MACROPHAGE OXYGEN-DEPENDENT ANTIMICROBIAL ACTIVITY

IV. Role of Endogenous Scavengers of Oxygen Intermediates*

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Our prior observations with activated mononuclear phagocytes have implicated oxygen intermediates as important effector molecules in both the intracellular destruction and the growth inhibition of *Toxoplasma gondii* (1–3). Because this complex oxidative interaction may be modulated by the presence of endogenous scavengers within either the parasite or host cell, we examined the roles of superoxide dismutase (SOD)[1], catalase, and glutathione peroxidase (GP). Together, these enzymes comprise a potent triad of intracelluar defense mechanisms against the toxicity of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (4). Their activity has been investigated in *T. gondii* and in various macrophage populations, obtained from in vivo and in vitro environments, that express different antimicrobial behavior. Our findings demonstrate that the levels of these three scavengers of oxygen intermediates vary from cell to cell during cultivation, and that they can be correlated with the effectiveness of macrophage antitoxoplasma activity. In particular, intracellular catalase appears to play an important modulating role in macrophage-toxoplasma interaction.

Materials and Methods

Parasites, Mice (NCS), Macrophages, and Spleen Cell Supernates (Lymphokines). These were obtained, cultivated, or prepared as in the accompanying report (3). Additional macrophages were from acatalasemic C3H/HeAnl/Cs^b mice bred from stock generously provided by Dr. Robert N. Feinstein, Argonne National Laboratory, Argonne, Ill. (5). Some of these mice were immunized and boosted with *T. gondii* as described for NCS mice (2, 3). Macrophage antitoxoplasma activity was assessed by microscopic enumeration of Giemsa-stained cover slips 18 h after infection with virulent RH strain toxoplasma trophozoites (2, 3).

Macrophage and Toxoplasma Enzyme Activities. 4–5 × 10^6 peritoneal cells were added to 35-mm round plastic tissue culture dishes for 1 h, and following washing, cultures were reincubated for 3–72 h in Dulbecco's medium containing 20% heat-inactivated fetal bovine serum, penicillin,

* Supported by grants 1 732 GM-07245 and AI 07012 from the U. S. Public Health Service, and The Rockefeller Foundation Grant GAHS 7716.

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§ Scholar of the Leukemia Society of America (U. S. Public Health Service grant CA 22090) and Research Career Scientist of The Irma T. Hirschl Trust.

[1] *Abbreviations used in this paper:* AT, aminotriazole; BU, Baudhuin units; D_{20}HIFBS, Dulbecco's medium containing 20% heat-inactivated fetal bovine serum, penicillin, and streptomycin; GO, glucose oxidase; GP, glutathione peroxidase; IB, toxoplasma immune-boosted macrophage; IM, toxoplasma-immune macrophage; KRPG, Krebs-Ringer phosphate buffer with 5.5 mM glucose; NADPH, nicotinamide adenine dinucleotide phosphate; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; PMN, polymorphonuclear neutrophil; SOD, superoxide dismutase.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/80/12/1610/15 $1.00
Volume 152 December 1980 1610–1624

and streptomycin (D_{20}HIFBS) alone or D_{20}HIFBS plus active or control lymphokines (3) in 5% CO_2 at 37°C. Media were changed daily. At various intervals, duplicate dishes were thoroughly washed, and lysates were prepared after application of 0.05% Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.). For parasite enzyme activities, 10^7–10^8 washed toxoplasma trophozoites in 0.2–0.5 ml of isotonic phosphate-buffered saline (PBS) were either lysed hypotonically (1) or twice sonicated for 60 s (Cell Disruptor, 50% duty cycle, output control 4; Heat Systems-Ultrasonics, Inc., Plainview, N. Y.). Macrophage and parasite lysates or sonicates were cleared at 4°C by centrifugation for 15 min at 8,000 *g* in an Eppendorf microcentrifuge, placed on ice, and the supernates were promptly assayed spectrophotometrically for enzyme activity. Examination of the lysed or sonicated parasite pellet revealed only a rare intact parasite.

Total SOD activity was assayed in the presence of 1 mM sodium azide (6) by the method of McCord and Fridovich (7). As defined (7), 1 U of SOD activity inhibited the rate of xanthine-xanthine oxidase-induced reduction of ferricytochrome *c* by 50%. Catalase activity was assayed by the method of Baudhuin et al. (8), and was expressed in Baudhuin U (BU) as described (1). We have previously reported that the inhibition of macrophage lysate catalase activity by aminotriazole (AT) can be abolished by ethanol (1), indicating the detection of native catalase by this technique. GP was assayed as described by Paglia and Valentine (9), and activity was expressed as nanamoles of nicotinamide adenine dinucleotide phosphate (NADPH) oxidized to nicotinamide adenine dinucleotide per min using the extinction coefficient for NADPH of 6.22×10^3 $M^{-1}cm^{-1}$. In certain GP assays, cumene hydroperoxide was substituted for H_2O_2 (10). Supernate protein was determined by the method of Lowry et al. (11), and enzyme activities have been expressed per milligram of adherent cell or parasite protein. Boiling the supernates of macrophage or toxoplasma preparations for 20 min ablated the activity of all three enzymes.

