

DESTRUCTION OF *LEISHMANIA MEXICANA AMAZONENSIS*
AMASTIGOTES WITHIN MACROPHAGES IN CULTURE
BY PHENAZINE METHOSULFATE
AND OTHER ELECTRON CARRIERS*

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The life cycle of *Leishmania* involves two distinct stages of the parasite: the flagellated promastigotes live extracellularly in the gut of insect Phlebotomes, and upon transmission to the vertebrate host they are taken up by macrophages and transform into nonflagellated amastigotes. The amastigotes are obligatory parasites of macrophages and lodge within modified phagolysosomes termed parasitophorous vacuoles (1, 2). Amastigotes obtained from tissues of infected animals readily infect primary macrophage cultures, macrophage lines, or Sticker sarcoma cells (3–6).

In a variety of in vitro models oxygen metabolites produced by macrophages stimulated by immune complexes, particles, or membrane-active drugs have been related to the killing of intracellular microorganisms by the phagocytes (7–9). The key metabolite is the superoxide anion (O_2^-), derived from the univalent reduction of molecular oxygen. O_2^- can undergo dismutation to hydrogen peroxide (H_2O_2) and can lead to the generation of other toxic oxygen metabolites such as the hydroxyl radical ($OH\cdot$) (10, 11). It has also been shown that a series of electron carriers, including phenazines, thiazines, and quinones, when in contact with living cells such as *Escherichia coli*, can be reduced to auto-oxidizable intermediates. Upon reoxydation, these intermediates can generate O_2^- and H_2O_2 (12). For this reason we chose to investigate the effect of 5-methylphenazinium methyl sulfate (phenazine methosulfate, PMS)¹ and other potential redox cycling agents on *Leishmania m. amazonensis*. We report here that intracellular amastigotes are killed by drug concentrations that do not appear to be toxic to the host phagocytes and that low concentrations of the drugs also inhibit the growth or kill *Leishmania* promastigotes in culture.

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¹ Abbreviations used in this paper: BCG, Bacillus Calmette-Guerin; DMEM, Dulbecco's modified Eagle's minimum essential medium; FCS, fetal calf serum; HS, horse serum; LCM, L cell conditioned medium; MM, Mitsuhashi and Maramorosch's medium; NBCS, newborn calf serum; PBS, phosphate-buffered saline; PMS, phenazine methosulfate; PPD, purified protein derivative.

Materials and Methods

Animals. Adult golden hamsters of both sexes were purchased from the Centre d'Élevage d'animaux de laboratoire (Ardenay, France). Outbred adult female OF₁ mice were obtained from Iffa Credo (L'Arbresle, France).

Media. Ca⁺⁺, Mg⁺⁺-free phosphate-buffered saline (PBS) contained 6 mM PO₄⁻⁻ buffer and 138 mM NaCl and was adjusted to pH 7.2. Modified Locke's solution contained 154 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 2 mM NaHCO₃, and 1.1 mM glucose. Earle's balanced salt solution was prepared as described previously (13). Dulbecco's modified Eagle's minimal essential medium (DMEM) was obtained from Laboratoires Eurobio (Paris, France). Horse serum (HS) and newborn calf serum (NBCS) were purchased from Gibco Laboratories (Grand Island Biological Co., Grand Island, N. Y.) and from Flow Laboratories (Asnieres, France). Conditioned medium (LCM) was collected from confluent L cell cultures after post-incubation for 7 d in serum-free DMEM and stored at -20°C. Thioglycollate medium was obtained from Institut Pasteur Production, Paris, France.

Chemicals and Dyes. The following were purchased from Sigma Chemical Co., St. Louis, Mo. (Stated dye contents are those given by the supplier). Basic blue 24 (80%); brilliant green (90%); cresyl violet acetate (75%); brilliant cresyl blue (81%); Thionine (90%); neutral red (92%); safranin 0 (90%); auramine 0 (70%); pyronin B (95%); acid fuchsin (75%); fast green FCF (90%); phenazine methosulfate; phenosafranin; light green SF; menadione bisulfite; methyl viologen; alizarine sodium sulfonate (90%); pararosaniline hydrochloride. Toluidine blue 0 was obtained from Serva (Accurate Chemical & Scientific Corp., Westbury, N. Y.), methylene blue from Fisher Scientific Co., Pittsburgh, Pa. and crystal violet from Allied Chemical Corp., Morristown, N. J. 1-methoxy-5-methylphenazinium methylsulfate was kindly provided by Dr. Tashuhiko Yagi, Shizuoka University, Shizuoka, Japan.

Leishmania Strain and Preparation of the Amastigote Inoculum. *Leishmania mexicana amazonensis* LV 79 was obtained from the Dept. of Parasitology, Liverpool School of Tropical Medicine, Eng., and serially passaged in the hamster. Granulomas were induced by subcutaneous injection of amastigote suspensions in the dorsal aspect of the paw. After 4-6 wk the granulomas were excised, minced, and disrupted with the help of a glass homogenizer fitted with a Teflon pestle (Bellco Glass Inc., Vineland, N. J.). Tissue debris was removed by slow centrifugation (40 g for 10 min) and the amastigotes recovered by spinning the supernatant at 1,000 g for 10 min. The amastigotes were washed three times in Locke's medium by centrifugation at 1,000 g for 10 min and resuspended in the appropriate tissue culture medium. One paw granuloma provided from 1×10^8 to 3×10^8 amastigotes.

Promastigote Cultures. These were established from hamster granulomas and grown in nutrient agar (Oxoid Ltd., London, Eng.) containing 15% whole rabbit's blood with Locke's overlay (14). For drug assays, culture tubes with 1 ml of Mitshuhashi and Maramorosch's (MM) medium (15) supplemented with 15% fetal calf serum (FCS) were seeded with 1×10^6 promastigotes and incubated at 25°C. Logarithmic growth was obtained between the 2nd and 4th d of culture. Drugs were added on the 2nd d and the numbers of promastigotes were determined twice a day by counting in a hemocytometer.

