MACROPHAGE OXYGEN-DEPENDENT ANTIMICROBIAL ACTIVITY

I. Susceptibility of *Toxoplasma gondii* to Oxygen Intermediates*

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The importance of cell-mediated immunity in resistance to infection caused by intracellular pathogens has been well established. Mononuclear phagocytes clearly play primary roles in the efferent limb of cellular control of such infections (1–7). Although the mechanism(s) by which monocytes and macrophages exert their antimicrobial activity have not been defined, it is nevertheless plausible to suggest a role for oxidative metabolites. First, microbicidal activity in polymorphonuclear phagocytes has been linked to potent oxygen intermediates generated during the phagocytic respiratory burst (8–10). Second, both monocytes and macrophages from animals and humans release such products under a variety of stimulatory conditions (11–15). Third, macrophage release of H_2O_2 has recently been correlated with the ability to destroy intracellular *Trypanosoma cruzi* (16). However, there is little evidence which directly implicates oxygen intermediates in the microbicidal mechanisms of macrophages.

The present study examines the role of products of oxygen reduction in macrophage control of infection caused by *Toxoplasma gondii*, an obligate intracellular parasite. The first portion of this study establishes methods which demonstrate the susceptibility of *T. gondii* to selected oxygen intermediates generated in a cell-free system. The accompanying report establishes conditions for the analysis of macrophage oxygen-dependent antimicrobial mechanisms.

METHODS AND MATERIALS

Parasites. The virulent RH strain of *T. gondii* was obtained from Dr. Thomas C. Jones (Cornell University Medical College, New York) and was maintained by biweekly intraperitoneal passage in Rockefeller NCS female mice. Parasites were collected from peritoneal exudate fluid in phosphate-buffered saline, pH 7.4, (PBS)[1] (Dulbecco's, Grand Island Biological Co., Grand Island, N. Y.) and centrifuged at 50 *g* for 5 min at 22°C to sediment cells and debris. The supernate was centrifuged at 50 *g* for 10 min to collect toxoplasmas. This low-speed second centrifugation was important in increasing the yield of viable parasites. Pelleted toxoplasmas were resuspended in PBS for microbicidal assays, or, for infection of macrophage monolayers,

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[1] *Abbreviations used in this paper*: PBS, phosphate-buffered saline; D_{10-20} HIFBS, Dulbecco's modified Eagle's medium containing either 10% or 20% heat-inactivated fetal bovine serum; HBSS, Hanks' balanced salt solution; SOD, superoxide dismutase; DABCO, diazabicyclooctane; AO, acridine orange; LPO, lactoperoxidase; GO, glucose oxidase; XO, xanthine oxidase; and PMN, polymorphonuclear neutrophil.

in Dulbecco's modified Eagle's medium (Grand Island Biological Co.) containing 20% heat-inactivated, Sabin-Feldman dye test negative fetal bovine serum (HIFBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (D_{20} HIFBS). Parasites were counted in a hemocytometer and used within 1 h of harvesting.

Cells. Resident (normal) peritoneal macrophages were obtained from female NCS mice using previously described methods (17). For exogenous catalase uptake experiments, 4–5 × 10^5 total peritoneal cells, of which 45–55% were macrophages, were added in D_{10} HIFBS to 35-mm round plastic tissue culture dishes (Nunclon, Copenhagen, Denmark). For macrophage toxoplasma infection experiments, 4–5 × 10^5 peritoneal cells were added directly to 12-mm round glass coverslips (Propper, Long Island City, N. Y.) placed in similar dishes. After 60 min at 37°C in 5% CO_2, nonadherent cells were removed by washing with Hanks' balanced salt solution (HBSS) (Grand Island Biological Co.) and cultures were incubated overnight in fresh medium.

