MACROPHAGE OXYGEN-DEPENDENT ANTIMICROBIAL ACTIVITY

III. Enhanced Oxidative Metabolism as an Expression of Macrophage Activation*

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In previous reports (1, 2) we demonstrated that *Toxoplasma gondii*, an obligate intracellular parasite, is susceptible to oxygen intermediates generated by the partial reduction of molecular oxygen. In a cell-free model, toxoplasmas are resistant to superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), but are readily killed by toxic products of O_2^--H_2O_2 interaction, presumably hydroxyl radical (OH·) and singlet oxygen ($^1O_2$) (1). We also implicated these latter two oxygen intermediates as probable mediators of the toxoplasmastatic and toxoplasmacidal activity of specifically immune peritoneal macrophages (2).

The present study extends these observations by examining in parallel the antitoxoplasma activity and oxidative capacity of peritoneal cells stimulated in vivo by inflammatory and immunologic agents, and in vitro by soluble products of sensitized lymphocytes (lymphokines). We have, thus, been able to characterize a spectrum of activated macrophages that differ quantitatively in their production of O_2^- and H_2O_2, and their capacity to carry out an intracellular anti-microbial act. Our observations also reemphasize the importance of macrophage oxygen-dependent antimicrobial mechanisms in both the in vivo and in vitro activated state.

Materials and Methods

Parasites. The virulent RH strain and the nonvirulent Pe strain of *Toxoplasma gondii* were maintained and harvested as previously reported (1). RH strain toxoplasma trophozoites were used to infect cultivated macrophages, whereas Pe strain brain cysts were used to produce chronically infected toxoplasma immune mice (2).

Macrophages. Normal macrophages were obtained from female NCS mice (The Rockefeller University, New York). Toxoplasma-immune macrophages (IM)[1] and immune-boosted macrophages (IB) were from NCS mice infected 3–8 wk before the Pe brain cysts. Immune-boosted mice received 5 × 10^6 heat-killed RH toxoplasma trophozoites intraperitoneally 3 d before harvest (2). Other NCS mice were injected intravenously or intraperitoneally with 2 × 10^7

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[1] *Abbreviations used in this paper:* BCG, Bacille Calmette-Guérin, Con A, concanavalin A; DABCO, diazabicyclooctane; D_{20}HIFBS, Dulbecco's medium containing 20% heat-inactivated FBS, penicillin, and streptomycin; FBS, fetal bovine serum; HIB, heart infusion broth; IB, toxoplasma immune-boosted; IM, toxoplasma-immune; NBT, nitroblue tetrazolium; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; PP, proteose peptone; SOD, superoxide dismutase; THIO, thioglycollate.

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viable Pasteur type Bacille Calmette-Guérin (BCG) (Trudeau Institute, Saranac Lake, N. Y.) or 0.2 ml (1.4 mg) of formalin-killed *Corynebacterium parvum* (Coparvax; The Wellcome Research Laboratories, Kent, England). Macrophages were harvested 1–3 wk later from intraperitoneal BCG-infected mice (3), 3–4 wk later from intravenous BCG-infected mice, and after 1–3 wk for *C. parvum*-immunized mice. In addition, some animals were boosted intraperitoneally with respective antigen (2×10^7 autoclaved BCG or 1.4 mg of *C. parvum*) 3 d before harvest. Macrophages were also obtained from NCS mice 4 d after intraperitoneal injection of 1 ml of phosphate-buffered saline (PBS) containing 1% proteose peptone (PP), 4% Brewer's thioglycollate (THIO), or 1% heart infusion (HIB) broths, all from Difco Laboratories, Detroit, Mich. Additional macrophages (provided by Dr. V. Freedman, The Rockefeller University) were from C57BL/6J mice injected intraperitoneally 3–4 wk before with 2×10^7 heat-killed BCG, and boosted intraperitoneally with dead BCG 4 d before harvest.

Macrophage Cultivation. Peritoneal cells were harvested by the method of Cohn and Benson (4). 4–5 $\times 10^5$ cells, suspended in Dulbecco's modified Eagle's medium containing 20% heat-inactivated fetal bovine serum (FBS) (HIFBS), 100 U/ml penicillin and 100 μg/ml streptomycin (D_{20}HIFBS), were added directly to 12 mm-round glass cover slips. Coverslips were placed in 35-mm plastic tissue culture dishes (Nunclon, Roskilde, Denmark) for toxoplasma infection experiments, or in 16-mm wells of tissue culture trays (Costar, Data Packaging, Cambridge, Mass.) for H_2O_2 and O_2^- assays. After 60 min at 37°C in 5% CO_2, nonadherent cells were removed by washing and cultures were reincubated for up to 72 h before infection or assay in D_{20}HIFBS alone or in D_{20}HIFBS plus spleen cell supernates. Fresh media were added daily. After 24 h in culture, polymorphonuclear leukocytes accounted for <1% of adherent cells for all macrophage populations.