Cell Cultures. L cells were grown in DMEM with 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 100 µg/ml kanamycin. Resident peritoneal macrophages were collected in PBS and allowed to attach onto 12-mm diameter coverglasses. The coverglasses were rinsed in PBS and distributed in the 16-mm diameter wells of Cosatar plates (Nunc, Roskilde, Denmark) in 0.5 ml DMEM with 10% NBCS. Approximately 10^6 macrophages were plated per well and used 1 or 2 d thereafter. Elicited macrophages were collected 5 d after the intraperitoneal administration of 2 ml thioglycollate medium. Bacillus Calmette-Guerin (BCG)-activated macrophages were harvested 3-4 wk after intraperitoneal inoculation with 4×10^6 viable organisms. The BCG was kindly provided by Dr. Philippe Lagrange, Institut Pasteur, Paris. Some normal or BCG-infected mice were injected intraperitoneally with 50 µg purified protein derivative (PPD) 4 d before killing. The PPD was a gift from Dr. J. Augier, Institut Pasteur, Paris. Elicited and activated macrophages (1×10^5 to 1.5×10^5 /well) were maintained in DMEM with 10% FCS and infected with *Leishmania* 1 or 2 d later. Marrow macrophages were obtained by seeding 10^6 bone marrow cells per well of Costar plates. The medium consisted of DMEM enriched with 10% HS and 10% LCM. After 5 or 6 d the macrophages matured and

were rinsed and used in the experiments. For electron microscopy, cells were grown directly on 35-mm tissue culture dishes (Corning Glass Works, Science Products Div., Corning, N. Y.).

Leishmania Infection of Macrophages. Macrophages were infected at an estimated multiplicity of 5 amastigotes/cell in the corresponding tissue culture medium. Under these conditions, in most experiments, there was little or no replication of the *Leishmania* at 37°C.

Drug Treatment of Infected Macrophages. Unless otherwise noted, cultures were exposed to the drugs 24 h after infection. In most experiments with PMS the drug was added in Earle's salts medium containing 13 mM bicarbonate, 1 mg/ml glucose, 10 mM Hepes buffer pH 7.6, and 2.5% HS. After the PMS pulse the medium was replaced by complete tissue culture medium appropriate for peritoneal or marrow macrophages (see above) except that in the case of marrow cultures, in order to limit macrophage multiplication, the LCM concentration was reduced to 2.5%. The other drugs were added in complete medium for 18–24 h. Since PMS and other compounds can be photoreduced, all compounds were added to the cultures under subdued light and the plates immediately wrapped in aluminum foil.

Light microscopic observations. Drug-treated and control cultures were fixed in Zenker's fluid with 5% acetic acid and stained with Giemsa for counts with the light microscope. Alternatively, macrophages were fixed in 2% glutaraldehyde in PBS and mounted in glycerol-gelatin for counts with the phase contrast microscope. Percent infection was determined by scoring 100 or 200 macrophages in each of two to four replicate coverslips. Macrophages were scored as infected when they contained at least one recognizable *Leishmania* amastigote. In studies with marrow derived macrophage cultures only well-spread cells situated in the periphery of the colonies were scored for infection.

Time-lapse Cinemicrography. Marrow-derived macrophages were grown on 25-mm round coverslips, infected, and mounted in Sykes-Moore chambers (Bellco Glass Inc.). The medium contained 10% HS, 2.5% LCM, 10 mM Hepes pH 7.3, and 1.4 mM sodium bicarbonate in DMEM. PMS (10 or 15 μ M) was added to the medium and filming started immediately or after 40–60 min, during which time the cultures were shielded from light. Control cultures were filmed for periods of 2–4 h. Recordings were obtained at six exposures/min with a Paillard camera and a Wild microscope (Wild Heerbrugg Instruments Inc., Farmingdale, N. Y.) which was equipped with a long distance condenser and a \times 50 oil phase contrast objective. The microscope was enclosed in a thermostatically controlled chamber adjusted to 37°C. The film used was 16-mm negative Agfa Gevaert Copex (Agfa-Gevaert Inc., Teterboro, N. J.).

Electron Microscopy. Cultures were fixed for 1 h at room temperature in 2.5% glutaraldehyde in 5 mM cacodylate buffer pH 7.2 containing 0.1 M sucrose. After washing in buffer the cultures were post-fixed in osmium tetroxide for 1 h at 4°C and treated at room temperature with 1% uranyl acetate in distilled water. The monolayers were dehydrated in ethanol, embedded in Epon, and sectioned (16). To follow the kinetics of *Leishmania* destruction by PMS, the numbers of morphologically intact or damaged parasites were counted in 80–150 thin sections of different macrophages.

Results

In most of the experiments, resident mouse peritoneal macrophages kept in culture for 1–3 d or in vitro-derived marrow macrophages grown for 5 d were infected with a 5:1 multiplicity of *L. m. amazonensis* amastigotes. The following day, 70–95% of the macrophages displayed large parasitophorous vacuoles containing one or more amastigotes attached to the inner surface of the vacuolar membrane. In a typical experiment, infected macrophages were given a 2-h pulse with 10 μ M PMS in Hepes-buffered Earle's salts medium enriched with glucose and 2% serum. At the end of the pulse, the medium was replaced by drug-free complete medium for an additional 18 h, at which time the cells were fixed and stained for microscopic determination of the percent of infected macrophages. Under these conditions, 0–5% of the PMS-treated macrophages contained recognizable *Leishmania*, the majority of the parasitophorous vacuoles had disappeared, and most macrophages returned to their usually elongated

configuration. In contrast, parallel cultures treated similarly but without PMS displayed 70% parasitized macrophages or more. The following experiments examined several features of the PMS-induced destruction of the intracellular amastigotes.

Effect of PMS Concentration and Duration of the PMS Pulse on the Leishmania Infection. Fig. 1 shows that the effect of PMS, as determined 18 h after a pulse, was related to both drug concentration and duration of the pulse. A relatively narrow range separated concentrations of PMS that were inactive from those that markedly reduced the infection, i.e., the dose-response curves were rather steep. Concentrations $>15 \mu\text{M}$ were toxic, as shown by reduction in the numbers of macrophages, by morphological changes in the cells, and by inhibition of ingestion of sheep erythrocytes coated with anti-sheep red cell IgG (not shown). Most of the succeeding experiments used 2-h pulses with $10 \mu\text{M}$ PMS. In additional experiments, infected macrophages were treated continuously for 24 h with different concentrations of PMS. Nearly complete cures were obtained with $5 \mu\text{M}$ of the drug (Table I).

Infection in Macrophages Fixed at Different Times after a Pulse with PMS. Representative microphotographs (Fig. 2) show that 2 h after a 2-h pulse with $10 \mu\text{M}$ PMS most macrophages displayed "empty" vacuoles, i.e., *Leishmania* could no longer be identified with the light microscope (Fig. 2B). After 6 h (Fig. 2C) fewer and smaller parasitophorus vacuoles were seen, whereas at 24 h most macrophages were free of vacuoles (Fig. 2D). In contrast, control macrophages, pulsed with drug-free medium and fixed at 24 h, contained numerous *Leishmania* (Fig. 2A).

