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Suramin Effects on Macrophage Phagolysosome Formation and Antimicrobial Activity

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The effects of suramin on phagolysosome formation and antimicrobial activity of mouse peritoneal macrophages cultivated in vitro have been studied. Prolonged in vitro pretreatment of macrophages with high concentrations of suramin caused macrophages to form large fragile phagolysosomes in which the concentrations of the various lysosomal enzymes were inferred to be diminished. In addition, suramin-treated macrophages demonstrated enhanced exocytosis of acid phosphatase during phagocytosis of polyvinyl toluene spherules. However, suramin was found not to inhibit formation of phagolysosomes in macrophages that had ingested *Listeria monocytogenes* when those cells were examined by the electron microscope. Suramin pretreatment did not alter the ingestion or intracellular killing of *Staphylococcus aureus* or of a strain of *L. monocytogenes* that was essentially avirulent for mice, but did protect macrophages from destruction by virulent *L. monocytogenes* ingested in vitro, an effect that appeared to have been mediated through enhancement of the bacteriostatic potential of the macrophages. However, at a single dosage level, the drug did not alter the mortality of mice challenged with virulent *L. monocytogenes*.

The mechanisms involved in the antimicrobial activity of macrophages have not been established (6). Lysosomal contents, for example myeloperoxidase, contribute to the antimicrobial activity of polymorphonuclear leukocytes (27), but the role of lysosomes in macrophage antimicrobial activity has not been defined. Although lack of fusion of lysosomes with phagosomes in macrophages correlates well with intracellular growth of ingested parasites in some circumstances (1, 15), it has been shown that enhancement of lysosomal fusion is not sufficient to promote enhanced macrophage antimycobacterial activity (2). If phagolysosome formation could be pharmacologically blocked, then the role of lysosomes in macrophage antimicrobial activity could be more accurately assessed. Numerous drugs such as colchicine (21, 34) and glucocorticoids (30) have been thought to stabilize lysosomes and/or prevent fusion of lysosomes with phagosomes, but only suramin (13), mycobacterial sulfatides (10), and concanavalin A (8) have been alleged to manifest that activity in intact phagocytes.

Suramin is a sulfonated derivative of urea, chemically related to the dye trypan blue, which has long been used in the therapy and prophylaxis of African trypanosomiasis (31). It is strongly protein bound and is concentrated within lysosomes, probably as a result of endocytosis of suramin-protein complexes (7). In prior studies of the effects of the drug on antibacterial immunity, suramin was shown to have a detrimental effect on host-cell antibacterial activity both in vivo (24, 33) and in vitro (11). However, a potentially beneficial effect on induction of cellular immunity by killed organisms has recently been described (29). Because suramin concentrates intralysosomally, and because other intralysosomally stored agents, e.g. trypan blue (17) and hydroxystilbamidine (9), may alter macrophage function, it seemed reasonable to assume that suramin's effects are indeed mediated by its effects on lysosomal function, primarily inhibition of phagolysosome formation (13). The experiments described here were undertaken to better define the effects of suramin on phagolysosome formation and antimicrobial activity of murine macrophages.

MATERIALS AND METHODS

Mice. Outbred male Swiss Webster mice weighing 25 to 30 g, obtained from Sutter Farms Springfield, Mo., were used in all experiments.

Macrophages. Unstimulated peritoneal macrophages were harvested by lavage with 0.01 M heparinized phosphate-buffered saline (PBS) (pH 7.4) using methods previously described (4). Macrophages were maintained as monolayers in Leighton tubes or tissue culture flasks in medium 199 supplemented with heat-inactivated newborn calf serum (20%) and penicillin G

(1,000 U/ml) in an atmosphere containing 95% air and 5% CO_2 at 37°C. The medium was changed at 1 and 24 h after harvest of cells and then every 2 to 3 days. Cell viability was determined by use of trypan blue (22) and acridine orange (25); live cells concentrate acridine orange and exclude trypan blue.

Suramin. Suramin (Mobay Chemical Corp.) was dissolved in PBS and sterilized by filtration through 0.22-μm membrane filters just before each day's use.

Microorganisms. A strain of *Listeria monocytogenes* (serotype I) (Lm), obtained from John Kasik (University of Iowa), was virulent for mice (50% lethal dose [LD_{50}], 10^4 colony-forming units) intravenously and caused in vitro destruction of macrophage monolayers obtained from nonimmune mice. Its virulence was maintained by continuous passage in mice. A second strain of *L. monocytogenes* (serotype IIb) (Lo), obtained from the Clinical Microbiology Laboratory of the University of Iowa Hospitals, was of low virulence for mice (LD_{50} >10^7 colony-forming units, intravenously) and did not replicate within normal mouse peritoneal macrophages in vitro. A strain of *Staphylococcus aureus* (coagulase positive and penicillin sensitive) was also obtained from the Clinical Microbiology Laboratory. *Saccharomyces* sp. were obtained as commercial desiccated bread yeast. Prior to use, bacteria were grown overnight in Trypticase soy broth at 37°C; the yeast was grown in Trypticase soy broth supplemented with 2% glucose.