Special Reagents. Hydrogen peroxide (H_2O_2, 30% solution) was obtained from Mallinckrodt, Inc., St. Louis, Mo. Superoxide dismutase (SOD, bovine blood, lyophilized powder, 2,900 U/mg), glucose oxidase (type V, *Aspergillus niger*, 1,460 U/ml), xanthine oxidase (milk, 65 mg/ml, 0.19 U/mg), mannitol, sodium benzoate, histidine hydrochloride, and trypsin (bovine pancreas, twice recrystallized type III) were from Sigma Chemical Co., St Louis, Mo., and all were used without further preparation. Xanthine (Sigma Chemical Co.) was prepared in 0.05 M potassium phosphate buffer (pH 7.8) and EDTA (10^{-4} M) at 10^{-3} M. For microbicidal assay experiments, Sigma catalase (beef liver, twice recrystallized, 25 mg/ml, 32,000 U/mg) was freed of minor contamination with SOD by filtration over Sephadex G-100 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) using 0.05 M potassium phosphate buffer with 0.1 M KCl, pH 7.8 (9). For macrophage uptake experiments, Sigma catalase was used without further preparation. Catalase was heated at 100°C for 20 min and SOD was autoclaved at 120°C for 30 min where indicated. Diazabicyclooctane (DABCO) was from Eastman Kodak Co., Rochester, N. Y. Lactoperoxidase (lyophilized powder, B grade, 45.7 IU/mg) was obtained from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif., and was assayed by the orthodianisidine method before use (18). Acridine orange (3,6-dimethylamino-acridine) was from Allied Chemical Corp., Specialty Chemicals Div., Morristown, N. J. 3-amino-1,2,4-triazole was from Mann Research Labs., Becton-Dickinson Co., Oxnard, Calif., and trypsin inhibitor (ovomucoid) was obtained from Worthington Biochemical Corp., Freehold, N. J. All special reagents were suspended in PBS, pH 7.4, unless otherwise indicated.

Microbicidal Assay Using Acridine Orange (AO) Fluorescent Microscopy. Toxoplasmas from mice infected 3 d earlier were prepared and resuspended in PBS. Components indicated in the legends to the tables and figures were added to 0.5-ml aliquots of parasite suspensions (5–10 × 10^6 toxoplasmas/ml) in 12 x 75-mm plastic tubes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.), vortexed vigorously, and incubated for varying periods. All experiments were carried out at 37°C and the final volume of reaction mixture was 1 ml. 10 min before the indicated time points, 0.2 ml of reaction mixture was removed, mixed with 10 μl of AO (final concentration, 5 μg/ml), and reincubated for an additional 10 min. Duplicate samples of AO-stained suspensions were then placed on glass slides, overlayed with coverslips, and sealed. Preparations were immediately examined for parasite fluorescent staining using a Zeiss Photomicroscope III with epi-illumination (exciter filter BG 12, barrier filter 53) (Carl Zeiss, Inc., New York).

Infection of Macrophages. Culture medium overlying coverslips on which macrophages had been cultivated for 24 h was replaced with 1 ml of D_{20} HIFBS containing 1–2 × 10^6 toxoplasmas for 30 min at 37°C in 5% CO_2. Coverslips were then washed to remove extracellular parasites, and reincubated in fresh medium. Immediately after (time zero), and 6 and 18 h after the infection period, coverslips were fixed in glutaraldehyde, stained, and mounted. The percentage of macrophages infected, the number of toxoplasmas per 100 macrophages, and the number of toxoplasmas per vacuole were counted in Giemsa-stained preparations.

Catalase Uptake by Macrophages. Various concentrations of Sigma catalase dissolved in D_{10} HIFBS were added to dishes containing 24-h cultures of resident macrophages for 1–6 h at 37°C. At selected times, duplicate dishes were washed eight times with HBSS, reincubated for 30 min in fresh medium, and then washed three more times. This extensive washing was necessary to remove all traces of extracellular enzyme (18). Monolayers were next overlayed with 0.05% Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.) and dishes were carefully scraped. 0.1-ml aliquots of cell lysate were promptly assayed for catalase activity at 0°C by the method of Baudhuin et al. (19). 1 ng of Sigma catalase (32,000 U/mg) yielded $1.2 \times 10^{-5} \pm 0.1$ (mean ± SE) Baudhuin units (BU). Cell lysate protein was measured by the Lowry procedure (20).

To be certain that lysate catalase activity reflected interiorized enzyme, three approaches were used. First, in the presence and absence of cells, there was no catalase activity in the final 3 of 11 total washes of culture dishes exposed to 1 mg/ml catalase for 3 h. Second, after incubating dishes without cells for 1–3 h with catalase and then applying detergent and scrapping, this lysate yielded a catalase activity of only 2.5 ± 0.6% (mean ± SE) of that measured for similarly treated but cell-containing dishes. Thus, dish-bound enzyme was negligible. Finally, trypsin (0.5 mg/ml) was added to cultures of well-washed macrophages which had been exposed to 1 mg/ml catalase for 3 h. After 20–30 min, trypsin was removed and trypsin inhibitor (0.5 mg/ml) was added for 5 min. Dishes were then rinsed and lysates prepared. Macrophage catalase activity was unchanged by exposure to trypsin. Because trypsin rapidly degrades catalase (21), inactivation of any surface-bound or accessible dish-bound enzyme should have decreased total cell lysate activity. This was not observed. Thus, as measured, macrophage lysate activity appeared to accurately reflect levels of interiorized enzyme.