Time-lapse Cinemicrographic Observations. The preceding experiments indicated that when infected macrophages were exposed to PMS, parasites identifiable in fixed preparations rapidly disappeared. Because it was difficult to exclude changes in the composition of cell populations over time, the effects of PMS on single infected

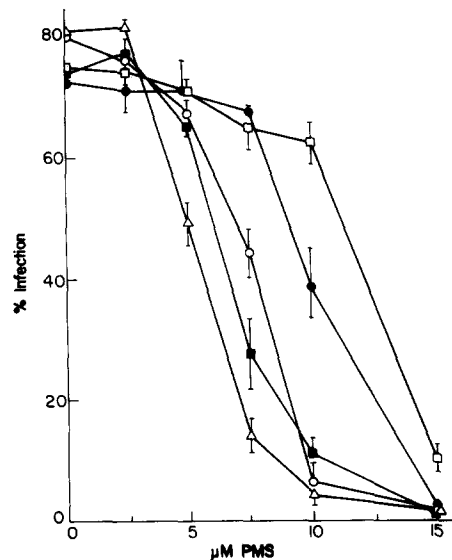


FIG. 1. Percent infection of marrow-derived macrophages as a function of PMS concentration and of the duration of the PMS pulse. After exposure to PMS-containing or control medium, cultures were placed in drug-free complete medium and fixed 18 h later. □, 15 min; ●, 30 min; ■, 1 h; ○, 2 h; △, 4 h.

TABLE I
Destruction of Intracellular Amastigotes and Growth Inhibition of Promastigotes by Electron Carriers

Drug	Chemical group	Concentration for 95% reduction in percent infection*	Concentration that reduced growth of promastigotes‡
		μM	μM
Crystal violet	Triphenylmethane	0.07 ± 0.1 (7)§	0.01
Brilliant green	Triphenylmethane	0.5 (2)	0.1
Brilliant cresyl blue	Phenoxazine	1.5 ± 0.2 (6)	0.5
Cresyl violet acetate	Phenoxazine	2.0	ND
Pyronin B	Xanthene	2.3 (3)	ND
Auramine O	Diphenylmethane	2.4 ± 0.1 (4)	ND
Basic blue 24	Phenothiazine	2.5	2.5
Safranin O	Phenazine	3.7 ± 1.0 (4)	1.0
Toluidine blue O	Phenothiazine	4.8 ± 0.4 (5)	1.0
Phenazine methosulfate	Phenazine	5.4 ± 0.2 (5)	2.5
Methoxy PMS	Phenazine	8.0 (2)	5.0
Methylene blue	Phenothiazine	20.0 ± 3.5 (4)	10-15
Menadione bisulfite	Quinone	24.0 ± 1.8 (5)	5
Neutral red	Phenazine	>30 (2)	20
Phenosafranin	Phenazine	>40 (3)	10

* Infected macrophages incubated with the drugs for 18-24 h.

‡ Promastigote cultures treated with the drugs for 24-48 h (average of one to three experiments).

§ Mean \pm SE (number of experiments); four coverslips counted per experiment.

|| Not determined.

macrophages were also examined by time-lapse cinemicrography. Fig. 3 shows frames from a continuous recording (at 6 exposures/min) of a macrophage treated with 10 μM PMS from zero time. No major changes were visible for ~40 min (Fig. 3A-C). Thereafter the *Leishmania* became morphologically altered and fragmented into granules or vesicles that could be seen in the lumen of the parasitophorous vacuoles (Fig. 3D-F). At 61 min most identifiable parasites had disappeared from the vacuoles. The cell partially rounded during the observation period. Upon projection of the films at 24 frames/sec (an acceleration of 240 times), an apparent rotation of the *Leishmania* was observed in most sequences; often vicinal parasites appeared to rotate in opposite directions. It is not clear whether the rotation is related to movement of the small vacuoles seen within the parasites or is due to the rotation of the *Leishmania* bodies. In cells exposed to PMS, the rotation often came to a stop just before the fragmentation of the amastigotes. This fragmentation conveyed the impression of an explosion or burst. Vacuoles similar in size to the vacuoles seen in intact *Leishmania* as well as smaller granules were seen to scatter and to freely move within the parasitophorous vacuoles. Different *Leishmania* within the same vacuole were destroyed at different times, and some parasites were still intact after several hours of exposure to PMS. Different cells also lost *Leishmania* at different rates. In control cultures, only occasional parasites were seen to "explode" after 2 h of observation. The effect of PMS was similar whether the macrophages were kept in the dark for 40 or 60 min, or filmed immediately after exposure to the drug.

Electron Microscopic Observations. Study of thin sections confirmed the damage and