Inhibition of Catalase Activity by AT. 5×10^6 washed toxoplasmas were suspended in 1 ml of PBS, pH7.4, and AT, which irreversibly inhibits catalase (12), was added in a final concentration of 5–50 mM for 15–60 min at 37°C. Parasites were then washed with PBS and resuspended in either (*a*) Krebs-Ringer phosphate buffer with 5 mM glucose (KRPG), pH7.4, for cell-free microbicidal studies, (*b*) D_{20}HIFBS for infection of macrophages, or (*c*) distilled water (lysis) for enzyme assays. Cultivated macrophages were also incubated with AT diluted in D_{20}HIFBS for varying periods, washed thoroughly, and either infected with toxoplasmas, lysed as described, or reincubated in medium alone for up to 72 h before detergent application. Mixing equal volumes of AT-treated and control macrophage or parasite preparations for 30 min at 37°C decreased catalase activity only by the expected dilutional proportion, indicating no free AT. 25 mM AT was also not toxic to macrophages as judged by trypan blue dye exclusion, microscopic appearance, or the ability to phagocytize toxoplasmas. In addition, in the concentrations employed, AT did not affect either macrophage or parasite GP activity.

Parasite Susceptibility to H_2O_2. After 60 min of exposure to PBS alone or PBS plus 5–50 mM AT, toxoplasmas were pelleted and resuspended in KRPG. Each 1-ml reaction vol contained KRPG, 5×10^6 toxoplasmas, and either reagent H_2O_2 (final concentration, 10^{-6} to 10^{-2} M) or varying amounts of glucose oxidase (GO) generating up to 20 nmol H_2O_2/min. After 60 min at 37°C, parasite viability was assessed by mixing 0.2-ml reaction mixture aliquots with either 10 μl of acridine orange (5 μg/ml) for 10 min (1), or 0.2 ml of 0.2% trypan blue for 90 s. Duplicate samples of stained suspensions were placed on glass slides, overlayed with cover slips, and promptly examined for trypan blue exclusion or for parasite acridine orange fluorescent staining patterns as described (1). To further corroborate toxoplasma viability, identically treated parasites were pelleted, resuspended in D_{20}HIFBS, and added to dishes containing cultivated resident macrophages from normal NCS mice. These cells digest and remove dead parasites within 6–18 h (1).

Macrophage Susceptibility to H_2O_2. After cultivation on glass cover slips for 3 or 24 h in D_{20}HIFBS or serum-free Neuman-Tytell medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.), macrophages were exposed to either 25 mM AT for 60 min or 1 mM azide for 10 min at 37°C. After thorough washing, cover slips in 35-mm dishes were overlayed with 1 ml of KRPG with or without added GO. After 60 min at 37°C (water bath), the medium was replaced by PBS containing 0.1% trypan blue dye for 90 s, followed by washing and glutaraldehyde fixation. Duplicate cover slips were mounted, and macrophages were scored as nonviable if they showed blue nuclear staining.

Assay for H_2O_2 Generation. Extracellular H_2O_2 release by phorbol myristate acetate (PMA)-triggered macrophages and that generated by GO was assayed by the fluorometric scopoletin technique (2, 3, 13).

Special Reagents. Reagents in addition to those described in the accompanying report (3) included reduced NADPH (type X), reduced glutathione (crystalline), glutathione reductase (type III), and AT (3-amino-1,2,4-triazole). All were from Sigma Chemical Co., St. Louis, Mo. Cumene hydroperoxide was obtained from Polysciences, Inc., Warrington, Pa.

Results

Levels of SOD and GP in Macrophages and Parasites. Both SOD and GP are efficient scavengers of O_2^- and H_2O_2, respectively (4, 9, 14, 15), and their levels were examined in macrophage and toxoplasma lysates. Fig. 1 A illustrates the SOD levels of in vivo-activated, unstimulated resident, and in vitro- (lymphokine) activated cells, and Fig. 1 B demonstrates the GP levels in the same populations. At the initiation of culture, the enzyme activities for all cells were quite similar. For both SOD and GP, lymphokine exposure of resident cells led to a moderate increase in enzyme specific activity, whereas cultivation of toxoplasma immune-boosted macrophages (IB) with or without lymphokine was accompanied by a consistent decrease. Toxoplasmas contained 6.1 ± 2.2 SOD U/mg of parasite protein, and GP levels of 117 ± 8 nmol/min per mg of protein. The substitution of cumene hydroperoxide for H_2O_2 did not yield additional macrophage or toxoplasma GP activity, suggesting the presence of only the selenium-dependent enzyme (10). These results do not indicate a significant role for either SOD or GP in the previously described decline in in vivo-activated macrophage production of O_2^- and H_2O_2, or the associated loss of antitoxoplasma activity after 48 h of cultivation (3).

Macrophage Catalase Activity. We next turned to catalase, an effective scavenger of