Preparation of Mitogen- and Antigen-Stimulated Spleen Cell Supernates (Lymphokines). 10^8 cells (1.7×10^7/ml) from spleens of (*a*) toxoplasma immune mice, (*b*) mice infected intravenously with BCG, or (*c*) normal mice were incubated with (*a*) 300 μg (50 μg/ml) of toxoplasma frozen-thawed antigen (2), (*b*) 1×10^7 (1.7×10^6/ml) autoclaved BCG, or (*c*) 3 μg/ml of concanavalin A (Con A) (Miles Laboratories Inc., Elkhart, Ind.), respectively, for 48 h at 37°C in 5% CO_2. In addition, each 6-ml culture contained Dulbecco's medium with 2% FBS, penicillin, and streptomycin. Control supernates consisted of cultures of spleen cells from normal mice incubated for 48 h with (*a*) toxoplasma antigen, (*b*) autoclaved BCG, or (*c*) alone, with Con A added at the end of the cultivation period. Collected supernates were centrifuged at 500 g for 20 min, sterilized by filtration, and stored at −70°C. Just before use, supernates were thawed and diluted in D_{20}HIFBS as follows: toxoplasma 1:20 (5%), BCG 1:8 (12.5%), and Con A 1:4 (25%) (5).

Macrophage Antitoxoplasma Activity. 1 ml of D_{20}HIFBS with 1–2 $\times 10^6$ viable toxoplasmas was added for 30 min to dishes containing cover slips, followed by washing and reincubation in fresh medium. At various intervals, duplicate cover slips were fixed, stained, and counted microscopically as described (2). Killing of intracellular toxoplasmas (microbicidal activity) was indicated by a decrease in both the percent of cells infected and in the number of toxoplasmas/100 macrophages, whereas failure of parasites to replicate as judged by the number of toxoplasmas/vacuole 18 h after infection indicated microbistatic activity. Resident macrophages from normal mice fail to kill or inhibit the multiplication of viable *T. gondii*, and 18 h after infection there are four to five toxoplasmas/vacuole. In contrast, IM macrophages inhibit parasite replication for 24–30 h, and IB cells display potent toxoplasmacidal activity (2). The latter macrophages also appear strikingly activated morphologically with circumferential spreading, pronounced plasma membrane ruffling, and increased numbers of lysosomes and vesicles (2).

Glucose-Free Medium, Oxygen Intermediate Scavengers, and Other Reagents. Glucose-free medium and scavengers including superoxide dismutase (SOD), catalase, mannitol, benzoate, diazabicyclooctane (DABCO), and histidine were obtained, prepared, and administered to macrophages as previously reported (2). Glucose oxidase (type V) and xanthine oxidase (milk, 65 mg/ml) were from Sigma Chemical Co., St. Louis, Mo. Xanthine was prepared in 0.05 M potassium phosphate buffer (pH 7.8) and EDTA (10^{-4} M) at 10^{-3} M.

Assays for H_2O_2 and O_2^- Release. After various periods of cultivation, cover slips in Costar tray wells were thoroughly washed, and 1.5 ml of Krebs-Ringer phosphate buffer with 5.5 mM glucose, pH 7.4, was added to each well for 90 min at 37°C (water bath). For H_2O_2 release, the 1.5-ml reaction mixture contained scopoletin (Sigma Chemical Co.), 10 nmol/ml, horseradish peroxidase (Sigma Chemical Co., 0.44 purpurogallin U/ml, and phorbol myristate acetate (PMA) (Consolidated Midland Corp., Brewster, N. Y.), 100 ng/ml (2, 3). For O_2^- release, each 1.5 ml contained 80 μM ferricytochrome *c* (Sigma Chemical Co., type VI) and 100 ng/ml PMA (6). For H_2O_2, the oxidation of scopoletin by H_2O_2 catalyzed by horseradish peroxidase was assayed fluorometrically (3). For O_2^-, the aspirates were cleared by centrifugation and the concentration of reduced cytochrome *c* was determined spectrophotometrically using the extinction coefficient $\Delta E_{550\ \mathrm{nm}} = 2.1 \times 10^4\ M^{-1}\ cm^{-1}$ (6). In the absence of PMA, there was no detectable release of H_2O_2 or O_2^-. The addition of 25 μg/ml of SOD to the O_2^- assay reaction mixture abolished cytochrome *c* reduction for all cell types tested. Cell protein was determined by the method of Lowry et al. (7) after treatment of cover slips with 0.5 ml of 0.5 N NaOH. Results are expressed as nanamoles of H_2O_2 or O_2^- released/90 min per microgram of adherent cell protein.