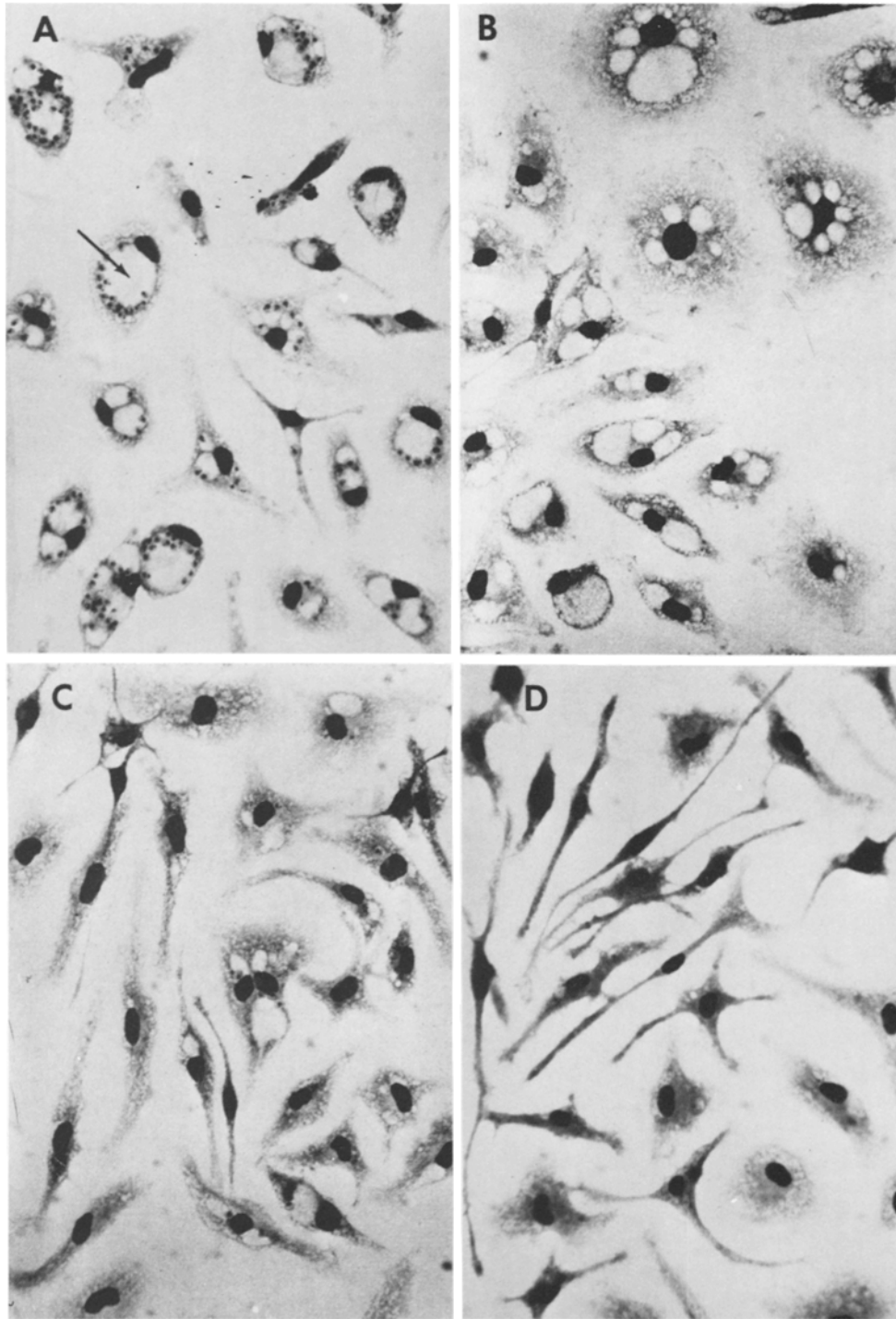


FIG. 2. Effect of PMS on marrow-derived *Leishmania*-infected macrophages. Giemsa stain. Macrophages were incubated for 2 h in either control medium (A) or in medium containing 10 μ M PMS (B, C, D). After the pulse cultures were incubated in complete medium and fixed 24 h (A), 2 h (B), 6 h (C), or 24 h (D) later. Arrow in A indicates parasitophorous vacuole. \times 500.

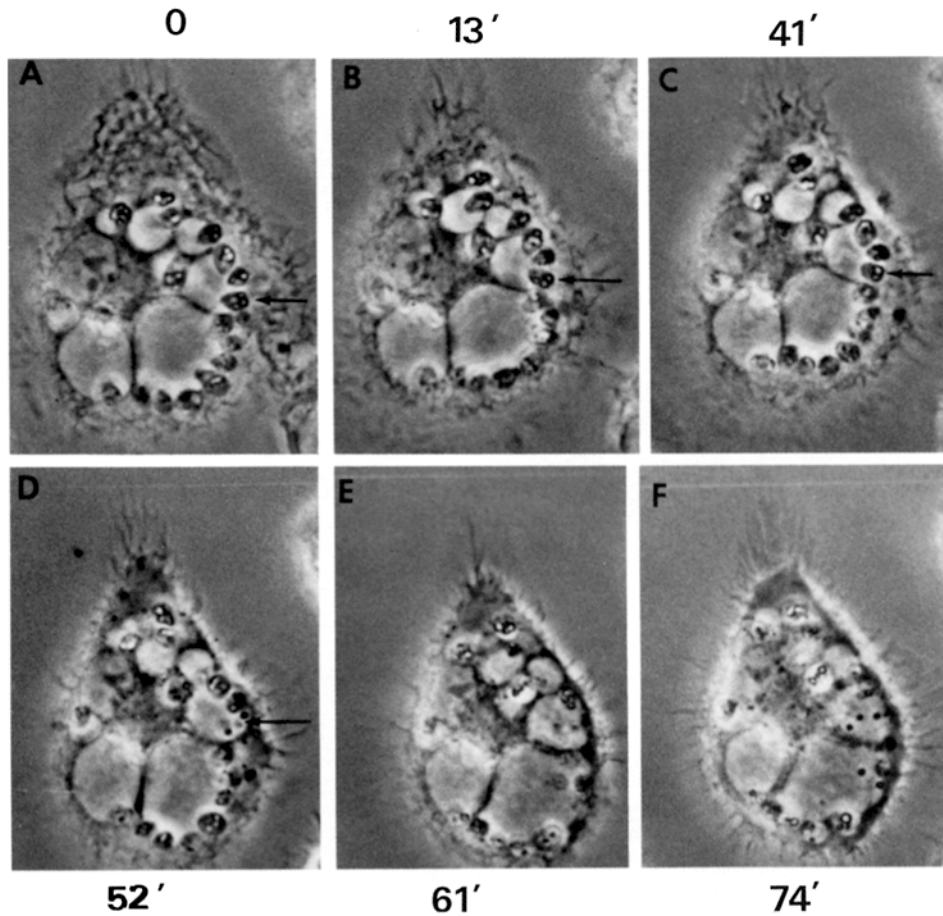


FIG. 3. Frames from cinemicrographic recording of marrow-derived macrophages treated with PMS at zero time. Time indicated in min. Arrows point to single *Leishmania*, which underwent fragmentation at 52 min. $\times 900$.

disappearance of *Leishmania* in macrophages treated with PMS and provided additional morphological information. The electron microscopic appearance of *Leishmania* within untreated macrophages is shown in Fig. 4 A. Changes were not detected after 1 h incubation in medium containing 10 μM PMS (Fig. 4 B). In contrast, after 2 h exposure to the drug, the parasite's envelope was fragmented and amastigote's contents were scattered within the parasitophorous vacuoles (Fig. 4 C). Often only a few vacuoles and the flagellar apparatus remained visible (Figs. 4 C and 5 A). Similar pictures were seen after exposure to 15 μM PMS for 1 h. As shown in Fig. 4 C, the alteration of the parasites did not involve all of the *Leishmania* at the same time. Intact and damaged *Leishmania* could often be seen within the same or in separate vacuoles belonging to the same macrophages. 6 h after a 2-h pulse with 10 μM PMS macrophages presented large vacuoles that were sometimes collapsed and were often filled with a dense or spongy material in which parasite debris was rare and difficult to identify (Fig. 5 B).