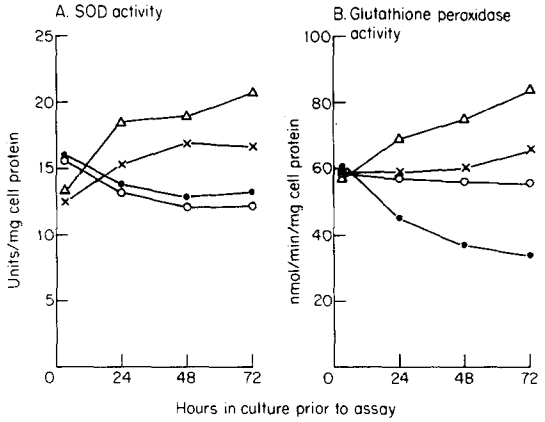

FIG. 1. SOD and GP activities of NCS macrophages cultivated for 3–72 h. Results are means of three duplicate experiments for each cell type, and GP data have been corrected for the nonenzymatic oxidation of NADPH by H_2O_2 (9). (A) SOD activity of resident cells from normal mice cultured in the presence (△) or absence (×) of 5% toxoplasma lymphokine; IB cells with (○) or without (●) similar lymphokine treatment. After 24 h of cultivation in D_{20}HIFBS, thioglycollate (THIO)-elicited cells contained 8.1 ± 1.4 SOD U/mg protein. (B) GP activity of resident (x), IB (●), and IM (○) cells cultivated in medium alone. 5% toxoplasma lymphokine did not alter the GP activity of IM or IB cells, but enhanced that of resident macrophages (△). Control lymphokine (3) had no such effect. In two experiments, the mean GP activities of IB cells from acatalasemic mice were 40.3 (3 h), 28.2 (24 h), 19.3 (48 h), and 24.1 (72 h). The GP activity of THIO cells first cultivated for 24 h was 89.2 ± 6.1 nmol/min per mg (mean ± SEM).

H_2O_2 (16), which is present in most mammalian tissues and oxygen-metabolizing microorganisms (4). During 3 d of cultivation in medium alone, while the H_2O_2 release and antitoxoplasma activity of IB and toxoplasma-immune macrophages (IM) declined in parallel (3), their catalase levels progressively increased (Fig. 2). By 72 h, catalase levels were eight- to ninefold higher than those of freshly-explanted macrophages. Daily lymphokine exposure, however, consistently blunted this rise, and as noted in the accompanying report (3), maintained the capacity of IB and IM macrophages to release enhanced H_2O_2 and exert antitoxoplasma activity. The catalase activity of normal resident cells from NCS mice also increased over 72 h (fourfold), but in contrast was enhanced an additional twofold by lymphokine treatment. Because of the correlation between IB and IM macrophage catalase levels, H_2O_2 release, and antitoxoplasma activity, further experiments were carried out to better characterize the role of intracellular catalase.

AT exposure effectively and rapidly inhibited the catalase activity of control and lymphokine-treated IB, IM, and resident NCS macrophages in both a time- and dose-related fashion (data not shown). After a 60-min exposure, inhibition reached >90% (Fig. 3 A). Similar results were achieved by a 10-min incubation with 1 mM azide. Recovery of enzyme activity was prompt (Fig. 3 B), and was suppressed by continuous treatment with 1–5 mM AT (Fig. 3 C).

The effect of catalase inhibition was explored by exposing resident NCS cells to an extracellular source of enzymatically generated H_2O_2. As indicated in Table I, 27% of freshly-explanted 3 h cells (low in catalase) remained viable after a 1 h exposure to 30 nmol H_2O_2/min as compared with 90% of cells first cultivated for 24 h in D_{20}HIFBS. These latter macrophages contained similar levels of GP (Fig. 1 B), but fourfold more catalase (Fig. 2), suggesting the importance of the latter scavenger in defense against high concentrations of H_2O_2. Exposing low catalase 3-h cell cultures to AT before the addition of GO yielded little additional cytotoxicity, but consistently increased the susceptibility of cells first cultivated overnight. A portion of the acquired resistance of 24-h cells to H_2O_2 appeared to be serum-related (Table I); however, the susceptibility of serum-free cultures was still enhanced by AT pretreatment. Thioglycollate-elicited macrophages were more resistant to H_2O_2, perhaps related to thioglycollate's scavenging of H_2O_2 (3). Appropriately, AT did not enhance the toxicity of H_2O_2 for macrophages from acatalasemic mice that contain 20- to 30-fold less catalase (see below).

Toxoplasma Catalase Activity. We have previously reported that *T. gondii* contain

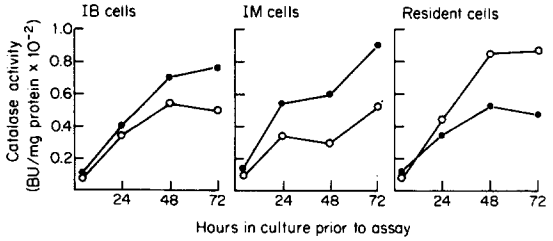

FIG. 2. Catalase activity of IB, IM, and resident macrophages from normal (NCS) mice cultivated in medium alone (●) or medium plus 5% toxoplasma lymphokine (○). The catalase activity of cells exposed to control lymphokine (3) was similar to that of cells cultivated in medium alone. Results are the mean of three to five duplicate experiments.

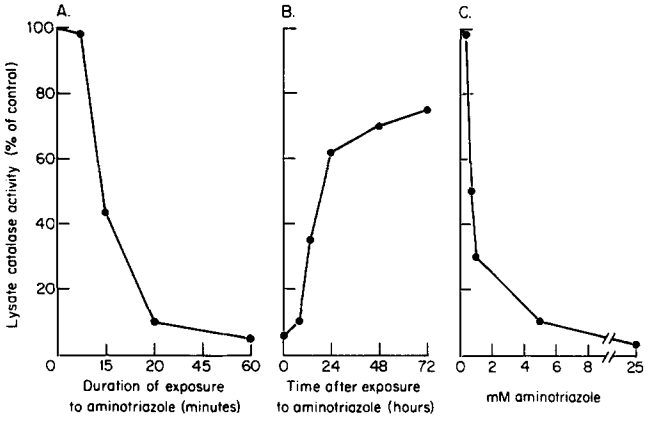

FIG. 3. Inhibition of IB macrophage catalase activity by AT (A) Overnight cultures were incubated with 25 mM AT for 5–60 min before thorough washing and lysis. (B) Recovery of macrophage catalase activity after a 1 h pulse of 25 mM AT (time zero), washing, and reincubation for up to 72 h in standard medium. (C) Effect of incubating macrophages with 0.5–25 mM AT for 18 h. Data in (A), (B), and (C) are the means of three experiments, each performed in duplicate. Similar results were obtained using normal resident cells.