Qualitative Nitroblue Tetrazolium (NBT) Reduction. Cultivated macrophages were exposed for 30 min at 37°C in 5% CO_2 to either 2×10^6 toxoplasmas or 2×10^6 opsonized zymosan particles suspended in 1 ml of D_{20}HIFBS containing 0.5 mg/ml of NBT (Sigma Chemical Co., grade III). Uningested parasites or particles were removed by washing, and cover slips were reincubated in medium alone for an additional 30 min. Intracellular toxoplasmas and zymosan were identified using phase contrast optics and then viewed by bright field microscopy. Macrophages were scored as positive if ingested parasites or particles were stained blue-black by precipitated formazan, the oxygen-dependent reduction product of NBT (8, 9).

Results

In Vivo-Activated Macrophages

OXIDATIVE CAPACITY AND ANTITOXOPLASMA ACTIVITY. The capacity to generate increased levels of oxidative metabolites appears to underlie the enhanced antitoxoplasma activity of IM and IB macrophages (2). We explored the general applicability of this biochemical-biologic relationship by examining 12 additional macrophage populations activated in vivo by stimuli unrelated to *T. gondii*. As judged by extracellular release of H_2O_2 (Fig. 1), there was a close correlation ($r = -0.9$) between

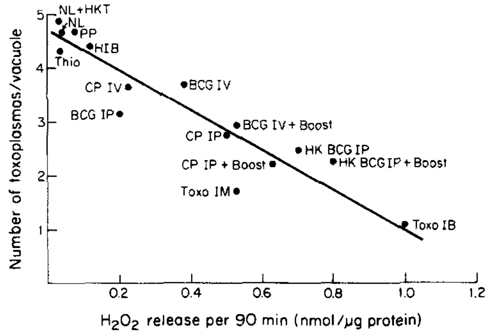

FIG. 1. H_2O_2 release vs. toxoplasmastatic activity of resident normal (NL), elicited (THIO, PP, HIB), and immunologically activated (BCG, *C. parvum* [CP], *T. gondii* [Toxo]) macrophages. See Materials and Methods for injection procedures. To minimize the contribution of any contaminating PMN to H_2O_2 release, cells were cultivated for 24 h before either triggering with PMA or challenge with parasites. Verticle axis indicates the mean number of toxoplasmas/vacuole 18 h after infection. After 24 h in culture, only IB were toxoplasmacidal (2). Additional abbreviations: NL + HKT (cells from normal mice injected intraperitoneally [IP] 3 d before with 5×10^6 heat-killed [HK] toxoplasmas); BOOST (IP injection of respective antigen 3 d before harvest); and HK BCG (cells from C57BL/6J mice as noted).

macrophage oxidative capacity and the ability to inhibit replication of intracellular toxoplasmas. Macrophages elicited by inflammatory agents (THIO, PP, HIB) were similar to normal resident cells both in terms of low H_2O_2 release and the failure to display antitoxoplasma activity. In contrast, most macrophage populations activated in vivo by variously delivered immunologic microbial stimuli (viable and dead BCG; killed *C. parvum*; viable *T. gondii*) demonstrated both enhanced H_2O_2 release and toxoplasmastatic activity. As illustrated in Fig. 1, immunologic specificity was not required for successful activation of macrophages to inhibit toxoplasma multiplication. Intraperitoneal boosting with respective microbial antigen before harvest heightened both oxidative and antimicrobial activity, and as previously reported for toxoplasma-immune mice (2), converted cells from microbistatic to strongly microbicidal. Intraperitoneal boosting of mice previously injected intraperitoneally (but not intravenously) with *C. parvum* or dead BCG also resulted in toxoplasmacidal activity if cells were infected shortly after explanting. At this time, however, up to 20% of adherent cells from these mice were polymorphonuclear leukocytes, making interpretation of H_2O_2 release difficult. If first cultivated for 24 h before infection, these boosted *C. parvum* and BCG macrophages displayed only toxoplasmastatic activity (Fig. 1). Macrophages from nonimmune mice injected intraperitoneally with a boosting dose of dead toxoplasmas 3 d before harvest displayed neither enhanced oxidative capacity nor antitoxoplasma activity.