Macrophages were free of recognizable parasites 20 h after treatment with PMS.

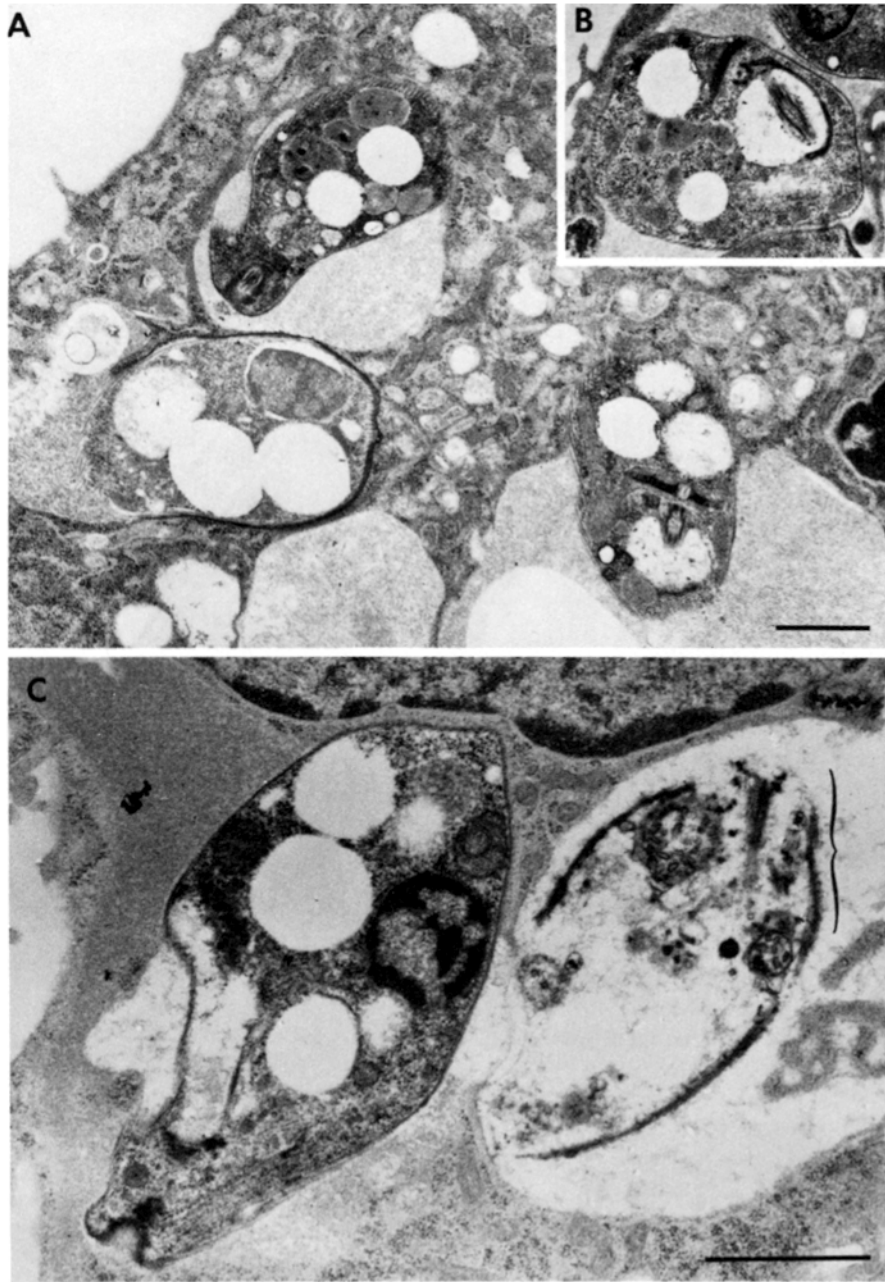


FIG. 4. Electron micrographs of thin sections of marrow-derived infected macrophages. A, control macrophages; B and C, macrophages treated for 1 or 2 h, respectively, with $10 \mu\text{M}$ PMS. Bracket indicates damaged amastigote. Bar equals $1 \mu\text{m}$.

Some macrophages contained one or two large secondary lysosomes filled with dense material. These lysosomes may have been derived from parasitophorous vacuoles. In infected, untreated macrophages, the numbers of intact *Leishmania* did not change