Other Procedures. Interiorized catalase was detected cytochemically using the diaminobenzidine technique at pH 9.5 (22). Peroxidatic activity of Sigma catalase, as determined by the *o*-dianisidine method, was generated by exposing catalase to trypsin for 60 min at 22°C in PBS, pH 7.4, followed by quenching in ice-cold sodium acetate buffer, pH 4.5 (21). 1 mg of trypsin-modified catalase yielded 89 ± 8.5 mU of peroxidatic activity. Undigested catalase had no detectable peroxidatic activity at pH 7.4. H_2O_2 generation by glucose-glucose oxidase interaction was measured fluorometrically at 37°C using the horseradish peroxidase-catalyzed oxidation of reduced scopoletin (11). Reagents for this assay were provided by Dr. Carl F. Nathan (Rockefeller University). For parasite catalase activity, toxoplasmas were lysed hypotonically by adding 0.2 ml of isotonic PBS containing $5–10 \times 10^6$ viable parasites to 0.8 ml of distilled water for 10 min with periodic vortexing. The suspension was centrifuged at 400 *g* for 10 min and the supernate was assayed for catalase activity (19). Examination of the pellet revealed no intact parasites. Where indicated, toxoplasmas in suspension were heat-killed by a 60-min incubation in a 56°C water bath.

Results

AO Fluorescence of T. gondii. To judge the effect of potentially lethal conditions on toxoplasmas in suspensions, a reliable method of assessing parasite viability was required. AO is selectively concentrated by lysosomes of intact cells, whereas damaged cells fail to demonstrate lysosomal fluorescence (23). Because toxoplasma lysosomes have been reported to fluorescence after exposure to AO (24), we examined the usefulness of this simple technique as an indicator of parasite viability.

Four preparations of toxoplasmas were initially studied. 90–95% of parasites obtained directly from peritoneal exudate fluid and exposed for 10 min to AO (5 μg/ml) exhibited two to three bright orange lysosomes against a homogenous dark green cytoplasm (Fig. 1). Lysosomes were often grouped at one end of the organism as

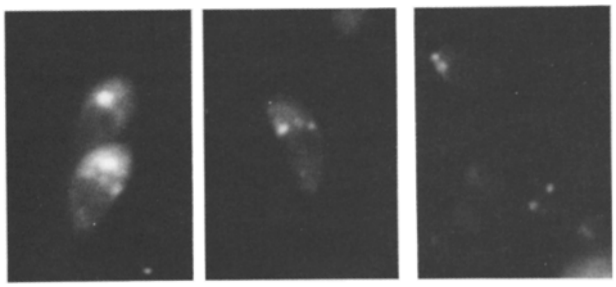

FIG. 1. *T. gondii* lysosomal fluorescence. Viable toxoplasmas from peritoneal exudate fluid were incubated with acridine orange (5 μg/ml) for 10 min at 37°C. Parasites show bright (orange-red) punctate lysosomal fluorescence (X 2,100).

previously noted (24). In contrast, heat-killed parasites, all of which failed to exclude trypan blue dye, exhibited light orange-green cytoplasmic fluorescence and no lysosomal staining. Toxoplasmas prepared in a standard fashion (3, 25) by centrifugation of peritoneal exudate at 30 *g* for 5 min and then at 120–600 *g* for 10 min yielded two parasite populations. 50–70% had diffuse orange cytoplasm and no lysosomal staining, compatible with dead cells incapable of concentrating the dye (23). The remainder (30–50%) appeared similar to toxoplasmas obtained directly from the peritoneal exudate. By reducing the second centrifugation to 50 *g* for 10 min, the proportion of parasites appearing viable was consistently increased to 80–85%.

Although resident peritoneal macrophages from normal mice do not restrict intracellular multiplication of viable toxoplasmas, these cells promptly digest parasites killed by heating or glutaraldehyde (26). To corroborate toxoplasma viability as judged by AO-staining patterns, normal macrophages were infected with various parasite preparations (Table I). The bulk of heat-killed toxoplasmas were degraded by 6 h and entirely so by 18 h, whereas approximately one-half the parasites prepared by high speed centrifugation were digested and removed. In contrast, with toxoplasmas obtained by low speed centrifugation, there was only a slight decline in the percentage of macrophages infected over 18 h. Thus, toxoplasma viability as indicated by AO staining appeared to correlate well with parasite survival in normal macrophages.