For in vitro studies utilizing bacteria, macrophage monolayers were first rinsed with medium 199 and incubated for 1 h in medium containing 100 U of penicillinase (Difco Laboratories) per ml; the medium was then aspirated, and fresh medium containing bacteria was added. Phagocytosis was terminated by aspiration of medium, rinsing three times with medium 199, and addition of fresh medium containing antibiotics. Lysostaphin (2 U/ml) (Schwartz/Mann) was used in experiments with staphylococci, and penicillin (2 U/ml) plus gentamicin (1 μg/ml) was used in experiments with *Listeria*. Bacteria were quantitated by determining the number of colonies forming on brain heart infusion agar from suitably diluted samples of medium and distilled water lysates of three-times-rinsed monolayers. For experiments with yeast, yeast suspensions (10^7/ml) were added to monolayers; phagocytosis was terminated at 15 min by rinsing five times with medium 199. Uptake of yeast was determined by microscopic counts of yeast liberated from macrophages by distilled water lysis, and viability of cell-associated yeast was determined by ability to exclude methylene blue.

Macrophage endocytosis. Uptake of [^{3}H]sucrose (specific activity, 2 Ci/mM; Amersham/Searle) present in the medium at a concentration of 5 μCi/ml was determined by liquid scintillation counting (20), and uptake of polyvinyl toluene (PVT) spherules (mean diameter, 2 μm, Dow Diagnostics), present in the medium at a concentration of 1 mg/ml, was determined by measuring the absorbance at 267 nm of PVT solubilized in *p*-dioxane (21). For both assays, macrophage monolayers, rinsed five times with PBS, were digested in 0.1 M sodium hydroxide. Bacterial uptake was assessed by light microscopy, using monolayers stained with Giemsa stain, and by determining the accumulation of viable particles in 1 h of phagocytosis as assessed by plate counts of distilled water lysates of macrophages. All results were normalized for cell protein determined by the Lowry et al. method (18), using crystalline egg-white lysozyme as the standard.

Phagolysosome formation. The effects of suramin on macrophage phagolysosome formation were assessed by fluorescent and electron microscopy and by assay of transfer of acid phosphatase to PVT-containing phagolysosomes and exocytosis of acid phosphatase. Macrophages to be studied with fluorescent microscopy were incubated in medium containing acridine orange (10 μg of base per ml) for 15 min and rinsed with medium 199; this resulted in intense fluorescent staining of lysosomes (13). Following addition of particles in fresh medium, transfer of dye to vacuoles containing the particles was monitored over time with a Leitz transmitted-light fluorescent microscope. The percentage of ingested particles that became stained was quantitated only in experiments using yeast cells, because interiorized yeasts were visible whether or not lysosomal fusion had occurred, whereas bacteria not coated with acridine orange were poorly resolved.

For electron microscopy, macrophages were allowed to ingest cadmium-free ferritin (5 mg/ml) (Miles Laboratories) for 24 h. Subsequent to removal of ferritin, macrophages were cultivated for an additional 6 days in the presence or absence of suramin (200 μg/ml). *L. monocytogenes* (Lo or Lm) was added at a final concentration of 5×10^7/ml to rinsed monolayers. At varying times after addition of bacteria, the medium was removed and the macrophages were rinsed with PBS and fixed overnight with 2% glutaraldehyde in cacodylate buffer (0.1 M) at 4°C. The cells were then scraped from flasks, sedimented at $250 \times g$ at room temperature, rinsed in cacodylate buffer, and postfixed in osmium tetroxide. Following dehydration in ethanol, the cells were embedded in Epon. Because uranyl acetate staining made recognition of ferritin more difficult, most preparations were not stained.

Exocytosis of acid phosphatase was assayed in monolayers that had ingested PVT (5×10^7/ml) for 2 h in Earle balanced salt solution without neutral red plus 20% newborn calf serum and penicillin. At the end of phagocytosis, medium was decanted and filtered and cells were rinsed with PBS. Both portions were subjected to five freeze-thaw cycles and assayed for acid phosphatase activity using α-naphthol acid phosphate (Dajac Laboratories) as a substrate. Triton X-100 was incorporated in the reaction mixture for the cell samples but was omitted for the medium samples (34). Labile enzyme activity in medium is expressed as percentage of total enzyme activity (enzyme activity in monolayer plus labile enzyme in medium).