TABLE I

*Susceptibility of Macrophages to H_2O_2**

Cells + additions		Percent cells viable‡		
		3 h	24 h	
		Serum	Serum	Serum-free§
Resident:	+ AT or azide alone	98 ± 1	97 ± 2	95 ± 2
	+ GO alone	27 ± 6	90 ± 4	55 ± 8
	+ AT + GO	23 ± 3	56 ± 4	31 ± 6
	+ azide + GO	21 ± 2	41 ± 7	—
THIO:	+ AT or azide alone	96 ± 3	95 ± 4	97 ± 3
	+ GO alone	62 ± 4	94 ± 2	75 ± 7
	+ AT + GO	59 ± 5	72 ± 4	60 ± 3
	+ azide + GO	56 ± 3	79 ± 2	—

* Resident and thioglycollate (THIO)-elicited macrophages from normal mice were cultivated in D_{20}HIFBS or serum-free Neuman-Tytell medium for 3 or 24 h before treatment with 25 mM AT for 60 min or 1 mM azide for 10 min. After washing, cells were incubated for 1 h at 37°C in KRPG with or without glucose oxidase (GO), which generated 30 nmol H_2O_2/min. Cover slips were then overlayed with 0.1% trypan blue dye and fixed. Including catalase (200 μg/ml) in the GO reaction, medium ablated cytotoxicity.

‡ As judged by trypan blue exclusion. Results are the mean ± SE for three to four experiments, each in duplicate.

§ Overnight cultivation in serum-free medium did not affect macrophage catalase or GP activity.

abundant catalase ($4.8 \pm 0.4 \times 10^{-2}$ BU/mg protein), and are resistant to a 1 h exposure to either 10^{-3} M reagent H_2O_2 or 20 nmol H_2O_2/min generated by GO (1). Parasite catalase was inhibited by AT in a time- and dose-related fashion (data not shown), and after exposure to 50 mM AT for 60 min at 37°C, catalase activity was reduced by 80 ± 6%. After such treatment, toxoplasma susceptibility to H_2O_2 was enhanced (Fig. 4 A) as judged by trypan blue dye exclusion, acridine orange fluores-

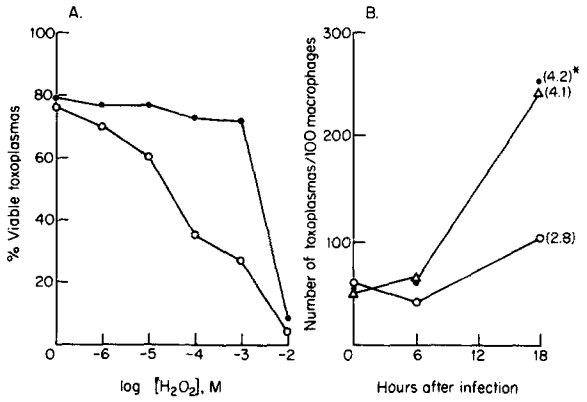

FIG. 4. Enhanced susceptibility of AT-treated toxoplasmas to exogenous H_2O_2. (A) 5×10^6 washed toxoplasmas were first incubated for 1 h at 37°C in 1 ml of PBS alone (●) or PBS plus 50 mM AT (○), and reagent H_2O_2 was then added in the indicated final concentrations for one additional hour at 37°C. Toxoplasmas viability was determined by acridine orange staining characteristics (1). In separate experiments, control parasites were resistant to a 1-h incubation with GO that generated 20 nmol H_2O_2/min (1); however, 50% of AT-treated parasites were killed. Catalase (200 μg/ml) abolished killing of treated toxoplasmas by both reagent and enzymatically generated H_2O_2. (B) Resident macrophages were infected with 50 mM AT-treated (○) or PBS-treated parasites (△), uningested toxoplasmas were removed by washing, and GO generating 6–8 nmol H_2O_2/min was added to the medium. After 1 h, the reaction medium was replaced by standard medium, and cultures were reincubated for an additional 17 h. (*) indicates the number of toxoplasmas/vacuole 18 h after infection. AT-treated parasites multiplied normally if GO was omitted (●) or if catalase (200 μg/ml) was included with GO. (A) and (B) are representative of three similar experiments.

cent staining characteristics (1), and the failure of treated parasites to survive and replicate in normal resident macrophages (1). Parasites exposed to AT alone multiplied normally, however, and showed four to five toxoplasmas/vacuole 18 h after infection. Enhanced parasite susceptibility to H_2O_2 after catalase inhibition was also confirmed in an intracellular environment by exposing normal macrophages to GO for 1 h after ingestion of AT-treated toxoplasmas. As illustrated in Fig. 4 B, a small proportion of catalase-depleted toxoplasmas were killed by 6 h, and at 18 h replication was consistently inhibited.