Toxoplasma Susceptibility to Hydrogen Peroxide (H_2O_2). Toxoplasmas were exposed to various concentrations of reagent H_2O_2 and to increasing amounts of H_2O_2 generated by glucose and glucose oxidase (GO). The latter reaction does not produce oxygen intermediates other than H_2O_2 (27). As demonstrated in Fig. 2, most toxoplasmas were quite resistant to reagent H_2O_2 (up to 10^{-2} M) and were completely insensitive to H_2O_2 formed by 3–56 mU of GO. These amounts of GO generated 1–19 nmol H_2O_2/min at 37°C. Toxoplasmas, preincubated with 10^{-3} M reagent H_2O_2 for 60 min, subsequently multiplied normally in resident macrophages. Because of these findings, toxoplasmas were lysed to detect intracellular catalase activity. One million parasites yielded $5.7 \pm 0.3 \times 10^{-4}$ BU (mean ± SE), equivalent to 47.5 ± 2.3 ng of Sigma catalase. Thus, endogenous parasite catalase may contribute to toxoplasma resistance to H_2O_2 toxicity.

Toxoplasma Susceptibility to H_2O_2-peroxidase-halide Systems. Because monocytes contain peroxidase (28), we examined whether its addition to H_2O_2 and an oxidizable

TABLE I
Viability of Toxoplasma Preparations

Preparation*	Percentage of toxoplasmas viable‡		Percentage of macrophages infected§			
	Trypan blue	AO	0 h	6 h	18 h	
Heat-killed	0	0	55	10	0	(0)‖
Fast spin	>90	30–50	52	30	24	(46)
Slow spin	>90	80–85	50	46	44	(88)

* Heat-killed (56°C for 60 min), fast centrifugation (50 *g* for 5 min, 150 *g* for 10 min), slow centrifugation (50 *g* for 5 min, 50 *g* for 10 min).

‡ Percentage of infecting parasite inoculum viable as indicated by trypan blue dye exclusion or AO staining.

§ Resident peritoneal macrophages from normal mice (representative experiment). Time 0 indicates percentage of cultivated macrophages with intracellular toxoplasmas after a 30-min infection period.

‖ Proportion (percentage) of original intracellular inoculum remaining 18 h after infection as judged by the percentage of macrophages still infected.

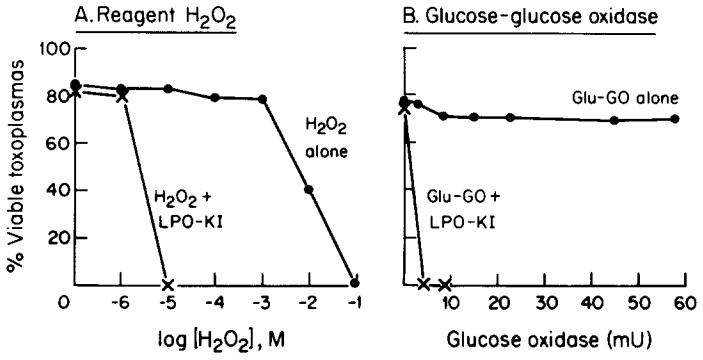

FIG. 2. Resistance of *T. gondii* to H_2O_2 and susceptibility to H_2O_2-peroxidase-halide. Parasites and reagents were diluted in PBS (pH 7.4) before use. (A) 5–10 × 10^6 toxoplasmas were exposed for 60 min at 37°C to reagent H_2O_2 in the final concentrations indicated with (×) or without (●) lactoperoxidase (LPO, 1.8 mU) and KI (0.05 μmol) present. (B) Reagent H_2O_2 was replaced by glucose (50 mM) and glucose oxidase (GO) alone (●) or with LPO (1.8 mU) plus KI (0.05 μmol) (×). Controls were incubated in PBS alone. Reaction vol was 1 ml. Parasite viability was determined by acridine orange criteria. Killing was abolished by the addition of catalase (200 μg).

halide could enhance toxoplasma killing. As indicated in Fig. 2, lactoperoxidase (LPO) in the presence of KI and either reagent H_2O_2 or glucose-GO killed all parasites by 60 min, and 80–90% were dead at 15 min (data not shown). With LPO and KI, the minimum lethal concentration of reagent H_2O_2 was reduced from 10^{-2} to 10^{-5} M.