The transfer of acid phosphatase to phagolysosomes containing PVT was quantitated using previously described methods (21). Briefly, macrophages that had ingested PVT were washed with PBS, removed from culture flasks by scraping, and disrupted with a Dounce homogenizer. Following sedimentation of nuclei and undisrupted cells by centrifugation at 2,500 $\times g$ for 10 min, the supernatant was layered onto a

discontinuous sucrose gradient (5 to 10 to 27.5% sucrose, wt/vol) and centrifuged for 1 h at 25,000 × *g* at 4°C. Unfused lysosomes settled at the 27.5 to 50% interface, while PVT-containing phagosomes and phagolysosomes floated to the 5 to 10% sucrose interface. Lysosomal contents released during processing remained in the 27.5% layer. Acid phosphatase activity in each fraction was expressed as percentage of total activity recovered from the gradient and was normalized for uptake of PVT per microgram of cell protein.

In vivo studies. Mice received suramin in 0.2 ml of PBS intravenously at 3-day intervals; two doses were given before infection and two after. *L. monocytogenes* (Lm) was also administered by tail vein injection. The LD_{50} of *Listeria* and of suramin was determined by the method of Reed and Muench (23), and then the interaction of the two agents was studied.

Data analysis. Microscopic counts were performed on coded slides or tubes. Unless otherwise specified, results represent means and standard errors of duplicate or triplicate values of three or more experiments. Straight lines were generated by the method of least-mean-squares linear-regression analysis. Differences among means of proportions were calculated by Student's *t* test for unpaired data, while differences among counts were determined by the χ^2 test with continuity correction. A two-tailed $P \leq 0.05$ was considered statistically significant.

RESULTS

Suramin was well tolerated by macrophages in culture as long as the concentration of the drug did not exceed 200 μg/ml and fresh drug was added no more frequently than every other day. However, extensive cell loss followed exposure to 400 μg/ml for 18 h or to 200 μg/ml within 3 days if fresh drug was added on a daily basis. Macrophages treated with suramin (100 to 200 μg/ml) developed numerous large phase-lucent vacuoles reminiscent of those seen after ingestion of undigestible carbohydrates by macrophages (5). At the electron microscopic level, the vacuoles contained membranous whorls and, in ferritin-loaded preparations, contained ferritin (Fig. 1).

Preliminary experiments indicated that prolonged in vitro pretreatment was necessary for suramin to alter transfer of acridine orange to phagolysosomes. The effect was best seen after 3 days of exposure and was not further enhanced by longer preincubation; there was no apparent difference among exposure times between 3 and 6 days, and data from these periods were pooled. As determined by fluorescent microscopy, pre-