PRESERVATION OF MORPHOLOGIC, BIOCHEMICAL, AND ANTIMICROBIAL PROPERTIES OF THE ACTIVATED STATE BY LYMPHOKINE. If cultivated in standard medium alone, the toxoplasmastatic and toxoplasmacidal activity of IM and IB macrophages, respectively, persists for 48 h and then is abruptly lost (2). This loss of activity was closely paralleled by a steep decline in oxidative capacity such that by 72 h these cells released no more H_2O_2 than resident cells from normal mice (Fig. 2). By this time, IM and IB macrophages no longer appeared activated morphologically, and closely resembled normal cells (2). Increased O_2^- release after PMA triggering also declined during 72 h of cultivation (data not shown). In contrast, daily exposure to as little as 5% fresh toxoplasma lymphokine maintained the enhanced capacity of IM and IB cells to release H_2O_2 (Fig. 2) and preserved their in vitro antitoxoplasma activity (Fig. 3 A). Control supernate failed to prevent the decline in either of these two functional markers of macrophage activation. Lymphokine-treated cells also remained activated

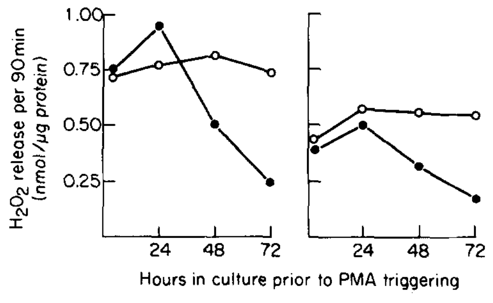

FIG. 2. H_2O_2 release by IB (left) and IM (right) macrophages. Cells were cultivated for 3-72 h before PMA triggering in medium alone (●) or medium plus 5% toxoplasma lymphokine (○). Media were changed daily. H_2O_2 release by cells exposed to 5% control toxoplasma lymphokine was similar to that of cells cultivated in standard medium. Results are the means of three to five experiments each performed in triplicate.

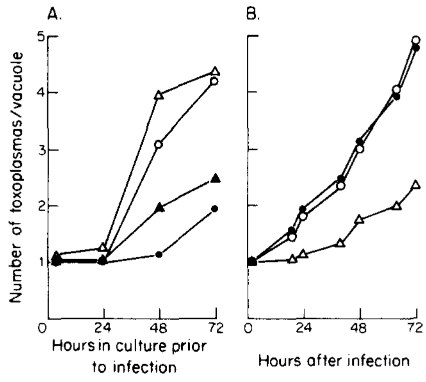

FIG. 3. A. Effect of lymphokine on IB (○) and IM (△) macrophage antitoxoplasma activity. Cells were cultivated as in Fig. 2 legend in medium alone (open symbols) or medium plus 5% toxoplasma lymphokine (closed symbols) for 3-72 h before challenge. Verticle axis indicates the number of toxoplasmas/vacuole 18 h after infection. Lymphokine-treated IB cells were toxoplasmacidal when challenged at 48 h, but at 72 h were toxoplasmastatic. Control supernate did not prevent the loss of IB and IM macrophage antitoxoplasma activity after 48 h. B. Freshly-explanted 3 h cultures of IM cells were infected and then cultivated in standard medium (●), medium plus 5% active toxoplasma lymphokine (△), or 5% control lymphokine (○). Fresh media were added daily. Results for A and B are the means of three experiments each performed in duplicate.

morphologically, showing circumferential spreading and plasma membrane ruffling. Daily addition of toxoplasma lymphokine after infection of freshly explanted 3-h cultures inhibited the multiplication of the few surviving parasites within IB cells for up to 72 h (2), and partially reversed the loss of IM macrophage toxoplasmastatic activity (Fig. 3 B).

In Vitro-Activated Macrophages

EFFECT OF LYMPHOKINE ON OXIDATIVE CAPACITY AND ANTITOXOPLASMA ACTIVITY OF NORMAL AND ELICITED MACROPHAGES. In vitro exposure to lymphokines has been reported to induce normal human monocytes and chemically-elicited (but not resident) mouse peritoneal macrophages to inhibit toxoplasma replication (10-13). Lymphocyte products can also activate resident or elicited macrophages to kill other intracellular pathogens such as *Leishmania enrietti* (14) and *Trypanosoma cruzi* (5). Although exposure to three separate lymphokines (toxoplasma, BCG, Con A) was sufficient to morphologically activate resident and PP-elicited cells (Fig. 4) and to augment their H_2O_2 release (Fig. 5 A), these cells failed to restrict toxoplasma multiplication. 18 h after infection of macrophages first preincubated with lymphokines for 24-72 h, there were four to five toxoplasmas/vacuole. