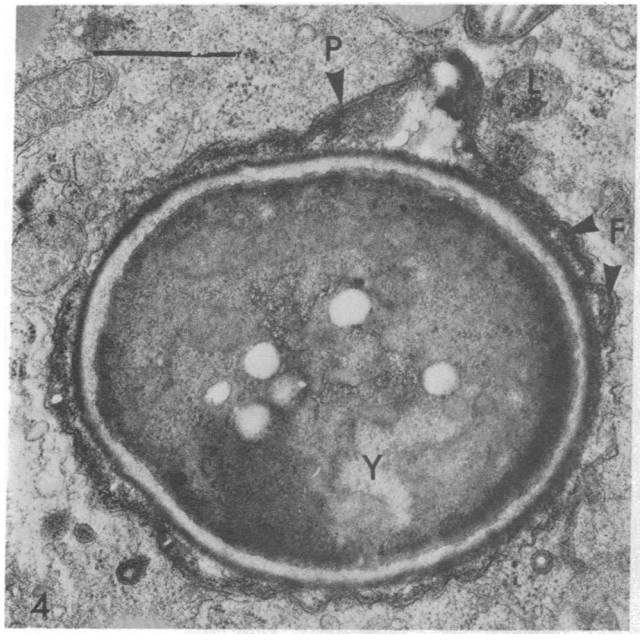

FIG. 4. Usual appearance of an intraphagosomal yeast (Y) 50 min after ingestion by a ferritin-labeled macrophage. Abundant ferritin (F) has entered the phagosome (P) as a result of fusion with labeled lysosomes (L). (Electron micrograph: bar represents 1 μm.)

or their limiting membranes, as the most likely sites for the sulfatide to act. However, if the effect were to block the general capability of membranes to fuse with those of other organelles we should be confronted with a paradox. For if SL droplets enter the cell by endocytosis, as strongly suggested by electron microscopy, why are successive pinosomes themselves not prevented from fusing with the secondary lysosomes? The situation is clearly different from that reported after treatment of macrophages with the plant lectin concanavalin A, which inhibits pinosome–lysosome fusion (17). It would be necessary to propose that the SL effect depends on some peculiarity of the lysosomal membrane, or perhaps on structural changes affecting the SL molecule that occur only in the lysosomal environment. An alternative hypothesis is that sulfatide, and perhaps suramin likewise (13), may exert its effects not by rendering membranes as such physically incapable of fusion, but through a more subtle disturbance of the recognition processes that in normal circumstances promote selective fusion of macrophage lysosomes with the membranes of internalized phagosomes.

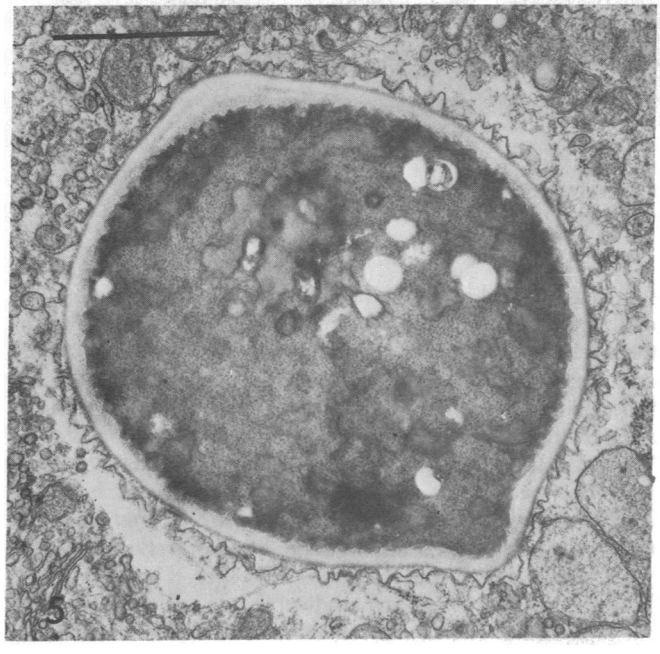

FIG. 5. Yeast cell 50 min after ingestion by a macrophage prelabeled with ferritin and treated 20 hr with SL-I at 100 μg/ml. Failure of lysosome–phagosome fusion is indicated by nonaccumulation of ferritin within the phagosome. (Bar represents 1 μm.)

Finally, extrapolation from the observations with suramin (a hexasulfonate) and sulfatide (in aqueous dispersion a micellar "polysulfate") suggests that polyanionic character could be a structural common denominator provoking the kind of membrane dysfunction we report—whether directly or through a mediator. Further, as yet unpublished experiments employing nontoxic levels of dextran sulfate and other substances with polyanionic structural features lend support to this idea.

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