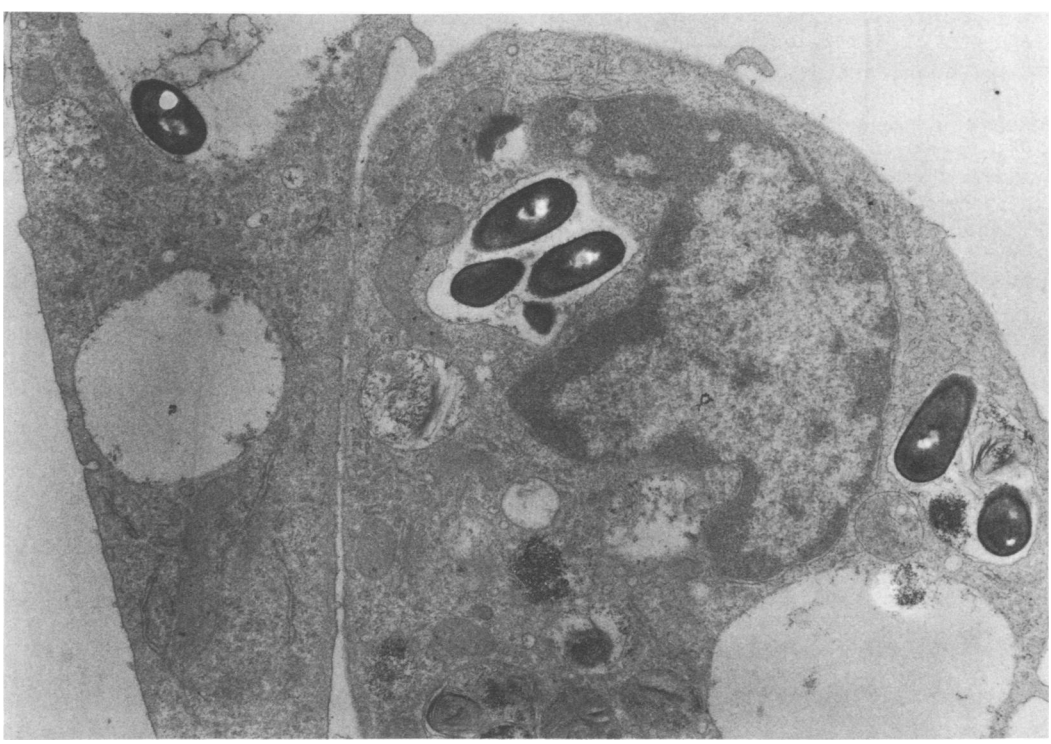

FIG. 1. *Electron micrograph of ferritin-labeled, suramin (200 μg/ml)-treated macrophages which had ingested the virulent strain of L. monocytogenes in vitro. Ferritin granules are visible in apparently normal secondary lysosomes, in those containing membranous whorls, in widely dilated lysosomes containing no recognizable structures, and in phagolysosomes containing the bacteria.*

treatment of macrophages with suramin (100 to 200 μg/ml) inhibited transfer of acridine orange to phagosomes in macrophages that had interiorized either *Saccharomyces* (Fig. 2A) (13) or either strain of *L. monocytogenes*. It was evident that extensive fusion had occurred in control macrophages ingesting *Listeria*. Suramin-treated macrophages ingesting bacteria normally but only rarely (less than 1/500 macrophages, under conditions in which every cell contained bacteria) contained a stained bacterium. There was no apparent difference between Lo and Lm in these observations.

The drug also altered transfer of acid phosphatase to PVT-containing phagolysosomes (Fig. 3), causing a marked difference in transfer of enzyme to phagolysosomes. The inhibition of transfer was not evident in cells that had been pretreated for less than 48 h, but was seen in cells that had been pretreated for at least 3 days. However, concomitant with inhibition of transfer of acid phosphatase to phagocytic vacuoles, an increase in the proportion of the enzyme that remained in the 27.5% sucrose layer was noted. In cells pretreated with suramin for 3 or more days, 18.5 ± 2.1% of total enzyme activity did not migrate, compared to 4.9 ± 0.9% in control preparations ($P < 0.01$). This fraction represents enzyme activity labilized during preparation and presumably indicates that the morphological changes in lysosomes evident microscopically (Fig. 1) were reflected in this assay in increased fragility of lysosomes and phagolysosomes. Whether or not there was true inhibition of phagolysosome formation could not be definitely determined.

In an attempt to gain additional biochemical

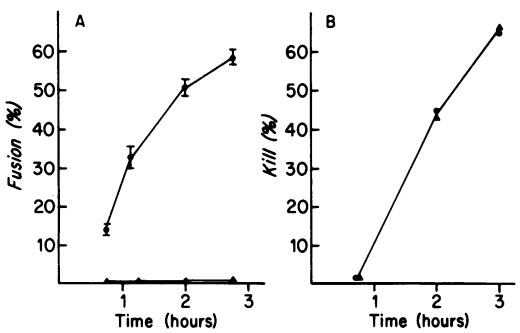

FIG. 2. *(A) Lysosomal fusion as evaluated by fluorescent microscope examination of transfer of acridine orange from lysosomes to phagolysosomes and (B) kill of interiorized Saccharomyces sp. as determined by ability of the yeast cells to exclude methylene blue in untreated macrophages* (●) *and in macrophages pretreated in vitro with 200 μg of suramin per ml* (▲).

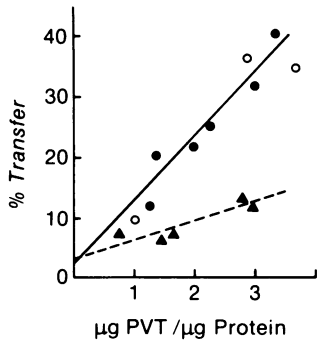

FIG. 3. *Transfer of acid phosphatase activity to containing phagolysosomes as percentage of total acid phosphatase activity recovered from the gradient and normalized for PVT uptake in normal macrophages* (●) *and macrophages pretreated in vitro with suramin (200 μg/ml) for 3 or more days* (▲) *or for 1 to 2 days* (○).

evidence of altered lysosomal fusion, the effect of the drug on exocytosis of lysosomal enzymes by macrophages ingesting PVT (5×10^7/ml) was studied. The drug did not affect the spontaneous loss of labile enzyme by cells that were not exposed to particles (≤5% in all groups), but at all concentrations tested (25, 50, 100, and 200 μg/ml) suramin caused significantly increased loss of enzyme by cells ingesting particles (49 ± 5% versus 29 ± 2%, $P < 0.02$). Over 98% of the cells in all groups were viable as determined by trypan blue exclusion and phase-contrast microscopy, and treated and control cells remained viable for 48 h after ingestion of particles.