H_2O_2 Release by AT-treated and Genetically Catalase-deficient Macrophages. The prior evidence suggested that increasing catalase levels were associated with a decline in both the capacity of IB and IM macrophages to release H_2O_2 and to exert activity against *T. gondii*. Cultivating these cells with lymphokine produced the opposite effects, and preserved oxidative and antitoxoplasma activity (3), and blunted the rise in catalase levels. As previously noted (3), normal resident macrophages released three- to fourfold more O_2^- and H_2O_2 after 72 h of lymphokine exposure. Nonetheless, their SOD, GP, and catalase levels were high, and they failed to display antitoxoplasma activity (3). We reasoned that inhibiting intracellular catalase might increase the availability (and release) of H_2O_2 or its reaction products and, thus, enhance macrophage antitoxoplasma activity. Exposing 48- and 72-h cultures of IB, IM, and lymphokine-stimulated normal cells to 25 mM AT, however, did not increase H_2O_2 generation after PMA triggering, as judged by the scopoletin assay (data not shown). Because the H_2O_2 detected extracellularly by this method may represent only a minor portion of the total produced during the phagocytic respiratory burst (17), we

investigated how much intracellular catalase is required to depress H_2O_2 release. As previously demonstrated (1), macrophages readily pinocytize exogenous soluble catalase, and in high doses, this scavenger inhibits the antitoxoplasma activity of IB and IM macrophages (2). Exposing IB cells to 2.5 mg/ml of catalase for 3 h increased intracellular levels by 17-fold (Table II), exceeding by nine times the highest amounts detected in untreated cells after 72 h of cultivation (Fig. 3). Although this loading technique decreased extracellular H_2O_2 release (Table II), it did not ablate it, suggesting that a portion of generated H_2O_2 may not be accessible to interiorized catalase.

To further explore this aspect, we examined the capacity of peritoneal macrophages from severely acatalasemic mice (5) to generate H_2O_2. Resident, IB, and IM cells from these mice were found to contain 20- to 30-fold less catalase than similar cells from NCS mice, and enzyme activity was not influenced by 3 d of cultivation with or without added lymphokine (data not shown). H_2O_2 release by acatalasemic IB and IM cells either did not decline or declined less steeply during cultivation, and at 72 h, these macrophages released up to 2.5-fold more H_2O_2 than comparably activated NCS cells (Table III). As with the latter macrophages (3), daily exposure to 5% toxoplasma lymphokine also increased acatalasemic macrophage H_2O_2 release (data not shown). Similarly, lymphokine enhanced the oxidative capacity of resident acatalasemic cells (Table III).

Fate of Toxoplasmas within AT-treated and Acatalasemic Macrophages. We pursued the potentially important biologic role of intracellular catalase by treating 48- and 72-h NCS IB and IM cells (which are high in catalase) with AT before challenge with toxoplasmas. This intervention resulted in maintenance of consistent toxoplasmastatic activity (Fig. 5A). Treated IB cells were also more active in inhibiting parasite replication, but under all conditions still lost their specific toxoplasmacidal activity (2, 3) after 48 h in culture. AT was similarly effective in inducing lymphokine-treated resident NCS cells to display toxoplasmastatic activity after inhibition of either parasite catalase, macrophage catalase, or both (Table IV). Continuous suppression of macrophage catalase activity by adding 5 mM AT to the culture medium after

TABLE II

*Uptake of Exogenous Catalase and Effect on Macrophage H_2O_2 Release**

Duration of exposure to catalase	Catalase activity (BU/mg × 10^{-2})‡	H_2O_2 release (% of control)§
0	0.42	100
10 min	—	94 ± 3
1 h	3.9	92 ± 4
2 h	6.0	—
3 h	7.4	49 ± 7

* Overnight cultures of toxoplasma IB macrophages were incubated with medium plus 2.5 mg/ml of soluble catalase for up to 3 h, thoroughly washed by previously described methods (1), and either lysed for catalase activity or triggered with PMA (100 ng/ml) for H_2O_2 release.

‡ Mean of two experiments, each performed in duplicate.

§ Mean ± SEM of three to five experiments, each in triplicate. Control H_2O_2 release was 0.80 ± 0.14 nmol/μg protein per 90 min. Exposing macrophages to 2.5 mg/ml of heated catalase for 3 h did not reduce H_2O_2 release.

TABLE III

H_2O_2 Release by Macrophages from Normal and Acatalasemic Mice

Cells‡	Mice§	H_2O_2 release during cultivation*		
		24 h	48 h	72 h
IB	Normal	0.95 ± 0.12	0.49 ± 0.09	0.22 ± 0.07
	ACAT	0.84 ± 0.10	0.64 ± 0.10	0.62 ± 0.09
IM	Normal	0.51 ± 0.12	0.31 ± 0.07	0.19 ± 0.06
	ACAT	0.41 ± 0.06	0.42 ± 0.10	0.46 ± 0.08
Resident	Normal	0.06 ± 0.03	0.13 ± 0.06	0.18 ± 0.07
	ACAT	0.14 ± 0.06	0.20 ± 0.08	0.23 ± 0.05
Resident + TLK	Normal	0.14 ± 0.05	0.34 ± 0.08	0.45 ± 0.05
	ACAT	0.24 ± 0.07	0.48 ± 0.03	0.59 ± 0.10

* Cells were cultivated for 24–72 h in medium or medium plus 5% toxoplasma lymphokine (TLK) before triggering with PMA, 100 ng/ml (3). Results (nmol $H_2O_2/\mu g$ protein per 90 min) are the mean ± SEM of three to six experiments each performed in triplicate. Comparative data for cells from normal mice are from the accompanying report (3; Figs. 2 and 5).