In the presence of pH 4–5 and low steady-state concentrations of H_2O_2, catalase augments the antibacterial activity of a glucose-GO-NaI system presumably by a peroxidatic mechanism (29). The finding that 90% of toxoplasmas were killed by exposure to pH 5 for 30 min and all were dead at pH 4 (data not shown) precluded testing this specific reaction. However, catalase may be cleaved to peroxidatic subunits under conditions of extreme pH or proteolysis (21, 30). Because mouse peritoneal macrophages contain catalase (see below), we explored the susceptibility of toxoplas-

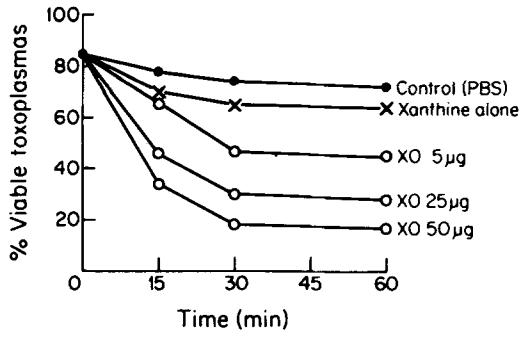

FIG. 3. Susceptibility of *T. gondii* to xanthine-XO. Toxoplasmas were incubated with PBS (control, [●]), xanthine 1.5×10^{-4} M alone (×), or xanthine (1.5×10^{-4} M) plus XO (○) for 15–60 min at 37°C in a total vol of 1 ml.

TABLE II

*Effect of Oxygen Intermediate Scavengers On Killing By Xanthine-XO**

Scavenger added	Percentage of parasites viable at 30 min‡
None	25.6 ± 2.1 (7)
Catalase, 200 μg/ml	68.0 ± 1.0 (3)
Catalase heated	28.0 ± 1.0 (2)§
SOD, 100 μg/ml	63.3 ± 3.3 (3)
SOD heated	29.5 ± 4.5 (2)§
Mannitol, 50 mM	65.3 ± 2.3 (3)
Benzoate, 10 mM	63.3 ± 4.4 (3)
DABCO, 1 mM	65.7 ± 2.3 (3)
Histidine, 10 mM	67.3 ± 4.1 (3)

* The reaction mixture contained $5–10 \times 10^6$ toxoplasmas, PBS (pH 7.4), 1.5×10^{-4} M xanthine, 50 μg XO, and scavengers in the indicated concentrations in a total vol of 1 ml. Viability of parasites in incomplete (control) reaction mixtures: PBS alone, 80.1 ± 1.5 (7); PBS + xanthine, 73.6 ± 1.7 (7); and PBS + XO, 78.3 ± 1.9 (3).

‡ As indicated by acridine orange fluorescence. Results are the mean ± SE of (*n*) experiments.

§ Mean ± range.

mas to H_2O_2-KI using trypsin-modified catalase as the peroxidatic agent. At pH 7.4, in the presence of glucose (50 mM), GO (6 mU), KI (0.05 μmol), and trypsin-modified catalase (6 mU), all toxoplasmas were killed within 30 min. Omission of any of the components of this system or addition of undigested catalase (200 μg) abolished parasite killing.

Toxoplasma Susceptibility to Other Oxygen Intermediates. More distal intermediates in the reduction of molecular oxygen generated by xanthine-xanthine oxidase (XO) were next examined. The dose-related killing of toxoplasmas in the presence of xanthine and XO is demonstrated in Fig. 3. In the absence of either xanthine or XO, no appreciable parasite killing occurred. That toxoplasma killing was related to species other than O_2^- and H_2O_2 was suggested from studies with oxygen intermediate scavangers (Table II) (31). Both catalase and SOD effectively reversed toxoplasma killing, whereas heated catalase and SOD had no protective effect. The requirement for both O_2^- and H_2O_2 suggested a role for products of their interaction (9, 31). The