The results of the experiments reported thus far were compatible with the hypothesis that suramin inhibited intracellular phagolysosome formation in normal peritoneal macrophages. However, electron micrographs of ferritin-loaded macrophages indicated that, although lysosomal morphology was altered by treatment with the drug and the ferritin was much less densely aggregated within dilated lysosomes, there was fusion of lysosomes with phagosomes following ingestion of living *Listeria* of either strain (Fig. 1). For these studies, macrophages treated with suramin (200 μg/ml for 6 days) were allowed to ingest *Listeria* for periods of time varying from 15 min to 2 h. Specimens were scanned electron microscopically, and 30 to 50 interiorized bacteria were identified and assessed for presence or absence of surrounding ferritin in each of six experiments. Photomicrographs were made of bacteria around which the presence of ferritin was questionable. All interiorized organisms in both control and suramin-treated macrophages were contained within vacuoles in

which ferritin was present, suggesting that phagolysosome formation had occurred virtually simultaneously with phagocytosis. There were no obvious differences in the behavior of macrophages toward either strain of *Listeria*; the virulent strain, which kills normal macrophages, was found to reside within phagolysosomes within 15 min (the earliest time studied) of initiation of phagocytosis both in control and suramin-treated preparations. In addition to these effects on lysosomal morphology, the drug apparently altered the ingestive process such that multiple bacteria were often noted to reside within single large vacuoles, a phenomenon seldom observed in untreated macrophages.

While these studies were in progress, the effects of suramin pretreatment on macrophage endocytosis and antimicrobial activity were evaluated. [^{3}H]sucrose uptake, a measure of pinocytosis (20), was unimpaired, as was ingestion of PVT (Fig. 4). In addition, treated macrophages accumulated as many bacteria (Fig. 5) and yeasts as did controls. There was no effect on macrophage fungicidal activity as determined by methylene blue dye exclusion (Fig. 2B), nor was the ability of the macrophages to inactivate *Listeria* (Lo) or *S. aureus* impaired (Fig. 5).

However, when macrophages were challenged in vitro with a dose of *Listeria* (Lm) sufficient to infect at least 95% of the cells within 1 h, macrophages pretreated with suramin were protected from death due to *Listeria* infection. As illustrated in Fig. 6, 90% of control macrophages were dead within 24 h after infection, but there was significantly decreased cell death if the cells had been pretreated with 100 to 200 μg of suramin per ml for 3 to 6 days. Examination of Giemsa-stained slides made at the end of 1 h of phagocytosis indicated that suramin had not inhibited uptake of Lm or altered the percentage of infected cells. To further evaluate an effect of

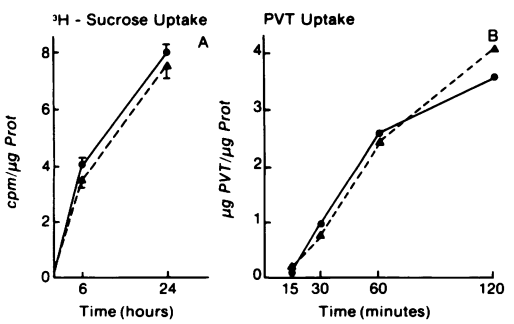

FIG. 4. *Pinocytosis of [^{3}H]sucrose (A) and phagocytosis of PVT (B) by control macrophages (●) and by macrophages pretreated in vitro with suramin (200 μg/ml) (▲) for 3 days.*

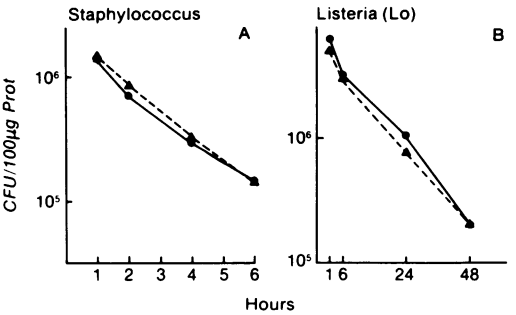

FIG. 5. *Kill of S. aureus 502A (A) and of L. monocytogenes (Lo) (B) by control (●) and suramin-treated (▲) macrophages as determined by plate counts of appropriate dilutions of distilled-water-lysed macrophage monolayers. Lysostaphin (2 U/ml) or penicillin (2 U/ml) plus gentamicin (1 μg/ml) were added at 1 h to monolayers challenged with Staphylococcus and Listeria, respectively.*