‡ IB and IM (toxoplasma immune-boosted and -immune macrophages) (2, 3).

§ ACAT, (acatalasemic) mice.

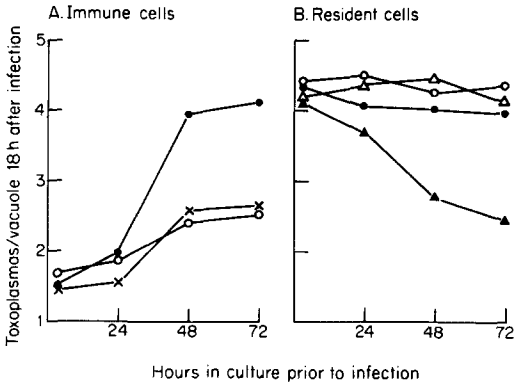

FIG. 5. (A) Enhancement of the toxoplasmastatic activity of IM macrophages from normal NCS mice by AT. Cells were cultivated for 3–72 h before infection. Untreated IM cells (●) lose activity after 48 h (2, 3); (×) same cells treated with 25 mM AT for 1 h before infection. Untreated IM macrophages from acatalasemic mice (○). AT maintained similar toxoplasmastatic activity at 48–72 h for IB cells from normal NCS mice; untreated IB cells from acatalasemic mice were similarly active. (B) Induction of toxoplasmastatic activity by lymphokine in resident macrophages from acatalasemic but not normal mice. Resident cells from normal NCS (circles) and acatalasemic mice (triangles) were cultivated for 3–72 h before infection in either medium alone (open symbols) or medium plus 5% toxoplasma lymphokine (closed symbols). Control lymphokine had no effect. Results of (A) and (B) are the means of three to four experiments each in duplicate.

infection (following an initial 25 mM pulse) did not further enhance the inhibition of toxoplasma replication for any cell type.

IB, IM, and resident macrophages from acatalasemic mice first cultivated for 3 or 24 h before infection were identical to those from NCS mice in their respective activities against *T. gondii*: IB cells were toxoplasmacidal, IM cells were toxoplasma-

TABLE IV

Effect of Inhibiting Macrophage and/or Parasite Catalase on Toxoplasma Replication

Conditions‡	Number of toxoplasmas/vacuole 18 h after infection			
	Control§	Preincubated with AT*		
		Cells	Parasites	Cells and parasites
Medium alone	4.3 ± 0.2	4.1 ± 0.1	4.2 ± 0.2	4.3 ± 0.3
Lymphokines:				
Toxoplasma (5%)	3.9 ± 0.2	2.5 ± 0.1	2.7 ± 0.2	2.2 ± 0.2
Concanavalin A (25%)	4.0 ± 0.1	2.6 ± 0.2	2.8 ± 0.1	2.3 ± 0.2

* AT was applied as described (Materials and Methods) for 1 h to either cells (25 mM), toxoplasmas (50 mM), or both. AT-treated and control macrophages were then challenged for 30 min with treated or untreated toxoplasmas. Uningested parasites were removed by washing, and cultures were reincubated in standard medium for 18 h. Results are the mean ± SEM of three to four experiments, each in duplicate.

‡ Resident macrophages from normal (NCS) mice were cultivated for 72 h before AT treatment or infection in medium alone or medium plus lymphokine in the indicated concentrations (3).

§ Control, neither cells nor parasites were exposed to AT before infection.

static, and resident cells supported parasite replication (2, 3). In contrast to in vivo-activated NCS macrophages (Fig. 3 A in the accompanying report [3]), however, acatalasemic IB and IM cells remained active against toxoplasmas even after cultivation for up to 72 h prior to infection (Fig. 5 A). In addition, although 72 h of lymphokine treatment failed to induce NCS resident macrophages to exert antitoxoplasma activity (3), similar stimulation was sufficient to induce resident acatalasemic cells to inhibit toxoplasma multiplication. This activity was apparent after as little as 24 h of lymphokine exposure, and was fully developed after 48 h (Fig. 5 B).

Discussion

The ability of macrophages to kill or inhibit the replication of intracellular *T. gondii* appears to depend upon the interaction of several key variables including (*a*) the effective triggering of the macrophage respiratory burst (18), (*b*) the magnitude of oxidative burst (2), (*c*) the generation of toxic oxygen intermediates beyond the production of O_2^- and H_2O_2 (2, 3), and, as suggested by the present studies, (*d*) the activity of competitive intracellular mechanisms that scavenge O_2^- and/or H_2O_2. These latter inhibitory systems in turn could result in diminished generation of products of O_2^--H_2O_2 interaction such as OH· (4, 19), a potent oxidant that is both bactericidal and toxoplasmacidal (1, 2, 20). For three reasons, a role for endogenous oxygen intermediate scavengers appeared plausible in macrophage-toxoplasma interaction. First, mononuclear phagocytes contain SOD, GP, and catalase (1, 6, 21–24); second, if administered before infection, SOD and catalase reverse the antitoxoplasma activity of IB and IM cells (2); and third, toxoplasmas are rich in all three enzymes and are resistant to O_2^- and H_2O_2 (1).