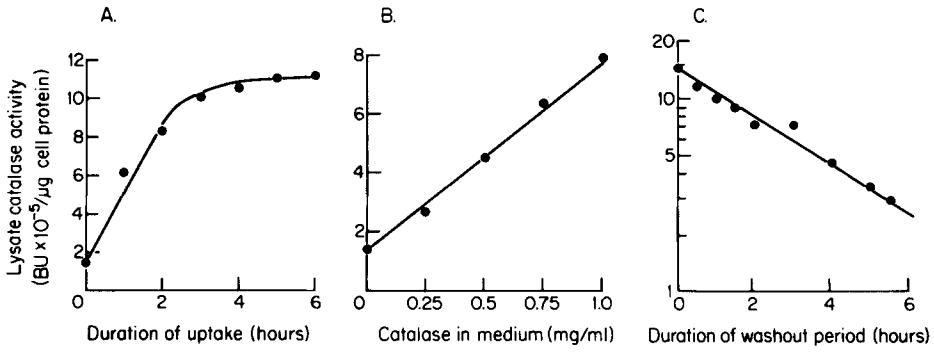

FIG. 4. The uptake and inactivation of catalase by resident macrophages. Overnight cultures of normal macrophages were exposed to exogenous catalase at 37°C. At the indicated times, duplicate culture dishes were removed, washed, and lysates were prepared and assayed as described. (A) Macrophage uptake after exposure to catalase (1 mg/ml) for 1–6 h. Vertical axis intercept indicates endogenous macrophage catalase level. (B) 2-h macrophage uptake of catalase as a function of enzyme concentration in the medium. (C) Exponential decay of interiorized catalase activity after a 3-h exposure to catalase (1 mg/ml), washing, and reincubation for indicated times in catalase-free medium. Half-life is ≅2.7 h.

effect of OH· scavengers (mannitol and benzoate) which do not reduce O_2^- or H_2O_2 production by xanthine-XO (32) and quenchers of $^1O_2$ (DABCO and histidine) was then examined. These scavengers were as efficacious as catalase and SOD in preventing xanthine-XO toxoplasma killing (Table II).

When preincubated with xanthine-XO (50 μg) for 30 min at 37°C, the bulk of toxoplasmas (80%) appeared dead by AO criteria, and the majority of these organisms were rapidly digested after administration to resident macrophages. The viable minority, however, multiplied normally showing four to five toxoplasmas per vacuole 18 h after infection. If catalase (200 μg) or SOD (100 μg/ml) was added to xanthine-XO during parasite preincubation, the subsequent intracellular survival and multiplication of treated organisms was similar to that of parasites preincubated in PBS alone or in PBS plus xanthine without XO (data not shown).

Catalase Uptake by Macrophages. In preparations for administering oxygen intermediate scavengers to macrophages with antitoxoplasma activity, we examined the interaction of soluble catalase with normal cells. Overnight macrophage cultures yielded an endogenous catalase activity of $1.8 \times 10^{-5} \pm 0.1$ (mean ± SE) BU/μg cell protein. This was equivalent to 120 ng of Sigma catalase/10^6 macrophages. Macrophage catalase activity was abolished by exposure to 50 mM aminotriazole, whereas 0.45 M ethanol inhibited catalase inactivation by aminotriazole, consistent with having detected native catalase (33). Upon exposure to 1 mg/ml of catalase, interiorized enzyme increased linearly for 2–3 h and then plateaued (Fig. 4A). Fig. 4B indicates the linear relationship between enzyme concentration and uptake. One million macrophages interiorized 0.06% of the administered load in 2 h. Uptake was temperature dependent and >90% inhibited at 4°C. The half-life of endocytosed enzyme was brief and decay was exponential (Fig. 4C). Interiorized catalase was inactivated an average rate of 19% per hour. Cytochemical techniques for detecting intracellular catalase by light microscopy (22) showed numerous diaminobenzidine-positive granules distributed in a perinuclear fashion typical of lysosomes (Fig. 5).