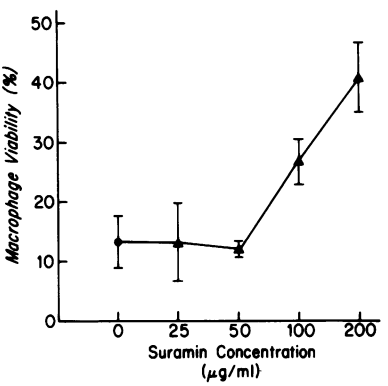

FIG. 6. *Survival of macrophages at 24 h after challenge with L. monocytogenes (Lm). Control (●) and suramin-treated (▲) macrophages were exposed to L. monocytogenes (Lm) at a concentration of 10^7 organisms per ml for 1 h. The medium was aspirated, the tubes were rinsed, and fresh medium containing penicillin (2 U/ml) and gentamicin (1 μg/ml) was added. The number of cells in each tube was determined by phase-contrast microscopy using an eyepiece grid at 1 h and 24 h, and the viability of cells at 24 h was determined by vital stains. Suramin at 100 and 200 μg/ml significantly ($P < 0.05$) reduced destruction of macrophages.*

the drug on fraction of cells infected (since a decrease in that parameter could falsely suggest protection), macrophages were exposed to differing inocula of *Listeria* (Lm), and the percentage of cells infected and subsequent formation of intracellular microcolonies (19) 7 h after termination of phagocytosis were assessed. Suramin pretreatment did not affect the percentage of cells infected (Fig. 7) or multiplicity of infection at either dose, but at 200 μg/ml effectively reduced the fraction of infected cells in which

microcolonies had formed by 7 h (Table 1).

Suramin at concentrations of 1,000 μg/ml did not inhibit growth of *Listeria* (Lo or Lm) either in Mueller-Hinton broth or tissue culture medium containing 20% newborn calf serum.

Although the concentrations at which in vitro protection was evident were much higher than those reported in the serum of patients or animals treated with suramin (28), some data suggest that suramin alters host responses to bacterial infections (24, 29, 33). In preliminary experiments, the LD_{50} of the drug when administered intravenously four times at 3-day intervals was found to be 8 mg per mouse (~300 mg/kg per dose). In an attempt to minimize nonspecific interactions between a toxic drug and infection (26), the dose was limited to 2 mg per injection (1/4 LD_{50}), which is similar to the doses used by others (24, 33). Suramin did not affect mortality or time to death of mice infected intravenously with *L. monocytogenes* (Lm) at a dose calculated to be 1 LD_{50} (Fig. 8).

Moreover, peritoneal macrophages lavaged from suramin-treated mice did not manifest the morphological changes observed in cells treated in vitro, transferred acridine orange to phagolysosomes normally, and were indistinguishable from normal macrophages in in vitro infection with *Listeria* (Lm).

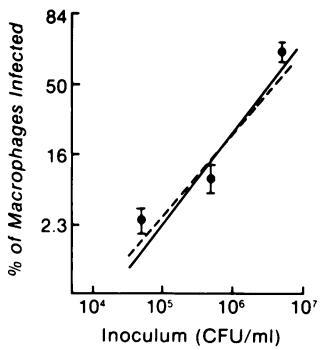

FIG. 7. *Fraction of macrophages ingesting L. monocytogenes (Lm) after a 30-min phagocytic pulse at three different inocula. The data are plotted as percentage of macrophages containing organisms (probit scale) versus inoculum size. Linear-regression lines are shown for macrophages treated with suramin (200 μg/ml) (---) and control macrophages (——). Means and standard errors are shown for the combined data.*

TABLE 1. *Formation of microcolonies of L. monocytogenes in macrophages in vitro*[a]

Listeria inoculum size (colony-forming units per ml)	% of infected macrophages containing microcolonies		
	Control	S50	S200
5×10^4	9.1	5.9	0[b]
5×10^5	23.7	14.9	0[c]
5×10^6	18.3	16.1	0.7[c]

[a] Macrophages were allowed to ingest *Listeria* at the stated inoculum in antibiotic-free medium for 1 h. Phagocytosis was terminated by aspiration of the medium, repeated rinsing, and addition of medium containing penicillin (2 U/ml) and gentamicin (1 μg/ml). After incubation for an additional 6 h, monolayers on coverslips were extracted, rinsed, air dried, fixed in methanol, and stained with Giemsa stain. At least 200 macrophages containing 1 or more *Listeria* were counted, and macrophages containing discrete clumps of organisms or over 10 organisms dispersed throughout the cytoplasm were considered to contain microcolonies. Macrophages pretreated with 200 μg of suramin per ml (S200) allowed significantly less replication than either control macrophages or those that had been pretreated with 50 μg of the drug per ml (S50).

[b] $P < 0.05$.

[c] $P < 0.01$.