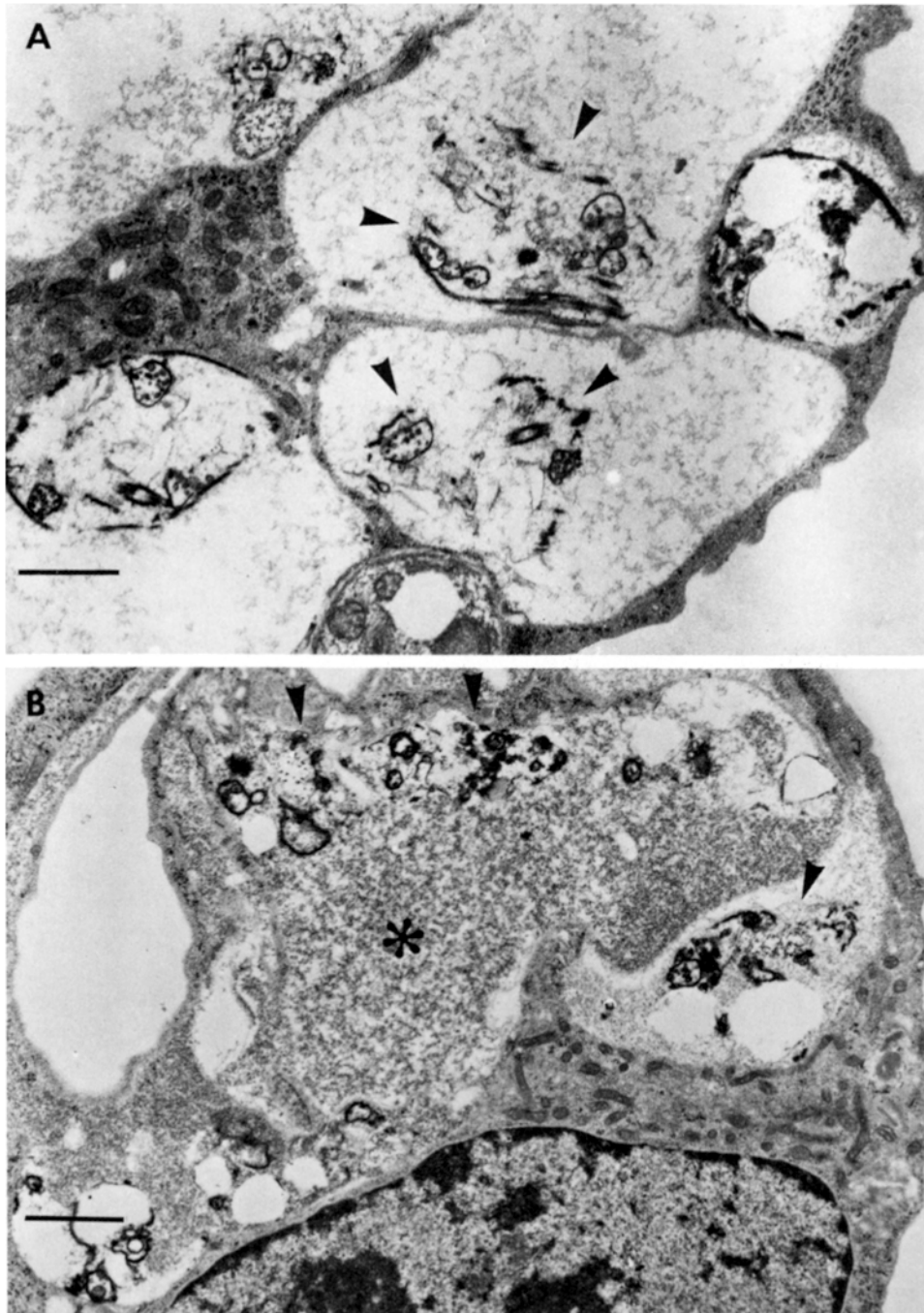


FIG. 5. Thin sections of infected macrophages fixed 2 h (A) or 6 h (B) after a 2-h pulse with 10 μ M PMS. Arrowheads point to amastigote debris. Asterisk indicates spongy material in parasitophorous vacuoles. Bar equals 1 μ m.

significantly during 20 h of incubation. The general ultrastructure of infected or uninfected macrophages treated with PMS did not show changes, although rarely lysed cells were present in some of the samples.

Treatment with PMS before Infection. We next asked whether exposure to PMS activated the macrophages to kill the intracellular parasites. Indeed, electron carriers such as methylene blue have been shown to increase the secretion of plasminogen activator by explanted macrophages (17). As an approach to the question, macrophages were pretreated with PMS and the drug was removed before infection with *Leishmania*. In a typical experiment, macrophages were incubated for 4 h with 10 μM PMS, maintained for an additional step-down period of 60 min in drug-free complete medium, washed, and infected with *Leishmania*. 24 h after infection, the percent infection was determined microscopically in comparison with untreated controls. The percent infection in the PMS-treated macrophages was 88% at 24 h, similar to that of infected controls not exposed to the drug. Analogous results were obtained when the 1-h step-down was omitted.

Treatment with PMS at Different Times after Infection. The parasitophorous vacuoles enlarge markedly during the first 24 h after infection. Because the microbicidal effect of PMS could be related to the size of the vacuoles, we examined the effect of PMS at different times after colonization of the macrophages with the parasites. In a typical experiment, PMS pulses (2 h, 10 μM) were given to macrophages infected 3, 6, 20, or 48 h previously. The percent infection was evaluated 24 h after the end of the pulse. In these four groups of treated cultures the percent infection varied between 3.3 and 5 (average of triplicates) compared with 77.8% in untreated controls (average of five samples). Thus PMS was also effective when given shortly after the uptake of *Leishmania* by macrophages. In other experiments macrophage cultures were treated with PMS on different days up to 7 d after infection. The percent infection was similarly reduced to low levels at all times of treatment in comparison with untreated controls (not shown).

Entry of PMS or of a Metabolite of PMS into the Parasitophorous Vacuoles. Because another phenazine dye, neutral red, is efficiently concentrated in the parasitophorous vacuoles (18), we investigated by fluorescence microscopy whether PMS (or a metabolite of PMS) could be likewise detected in the vacuoles. Infected macrophages were treated with 10 μM PMS in complete Earle's salts medium for different time periods at 37°C. The coverslips were rinsed in cold PBS and examined without fixation under a fluorescence microscope. Fluorescence in the vacuoles was detected as early as 20 min after incubation with PMS. In step-down experiments, infected macrophages were loaded with PMS for 60 min, rinsed, and incubated in drug-free complete medium at 37°C. Vacuolar fluorescence was undetectable at the end of 1 h.

Effect of pH of the PMS Medium on the Destruction of Leishmania. PMS is a quaternary aminium salt that is ionized but in equilibrium with its pseudobase. Formation of the latter should be pH dependent (19, 20). It can be assumed that the uncharged pseudobase easily crosses the plasma and vacuolar membranes. The following experiment was designed to determine the effect of medium pH on the destruction of *Leishmania* by PMS.

Infected macrophages were treated with PMS in two kinds of media: (a) PBS with Ca^{++} and Mg^{++} , 5 mM glucose, and 2% HS, adjusted to a range of pH; (b) Earle's salts with glucose and serum containing a range of concentrations of sodium bicar-

bonate between 0 and 13 mM. In the first instance, cultures were incubated in room air; in the second, in a 10% CO₂-room air atmosphere. Samples of the media were rapidly monitored with a Radiometer (Copenhagen) pH meter.

Fig. 6 shows that in both phosphate- and bicarbonate-buffered media the effect of PMS was markedly dependent on the medium pH. There was a negligible effect of PMS at pH 6.3 while maximal reduction of infection occurred between pH 7.5 and 8.0. The pH at which control infection was reduced by 50% was ~7.0. The pK_{R+} for pseudobase formation by the PMS cation has not been reported, but can be expected to be >6.8 (Dr. J. W. Bunting, Dept. of Chemistry, University of Toronto, Canada, personal communication).

Studies with Elicited and Immunologically Activated Macrophages. Activated, and in certain instances, inflammatory macrophages have been shown to have enhanced microbicidal abilities in several systems (e.g., 7-9). Activated macrophages, when adequately stimulated, release higher concentrations of oxygen reduction products. It was of interest to compare the effect of PMS on infected inflammatory or activated macrophages. Cultures were established with resident cells, cells from mice injected intraperitoneally with thiglycollate medium, from mice injected intraperitoneally with living BCG 3-4 wk before killing, or from similarly BCG-inoculated mice challenged intraperitoneally with PPD before killing (elicited and activated macrophages). Macrophages were infected with *Leishmania* and on the next day exposed to a range of PMS concentrations. Cultures were fixed 18 h after treatment and scored for percent infection. Fig. 7 shows that the dose-response curves obtained with these macrophage populations were quite similar. Thus, the effect of PMS on the *Leishmania* infection was unrelated to the functional state of the macrophages.