If unopposed, endogenously or exogenously derived O_2^- or H_2O_2 may inflict oxidant injury on a variety of targets either directly or via their reaction products (4). Indeed, phagocytosis itself may cause irreversible, oxidatively-mediated damage to polymorphonuclear and mononuclear phagocytes (25, 26). Thus, most cells and oxygen-

metabolizing microorganisms possess intracellular enzymatic mechanisms to detoxify these highly reactive agents (4). The beneficial roles of SOD, GP, and catalase in oxidant-mediated injury have recently been reemphasized in such diverse clinical settings as the cardiotoxicity of doxorubicin (27) and the pulmonary toxicity of hyperoxia (28). Although these enzymatic mechanisms are viewed as protective for most tissues and cells, our observations suggest that, under certain conditions, they may interfere with effective antimicrobial activity by phagocytes.

The spontaneous loss of IB and IM macrophage antitoxoplasma activity after 48 h of cultivation in medium alone, which correlated with declining H_2O_2 release (3), was accompanied by a progressive increase (eight- to ninefold) in intracellular catalase levels. In contrast, there was no change or a decline in IB and IM macrophage SOD and GP activities. Lymphokine-treated normal resident cells contained the highest catalase concentrations, and as noted (3), failed to display intracellular activity against toxoplasmas despite enhanced extracellular release of O_2^- and H_2O_2. Because mononuclear phagocytes appear to generate OH· (29–31), and in most systems catalase inhibits its production (30–36), our findings raise the possibility that increased intracellular catalase levels may diminish macrophage antitoxoplasma activity by enhancing H_2O_2 decomposition and perhaps by ultimately inhibiting OH· formation. Several lines of evidence, derived from experiments using cells in which catalase was either genetically deficient or chemically inactivated, supported this view. Treating IB and IM cells cultivated for 48–72 h in medium alone with AT before infection maintained definite toxoplasmastatic activity otherwise lost after 48 h. AT exposure also induced catalase-rich, lymphokine-treated normal cells to display antitoxoplasma activity. Moreover, in the absence of AT, IB and IM macrophages from acatalasemic mice were consistently active against *T. gondii* for up to 72 h, and showed comparatively little decline in H_2O_2 releasing capacity during 3 d in culture. Further, constant lymphokine exposure, which preserved the oxidative and antitoxoplasma activities of normocatalasemic IB and IM macrophages (3), was associated with a less pronounced increase in intracellular catalase concentrations. Finally, in the absence of heart infusion broth (3), lymphokine did induce resident cells from acatalasemic but not normal mice to inhibit toxoplasma replication. Thus, intracellular catalase appears to participate closely in macrophage-*T. gondii* interaction.

Although the enhanced capacity of AT-treated cells to inhibit toxoplasma replication was not paralleled by a detectable increase in H_2O_2 generation, the scopoletin assay detects only that portion released into the extracellular medium (13, 17). Whether scopoletin-detectable H_2O_2 is derived primarily (or only) from released O_2^- (13), from intracellularly generated H_2O_2 that traverses the cytosol and escapes (17), or by both mechanisms is not clear. However, inhibition or genetic absence of H_2O_2 degrading systems has been shown by others to result in increased phagocyte H_2O_2 production. Thus, polymorphonuclear neutrophil (PMN) generation and release of H_2O_2 is enhanced in both myeloperoxidase and glutathione synthetase-deficient cells (37, 38), and by azide or cyanide treatment that inhibit catalase and myeloperoxidase (17, 39). In alveolar macrophages, inactivation of glutathione peroxidase appears to increase intracellular H_2O_2 (22), and as we have shown, in vivo- and in vitro-activated macrophages from acatalasemic mice release more H_2O_2 than similar cells from normal mice. In addition, AT augments PMN hexose monophosphate shunt activity (40), presumably by inhibiting the degradation of H_2O_2 by catalase.

Our observations raise the question of how intracellular catalase could gain access to H_2O_2, especially if O_2^- and H_2O_2 production is initiated within plasma membrane-lined phagocytic vacuoles (41). With the exception of peroxisomes (microbodies), which have been identified in only a limited number of mammalian cells and microorganisms (42), the location of intracellular catalase has not been clearly defined. In guinea pig alveolar macrophages sonicates (6), the bulk of catalase activity is found in the 100,000 *g* supernate, suggesting free cytoplasmic enzyme. However, by cytochemical electron microscopic techniques, catalase in human PMN and rabbit monocytes appears to be primarily in cytoplasmic granules (42, 43). Although catalase may be transferred to phagosomes during phagocytosis in certain cells (44), such a large protein would not be expected to enter parasitopherous vacuoles of normal or IM macrophages that fail to fuse with pinosomes and lysosomes (45). However, exogenously administered catalase, which is readily endocytosed and apparently incorporated into macrophage secondary lysosomes (1), does reverse the antitoxoplasma activity of IB and IM cells (2). Exogenously provided SOD as well as scavengers of OH· and $^1O_2$ are also similarly effective (2). We postulated that interiorized catalase or other scavengers may quench oxygen intermediates generated from an extravacuolar source or perhaps act as extraphagosomal traps for intermediates such as H_2O_2 that traverse vacuolar membranes and enter the cytosol (2). Increasing intracellular catalase by 17-fold after a 3-h exposure to exogenous enzyme reduced IB macrophage H_2O_2 release by 50% suggesting that, at best, intracellular (lysosomal?) catalase may have access to only one-half of that portion of H_2O_2 detected in the extracellular medium. These data were, however, derived after PMA triggering, which presumably stimulates the entire plasma membrane. With the more limited stimulus of a single ingested parasite, high levels of intracellular catalase might reduce H_2O_2 generation more effectively.