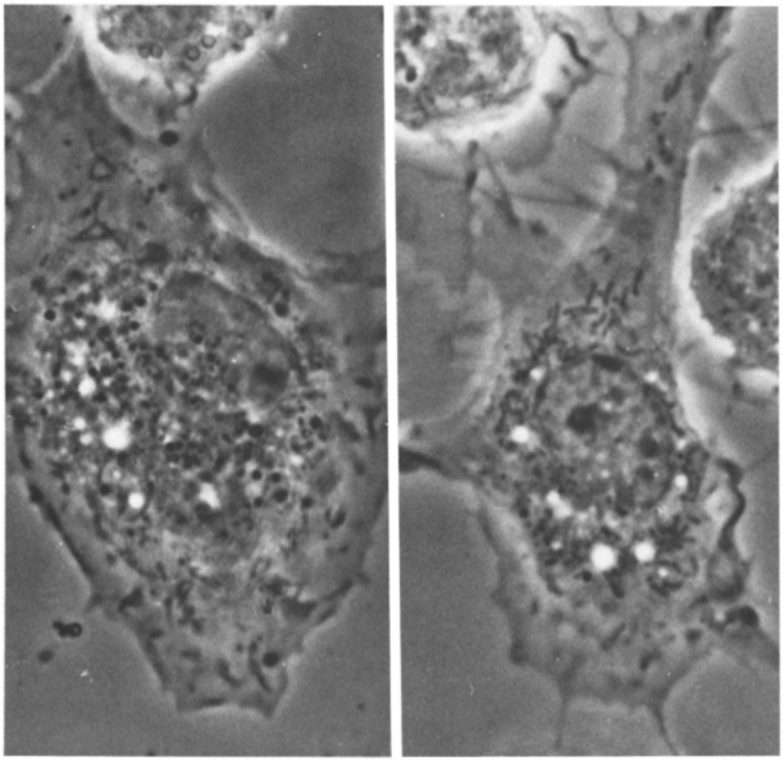

FIG. 5. Cytochemical localization of interiorized exogenous catalase. Macrophages were exposed for 3 h to catalase (1 mg/ml), washed, fixed, and stained with diaminobenzidine (DAB), pH 9.5 (22). Left, macrophage with numerous darkly-stained perinuclear DAB-positive granules. Right, control macrophage (phase-contrast microscopy, X 2,000).

Discussion

The in vitro interaction of *T. gondii*, a coccidian protozoan, with animal and human mononuclear phagocytes has been used extensively to investigate the role of cell-mediated immunity in resistance to intracellular pathogens (3, 25, 26, 34, and 35). The mechanism(s) by which these phagocytes exert antitoxoplasma activity, however, have not been defined. Because macrophages and monocytes release O_2^- (13–15), H_2O_2 (11, 15), and OH· (12), we examined toxoplasma susceptibility to these agents.

AO fluorescence was found to be an accurate indicator of parasite viability. This vital dye diffuses into cells and microorganisms such as *T. gondii*, and is concentrated in lysosomes (23, 24). Examination of freshly-harvested parasites established a clear-cut AO fluorescent pattern indicating viability. 50–70% of toxoplasmas however, were killed by centrifugation procedures typically employed in preparing them for infection of cell monolayers (3, 25). Dead parasites exhibited diffuse cytoplasmic fluorescence and no lysosomal staining. Norrby also found that 50% of rapidly centrifuged toxoplasmas failed to show lysosomal fluorescence (24). Thus, it is not surprising that Jones and colleagues (25, 26) observed that normal macrophages degraded and removed one-half of phagocytized parasites. Further, Remington and co-workers have reported that although 90% of parasites in the prepared inoculum excluded trypan

blue dye, only 5–20% incorporated tritiated uridine (3). Although parasite demise has been attributed to time-consuming preparative procedures, the yield of viable toxoplasmas was increased to 80–85% simply reducing the speed of centrifugation.

In a cell-free environment, virulent toxoplasmas were strikingly resistant to H_2O_2. At 10^{-2} M H_2O_2, almost one-half of parasites were viable. In contrast, intracellular pathogens such as trypanosomes are killed in cell-free systems by exposure to 3×10^{-4} M H_2O_2 (36, 37) or incubation with glucose-glucose oxidase (16). A previous study demonstrated no decrease in toxoplasma respiration upon exposure to 10^{-4} M H_2O_2, and provided indirect evidence supporting the presence of endogenous catalase (38). Parasites such as *T. cruzi* which are susceptible to H_2O_2 (37) do not contain catalase (39), whereas certain bacteria rich in catalase appear to be more resistant to PMN killing (40). Our finding of catalase activity in supernates of lysed toxoplasmas is consistent with parasite resistance to H_2O_2. In light of similar resistance to O_2^-, which may be directly toxic by itself to bacteria (41), it would be of interest to measure toxoplasma superoxide dismutase.

Lacking peroxidase, it is unlikely that macrophages possess an antimicrobial mechanism similar to the PMN H_2O_2-myeloperoxidase-halide system (31). Monocytes, however, contain myeloperoxidase (28), and it was of interest to observe that toxoplasma susceptibility to H_2O_2 was greatly augmented in the presence of lactoperoxidase or trypsin-modified catalase and KI. Microorganisms other than bacteria including fungi, viruses, and mycoplasmas are also susceptible to H_2O_2-peroxidase-halide exposure (8).