DISCUSSION

These studies were conducted in an attempt to confirm and further define the inhibition of phagolysosome formation first observed in suramin-treated macrophages by Hart and Young (13). The mechanisms involved in macrophage antimicrobial activity have not been delineated (6), and satisfactory hypotheses to explain the increase in activity that accompanies macrophage activation do not exist. Because polymorphonuclear leukocytes kill bacteria and yeasts by mechanisms which require participation of lysosomal contents (27), attention has been focused on lysosomes in macrophages. Fusion of

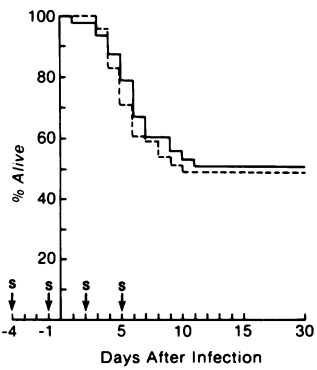

FIG. 8. *Survival of mice infected intravenously with 10^4 L. monocytogenes (Lm) on day 0. Twenty-four mice treated before and after infection with 2 mg of suramin (----), administered intravenously on days indicated by "S," responded indistinguishably from 23 control animals (——) not receiving suramin in two separate experiments.*

lysosomes with phagosomes is closely associated with intracellular killing of *Toxoplasma* (15), but for other intracellular parasites, the association of phagolysosome formation with intracellular inactivation of pathogens is much less clear. Although virulent *Mycobacterium tuberculosis* usually grows within phagosomes that have not fused with lysosomes, mycobacteria coated with specific antibody no longer inhibited phagolysosome formation but remained viable and grew intracellularly (2). In addition, there are examples of intracellular parasites that grow within phagolysosomes (3) and of those that are able to escape from the phagolysosomes to grow within the cytoplasm (16).

If lysosomal fusion could be blocked, the role of lysosomal contents in macrophage antimicrobial activity could be better defined. Many of the drugs that had been thought to inhibit phagolysosome formation, for example colchicine and glucocorticoids, because of their effects on stability of isolated lysosomes or on exocytosis of lysosomal enzymes, have been shown not to inhibit phagolysosome formation in intact macrophages (13, 21, 30). Suramin, however, was shown to inhibit phagolysosome formation by macrophages that had ingested live *Saccharomyces* (13), but the effects of the drug on macrophage phagolysosome formation and bactericidal activity following ingestion of an intracellular parasite have not been previously described.

Previous in vivo and in vitro studies with suramin and its analogs have suggested that at least part of its many pharmacological properties may result from effects on lysosomes. It has been shown to accumulate within lysosomes, for example, in renal tubular epithelial cells in animals given suramin (14), and it is capable of inhibiting the action of a wide variety of enzyme systems, lysosomal and other (32). In prior studies of the effects of suramin on host defenses, macrophages treated in vitro with suramin (dose and timing not specified) allowed more rapid replication of interiorized *M. tuberculosis* (11), and the drug enhanced mortality in murine tuberculosis (24). The recent demonstration (13) of inhibition of phagolysosome formation following ingestion of live yeast by suramin-treated macrophages further strengthened the hypothesis that effects on host defenses were secondary to effects of the drug on phagolysosome formation.

However, the studies of ferritin-loaded macrophages reported here clearly demonstrate that phagolysosome formation was not blocked in suramin-treated macrophages that had ingested the intracellular pathogen *L. monocytogenes*. The drug did cause formation of "loose" phagolysosomes, as had been previously described (6), resulted in formation of autophagic vacuoles, and seemed to increase the number of phagolysosomes containing multiple bacteria. The electron microscope observations thus did not confirm those made with the fluorescent light microscope concerning the behavior of lysosomes in suramin-treated cells ingesting *Listeria*. The most likely explanation for this difference seems to be that the concentration of transferred dye was insufficient to cause visible fluorescence in phagolysosomes in treated cells as well as in a significant percentage of phagolysosomes in control cells. Two observations support this concept. First, when the dye was dissolved in saline and placed on a microscope slide, the characteristic fluorescence was not microscopically visible until the concentration of the dye exceeded 500 μg/ml; thus, the dye had to have been concentrated in phagolysosomes at least 50-fold over its initial concentration in the medium for fluorescence to be microscopically detectable. In addition, in control macrophages, it was noted that even after in vitro incubation with yeast cells for up to 6 h, approximately one-third of the interiorized yeast had not acquired the fluorescent label. It is difficult to imagine that yeast, over 90% of which were dead by that time, could long remain within normal macrophages without lysosomal fusion having occurred, either prior or subsequent to death of the yeast. Thus, the acridine orange technique is probably useful only as a screening tool for phagolysosome formation, and is valid only if it indicates that fusion occurs in treated cells to the same extent as that observed in control cells.