Because SOD and GP activities remained constant or decreased during 72 h in culture, it appeared that these scavengers played relatively little role in the declining oxidative capacity of cultivated IB and IM cells. Although it has been suggested that GP, which is primarily cytoplasmic in location (6), is quantitatively more important than catalase in H_2O_2 catabolism (15, 22, 46), other studies have assigned a major role for catalase in cellular defense against exogenous and endogenous H_2O_2 (16, 47). In our experiments, intracellular catalase appeared to protect macrophages from the toxicity of high-dose exogenous H_2O_2.

Lymphokine-treated normal resident macrophages displayed the highest SOD, GP, and catalase activities amongst the populations examined; thus, the failure of these in vitro-activated cells to inhibit or kill toxoplasmas despite a clearly enhanced oxidative capacity may reflect the influences of all three enzymatic systems. Moreover, because the scavenging of either O_2^- or H_2O_2 inhibits OH· formation (29–36), the presence of increased levels of SOD, catalase, or GP might act alone or in concert to depress OH· (or $^1O_2$) formation (19) and abrogate macrophage antitoxoplasma activity. A role for endogenous catalase in this macrophage population was suggested by the induction of antitoxoplasma activity following AT treatment.

We also explored what role SOD, catalase, and GP, which are present within *T. gondii*, play in this parasite's resistance to O_2^- and H_2O_2. A fair correlation between virulence and catalase activity, but not SOD levels, has been demonstrated for other microorganisms, including *Pasturella* and *Brucella* species, *Staphylococcus aureus*, and

Mycobacterium tuberculosis (48–50). *T. gondii* also contains SOD and is resistant to O_2^- (1). Amongst intracellular protozoa, trypanosomes contain little or no catalase (51, 52) and are susceptible to H_2O_2 (48). The related hemoflagellates, *Leishmania*, are similarly susceptible to H_2O_2 and contain low levels of catalase and GP (H. Murray. Unpublished observations.). Although the extracellular protozoan *Entameba histolytica* has some GP activity, it is virtually acatalatic and readily killed by exogenous H_2O_2 (H. Murray and S. Aley. Unpublished observations.). In contrast, toxoplasmas are richly endowed with catalase and GP, and are entirely resistant to fluxes of H_2O_2 that kill 100% of *Trypanosoma cruzi* (48), *L. donovani*, and *E. histolytica* (H. Murray. Unpublished observations.). A protective role for toxoplasma catalase was indicated by the AT-induced enhancement of parasite killing by exogenous H_2O_2. Under the conditions employed, AT did not affect GP activity, nor was it toxic to the parasites which multiplied normally in resident macrophages. However, oxidatively active, lymphokine-stimulated normal cells were consistently able to inhibit the replication of catalase-depleted toxoplasmas.

Although our studies suggest a particularly influential role for catalase within both target (*T. gondii*) and effector cells (activated macrophages), it is likely that the activity of this enzyme alone is only one of several important determinants of phagocyte-parasite interaction. Simultaneous modification of macrophage or toxoplasma SOD and/or GP might produce more striking results. It should also be pointed out that mononuclear phagocytes may possess oxygen-independent antitoxoplasma mechanisms as well (18).

Summary

The activities of the endogenous O_2^- and H_2O_2 scavenging enzymes, superoxide dismutase (SOD), glutathionine peroxidase (GP), and catalase, were measured in lysates of the intracellular parasite, *Toxoplasma gondii*, and in various macrophage populations. During 72 h of cultivation in standard medium alone, the catalase activity of in vivo-activated toxoplasma-immune macrophages (IM) and immune-boosted macrophages (IB) progressively increased by eight- to ninefold, and correlated with the previously observed parallel decline in these cells' antitoxoplasma activity and capacity to release H_2O_2. SOD and GP activities either remained constant or decreased during this 3-d period. Lymphokine exposure, which preserved the antitoxoplasma activity and oxidative capacity of 48- and 72-h cultures of IB and IM cells, blunted the rise in catalase levels and had no effect on SOD or GP. Inhibition of IB and IM macrophage catalase by aminotriazole maintained toxoplasmastatic activity otherwise lost after 48 h of cultivation. In addition, IB and IM cells from acatalasemic mice contained 20- to 30-fold less catalase, and showed comparatively little decline in either H_2O_2 release or antitoxoplasma activity during 72 h in culture.

In vitro-(lymphokine) activated resident macrophages from normal mice had the highest levels of SOD, GP, and catalase, and these cells failed to kill or inhibit *T. gondii* despite enhanced extracellular release of O_2^- and H_2O_2. Toxoplasmas were also found to contain all three enzymatic scavengers. Aminotriazole inhibition of lymphokine-activated cells' catalase or of toxoplasma catalase was effective in inducing these macrophages to display antitoxoplasma activity. Moreover, and in contrast to normocatalasemic resident cells, those from acatalesemic mice were readily induced by lymphokine to inhibit the replication of untreated virulent toxoplasmas.

These results suggest that endogenous O_2^- and H_2O_2 scavenging enzymes, which function within both *T. gondii* and activated macrophages as host cell antioxidant protective mechanisms, may reduce the effectiveness of phagocyte antimicrobial activity. Thus, the presence of SOD, GP, and especially catalase within both target and effector cell may be important determinants of macrophage oxygen-dependent processes.

We thank Ms. Margaret Kielian for advice with the acridine orange fluorescence studies and Ms. Judy Adams for photographic assistance.

Received for publication 9 July 1980.

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