In contrast to our finding of *T. gondii* resistance to H_2O_2 alone, most toxoplasmas were rapidly killed by xanthine-XO. The aerobic oxidation of xanthine and other substrates catalyzed by XO generates O_2^-, H_2O_2, OH·, and probably $^1O_2$ (31). Although the precise pathway(s) by which OH· and $^1O_2$ are subsequently produced have not been fully clarified, it appears that formation of both these highly reactive species depends upon the initial interaction of O_2^- and H_2O_2 (for review see 31). The failure of the XO system to kill all toxoplasmas in suspension may reflect (*a*) varying parasite susceptibility, (*b*) rapid substrate depletion at 37°C as suggested by no increase in killing at 60 min compared to 30 min, (*c*) the quenching effect of xanthine and urate on OH· and $^1O_2$ (31), or (*d*) the autoinactivation of xanthine oxidase (42). By employing scavengers or quenchers of oxygen intermediates, it appeared that OH· and $^1O_2$ were primary toxoplasmacidal agents, whereas O_2^- and H_2O_2 played roles as precursors. Inhibition of xanthine-XO parasite killing by both SOD and catalase indicated that neither O_2^- nor H_2O_2 were toxic because O_2^- production is not diminished by catalase nor is H_2O_2 formation decreased by SOD (31). Further, toxoplasmas were not killed by direct exposure to reagent H_2O_2 (up to 10^{-3} M). A toxoplasmacidal role for OH· and $^1O_2$ was suggested, however, by the requirement of both O_2^- and H_2O_2 for parasite killing and the protective effect of scavengers of OH· (mannitol and benzoate) and $^1O_2$ (DABCO and histidine). Evidence for the generation of OH· (43) and $^1O_2$ (44) by XO systems has been reviewed elsewhere (31). Although the specificity of inhibition produced by OH· and $^1O_2$ scavengers has not been adequately determined (31), our findings suggest a primary toxoplasmacidal role for OH· and $^1O_2$ in this microbicidal system.

Characterizing the interaction of soluble catalase and macrophages was pertinent

to experiments in which oxygen intermediate scavengers were administered to toxoplasma immune macrophages. Normal macrophages contained measurable catalase as noted previously (45) and readily endocytosed exogenous catalase in a dose-related fashion by a cold-inhibitable mechanism. Both findings suggested that uptake occurred by fluid-phase (nonadsorptive) pinocytosis similar to macrophage uptake of horseradish peroxidase (18). A 3- to 4-h exposure was required to reach maximal (plateau) intracellular enzyme levels, and interiorized catalase was located in perinuclear granules consistent with secondary lysosomes. The half-life of pinocytosed catalase was brief (2.7 h), and its rate of inactivation (19% per hour) was three times faster than similarly administered horseradish peroxidase (18). These findings presumably reflect the action of lysosomal hydrolases, proteases, and low pH to which catalase appears more sensitive (46). These observations led us in the accompanying report to preincubate macrophages with oxygen intermediate scavengers before infection with toxoplasmas. Moreover, although 0.06% of the administered load of catalase was interiorized by 10^6 macrophages in the first 2 h, we administered exogenous scavengers to macrophages both during and after toxoplasma infection. This constant-exposure technique would presumably maximize scavenger success in reversing macrophage antitoxoplasma activity if oxidative metabolites were involved.

Summary

A sensitive method for evaluating extracellular parasite viability was used to determine the in vitro susceptibility of virulent *Toxoplasma gondii* to selected oxygen intermediates. By acridine orange fluorescent staining criteria, toxoplasmas were resistant to up to either 10^{-3} M reagent H_2O_2 or H_2O_2 generated by glucose-glucose oxidase. In keeping with a lack of sensitivity to H_2O_2, toxoplasmas contained endogenous catalase (5.7×10^{-4} Baudhuin units/10^6 organisms). The addition of a peroxidase and halide, however, markedly accelerated killing and lowered the H_2O_2 requirement by 1,000-fold. In contrast, toxoplasmas were promptly killed after exposure to products generated by xanthine (1.5×10^{-4} M) and xanthine oxidase (50 μg). The inhibition of this system's microbicidal activity by scavengers of O_2^- (superoxide dismutase) and H_2O_2 (catalase) indicated that although neither O_2^- nor H_2O_2 were toxoplasmacidal, their interaction was required for parasite killing. Quenching OH· and $^1O_2$, presumed products of O_2^--H_2O_2 interaction, by mannitol, benzoate, diazabicyclooctane, and histidine, also inhibited toxoplasma killing by xanthine-xanthine oxidase. These findings suggested that O_2^- and H_2O_2 functioned in precursor roles and that OH· and $^1O_2$ were toxoplasmacidal.

The capacity of normal peritoneal macrophages to pinocytose an oxygen intermediate scavenger, soluble catalase, was also demonstrated. Appreciable extraphagosomal concentrations of catalase were achieved by exposing macrophages to 1 mg/ml of the enzyme for 3 h. Maintenance of high intracellular levels required constant exposure because interiorized catalase was rapidly degraded.

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