Similarly, the studies of transfer of acid phosphatase to PVT-containing phagolysosomes were difficult to interpret in suramin-treated macrophages. One of the assumptions of that technique is that the procedures used to disrupt the cells cause similar damage to treated and to control macrophages. This assumption was not valid in these experiments. The widely dilated vacuoles in treated macrophages, loosely applied to ingested particles, were excessively fragile. This resulted in a high fraction of the total enzyme activity remaining in the 27.5% sucrose layer. Even accounting for that loss of potentially transferable activity, the amount of enzyme activity transferred to the phagolysosome layer at high particle loads seemed diminished, although the data do not allow firm conclusions to be drawn.

The other biochemical technique used to evaluate the effect of suramin on lysosomal fusion was the study of exocytosis of lysosomal enzymes accompanying ingestion of latex beads. Since both phagolysosome formation and exocytosis involve fusion of lysosomes with portions of the

cell membrane, it has been assumed that drugs that inhibit exocytosis also inhibit phagolysosome formation (34). At all concentrations tested, including those which had no effect on the behavior of virulent *Listeria* infections in vitro or on acridine orange transfer, suramin pretreatment enhanced exocytosis. Although these results may seem disparate with some of the former observations, they can be rendered compatible if it is assumed that, instead of inhibiting phagolysosome formation, suramin actually accelerated the process. Thus, during phagocytosis, lysosomes may have fused with plasma membranes prior to complete encirclement of the particles, with resultant leakage of the enzyme into the medium. This would result in the relatively sparse concentrations to ferritin seen in electron micrographs, and would also result in diminished intralysosomal concentrations of acridine orange and loss of fluorescence. Thus, it remains probable that phagolysosome formation is altered in suramin-treated macrophages such that ingested particles were exposed to lesser concentrations of active lysosomal contents; inhibition, in the sense that particles do not contact lysosomal contents at all, was not observed.

Since the drug did not completely block lysosomal fusion with phagocytic vacuoles, the lack of effect of suramin treatment on the behavior of bacteria of low virulence for murine macrophages is not unexpected, nor does the observation aid in discerning mechanisms of macrophage antimicrobial activity. However, the protection of macrophages against destruction by virulent *Listeria,* which resulted from prolonged in vitro pretreatment of macrophages with high concentrations of suramin, was unexpected both from prior published data on the drug's effects and from the other results reported here. There were certain analogies between the protective effect and the effects of acridine orange transfer, suggesting that the drug had to have been interiorized by macrophages to be effective. For the protection to be evident, high concentrations present in medium for at least 3 days were required; drug added to untreated macrophages after the cells were infected was without effect, and continued presence of suramin in the medium was not required for the effects to be manifest. These observations suggest that the protective effect was mediated through effects of the drug on lysosomes, but do not rule out other possibilities, for example alterations in other enzyme systems or in the cell membrane.

Because suramin pretreatment protected macrophage monolayers in vitro, in vivo trials were attempted. Although the concentrations of drug used in vitro would have been rapidly fatal in vivo, it was hoped that perhaps at least some of the effect would manifest itself in vivo. Because it is well known that suramin's toxic-therapeutic ratio is relatively low (31), and because it has been shown that even definitely beneficial drugs, such as antibiotics, can be harmful to mice with bacterial infections if used at doses too near their LD_{50} (26), dosage of suramin was limited to one-fourth the LD_{50}. At those doses, the drug was neither helpful nor detrimental. It remains possible that the in vitro effects could be produced in vivo if the drug is administered chronically to allow greater intralysosomal accumulation in macrophages, since continued pinocytosis of low levels could conceivably, over much longer periods of time, result in accumulation of effective intracellular concentrations of the drug.

In conclusion, the effects of suramin on murine macrophage function were found to be complex and not mediated by drug-induced blockade of phagolysosome formation. Although the transfer of lysosomal contents to phagosomes in suramin-treated macrophages may be somewhat diminished compared with that in untreated cells, the drug did not block phagolysosome formation such that the intracellular fate of *Listeria* not exposed to lysosomal enzymes could be determined. Inhibition of phagolysosome formation in intact cells remains an elusive goal. At doses of suramin that seemed to diminish the concentration of lysosomal contents in phagolysosomes (as inferred from acridine orange transfer and ferritin studies), intracellular growth of a virulent strain of *L. monocytogenes* was suppressed. Whether this reflects activation of macrophages, an alteration in lysosomal contents that *Listeria* somehow utilizes in its intracellular growth, alterations in macrophage membrane permeability to antibiotics, or other mechanisms is at this time unknown.

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