Destruction of Intracellular Leishmania by Other Electron Carriers. The following experiments examined the effect of a series of electron carriers on intracellular *Leishmania* in comparison with the effect of PMS. Infected macrophages were treated with a range of concentrations of the drugs in complete tissue culture medium. After 18-24 h of

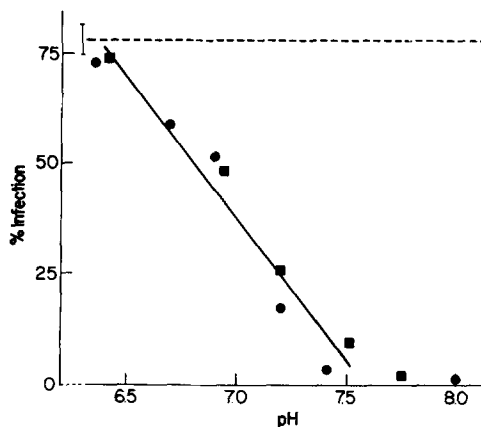


FIG. 6. Effect of medium pH during exposure to PMS on percent infection. Macrophages were treated for 2 h in phosphate- or bicarbonate-buffered media adjusted to a range of pH and containing 10 μ M PMS. At the end of the pulse media were replaced by complete DMEM, pH 7.3, and the cells fixed 18 h later. Controls were given a mock pulse without PMS. Control infection is indicated by the dotted horizontal line (bar represents standard error of the mean). Three replicates per point. ■, PBS-glucose 2% HS + PMS; ●, Earle's-glucose 2% HS + PMS.

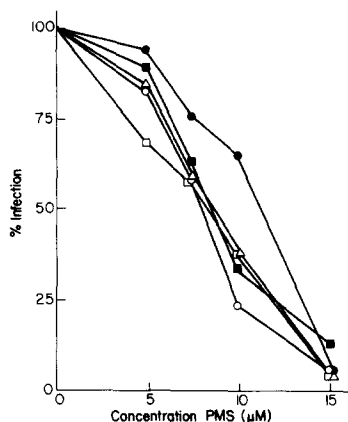


FIG. 7. Percent infection in resident, elicited, or activated macrophages given a 2-h pulse with a series of PMS concentrations and fixed 18 h later. □, resident; ○, thio; △, BCG-PPD; ■, BCG; ●, PPD.

continuous treatment the macrophages were fixed and stained for the determination of the percent infection. Untreated infected macrophages served as controls. Table I summarizes the results obtained. *Leishmania* were killed by compounds belonging to different structure groups. Because PMS is easily photoreduced, it is of interest that the more stable 1-methoxy derivative (21) was also active. The most effective of the drugs examined was crystal violet, an hexamethylated pararosanilin. Crystal violet reproducibly cured infected macrophages at 50–100 nM concentrations and exerted little toxicity on the phagocytes at concentrations 10–20-fold higher. The unsubstituted parent compound pararosanilin was inactive (not shown). The requirement for lipophilic moieties (methyl or ethyl substituents) is also illustrated by comparison of unmethylated phenosafranin with tetra- or pentamethylated safranin 0 (22). The former dye was incompletely active at 40 µM, whereas safranin 0 reduced infection by >95% at 2.5 µM. Sulfonated triphenylmethanes such as fast green FCF or light green SF were also inactive (not shown). Other compounds inactive at nearly toxic concentrations were alizarine sodium sulfonate, acid fuchsin, thionin, and methyl viologen.

Effect of Electron Carriers on Leishmania Promastigote Cultures. The curative effect of PMS and other agents on intracellular *Leishmania* amastigotes may result from a direct action on the parasites, from an effect on the macrophages, or from a combination of the two kinds of mechanisms. Because the amastigotes do not survive well extracellularly, we chose to evaluate the effect of the electron carriers on promastigote cultures. Promastigotes (4×10^5) were seeded in 1 ml of medium, and the drugs added 24 h later. The organisms were enumerated 24 and 48 h after addition of the drugs. Fig. 8 shows that graded concentrations of crystal violet, methylene blue, or PMS reduced the growth or killed the promastigotes in an approximately dose-dependent fashion. Table I lists the growth inhibitory concentrations for other compounds. It can be seen that there is a correlation between the concentrations that killed amastigotes and those that inhibited the growth of promastigotes ($r = 0.96$; 8 d.f., $P < 0.001$).

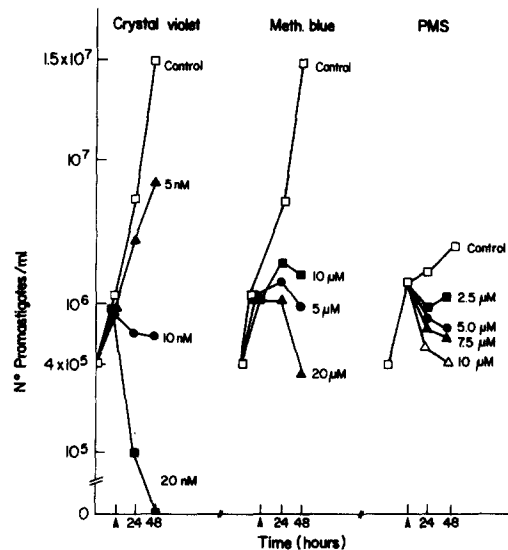


FIG. 8. Effect of crystal violet, methylene blue, or PMS on the numbers of promastigotes in culture. Promastigotes allowed to multiply for 24 h before the addition of the dyes (arrows on the abscissae). Drug concentrations are given in the figure.

Discussion

Exposure of *L. mexicana amazonensis*-infected macrophages to PMS or other electron carriers resulted in rapid destruction and disappearance of the parasites without obvious damage to the host cells. The drugs also inhibited the growth or killed *Leishmania* promastigotes in culture.

