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

(electron microscopy/endoctysis/membranes/polyanions)

MAYER B. GOREN‡‡, P. D'ARCY HART‡, M. R. YOUNG‡, AND J. A. ARMSTRONG‡

*Division of Molecular and Cellular Biology, National Jewish Hospital and Research Center, and Department of Microbiology, University of Colorado School of Medicine, Denver, Colo. 80206, and ‡National Institute for Medical Research, Mill Hill, London NW7 1AA, England

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ABSTRACT

Intracellular parasites (e.g., *Mycobacterium tuberculosis*, *Toxoplasma gondii*, and some *Chlamydiaceae*) 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 phagolysosome formation, 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 “infecitivity” (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 me-
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.
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, 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 Al3+. Introduced thus, directly into phagosomes with the target yeasts, sulfatide again induced al-
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,
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.

Finally, extrapolation from the observations with suramin (a hexasulfonyl) 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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