The first question to be considered is whether the destruction of the amastigotes was macrophage dependent or resulted from a direct effect of the drug on the parasites. Several findings favor the latter possibility. Thus, (a) promastigotes in culture were either killed or their growth reduced by drug concentrations often lower than those required to destroy the intracellular organisms; for instance, PMS was active on the flagellated forms at 2.5 μM and crystal violet at 10 nM (Fig. 8), whereas destruction of the amastigotes required 5.0 μM and 50 nM, respectively (Table I). (b) PMS or a metabolite of PMS was detected by fluorescence microscopy in the lumen of the parasitophorous vacuoles, and was therefore available for interaction with the parasites; in addition, the vacuolar fluorescence was not detected in macrophages incubated with PMS at pH 6.0, a pH at which little or no destruction of *Leishmania* was observed (Fig. 6 and data not shown). (c) The percent infection was not reduced in experiments in which noninfected macrophages were exposed to PMS for several hours followed by drug removal before infection. (d) Dose-response curves were similar for resident as well as for elicited or BCG-activated macrophages exposed to PMS (Fig. 7); this stands in contrast with the ability of activated macrophages to kill microorganisms after triggering with phorbol esters or phagocytic stimuli (7-11).

The mechanism of destruction of *Leishmania* is not yet clear. Table I shows that the active drugs belong to several structural classes. The most potent was the triphenyl-methane crystal violet, an hexamethylated pararosanilin, active on the amastigotes at 50 nM. Less active, i.e., requiring from 1 to 40 μM concentrations, were a series of

heterocycles, as well as the naphthoquinone menadione (structures of these compounds are given in refs. 22 and 23). In addition, whereas only a small number of congeners were examined, the results show that apolar substituents increased or were required for leishmanicidal activity, presumably because they enhanced the permeation of the compounds. In contrast, sulfonated derivatives, which are not expected to easily cross the plasma membrane, were inactive.

Several of the active drugs, including PMS, contain a quaternary ring nitrogen and possibly penetrate the macrophages or parasites in the form of the more permeant, uncharged pseudobases (19). This may account for the marked pH dependence of the destruction of amastigotes by PMS, although we were unable to find information on the pK_{R+} for PMS (pK_{R+} denotes the pH at which the heterocycle cation and pseudobase are present at equal concentrations, c.f. ref. 20). Pseudobase formation does also occur in triphenylmethane dyes (19).

A property common to most if not all of the active compounds is their readiness to reversibly accept and donate electrons (and protons). However, we found no apparent correlation between the leishmanicidal activity of the drugs and their redox potentials (24). Such correlation may be obscured by the additional structural requirements for drug permeation. Work with phenazines, oxazines, thiazines, and quinones has emphasized that under appropriate conditions these compounds can be reduced by cellular flavin- or pyridine nucleotide-dependent enzymes such as reductases or dehydrogenases. The reduction products can be reoxidized by oxygen, and in the process O_2^- or H_2O_2 may be generated (10, 12, 25). Indeed, the fact that H_2O_2 can be formed upon reoxydation of reduced methylene blue or PMS has been known for many years (26, 27). Thus the electron carriers, as also recently shown for the anthracycline drugs, may undergo redox cycling and generate toxic oxygen metabolites (12, 28-30). Furthermore, univalent reduction of PMS and of some of the other agents can generate toxic-free radical species of the drugs (29). Thus, damage to the *Leishmania* could result from free radical intermediates of drug reduction as well as from active oxygen metabolites produced in the course of reoxydation of the reduced species. PMS and other electron carriers also oxidize NADH and NADPH, and accumulation of $NADP^+$ leads to stimulation of the HMP (hexose monophosphate) shunt by the drugs (31). Finally, diversion by PMS of electron transport chains, inhibition of glycolysis, or of other intermediary metabolism pathways (32), could also lead to damage of the parasites.

We have so far been unable to exclude or support any of these possible mechanisms. In results not included in this paper we have found that the effect of PMS was unaffected by glucose starvation before and during the PMS pulses. Furthermore, destruction of the amastigotes by PMS was not modified by maximally tolerated doses of mannitol, histidine, benzoate, ethanol, or by catalase or superoxide dismutase. These negative results do not exclude the participation of oxygen metabolites, because the extent to which the scavengers have access to the parasitophorous vacuoles remains unknown.

Triphenylmethanes such as crystal violet or the phenothiazine methylene blue have long been known to inhibit the growth of certain bacteria, fungi, or parasites (33-35). We have shown that these and other molecules can kill intracellular *Leishmania* amastigotes as well as culture promastigotes. Besides the implication of the results for

the development of chemotherapeutic agents, the rapid leishmanicidal activity of the electron carriers provides a tool for the analysis of the parasitophorous vacuole's functions, which are dependent on the viability of the parasites.

Summary

Exposure of macrophages infected with *Leishmania mexicana amazonensis* to phenazine methosulfate (PMS) resulted in rapid damage and disappearance of the intracellular amastigotes without obvious ill effects to the host cells. The reduction of the percent infection was related to the concentration of PMS and to the duration of the pulse. Most *Leishmania* disappeared within 2 h of a 2-h pulse with 10 μM of the drug. In contrast, pretreatment of the macrophages with PMS followed by removal of the drug before infection did not result in disappearance of the parasites. The pH of the PMS medium markedly influenced the disappearance of *Leishmania*: maximum effect was observed at pH 8.0, while the effect was negligible at pH 6.3. The pH effect may be related to pseudobase formation by the PMS cation. Dose-response curves for PMS were similar for resident, elicited, or activated macrophages. Observations by time-lapse cinemicrography documented the explosion-like fragmentation of the amastigotes within 1–2 h of exposure of infected macrophages to the drug. Parasite-derived granules and vacuoles were seen to scatter within the parasitophorous vacuoles. This early damage to the parasites was confirmed by transmission electron microscopic observations. Infected macrophages incubated with PMS displayed detectable vacuolar fluorescence, indicating that PMS or a metabolite of PMS had access to the vacuoles.

A series of other electron carriers, including phenyl methanes, phenazines, oxazines, a xanthene, and a naphthoquinone, given continuously for 18 h, also induced the disappearance of the *Leishmania*. The most potent was crystal violet, active at 70 nM. The presence of apolar substituents enhanced activity and this is probably related to increased permeation of the dyes. Finally, PMS, as well as other electron carriers examined, also reduced the growth of *Leishmania* promastigotes in culture.

The results are compatible with a direct effect of the drugs on the intracellular amastigotes, involving only a permissive participation of the macrophages. We propose that the diverse agents destroy the amastigotes by redox-cycling generation of active oxygen metabolites at or near the parasites. Alternatively, the effect of the drugs could be mediated by toxic free radical reduction species of the drugs or by interference with electron flow or with the intermediary metabolism of *Leishmania*.

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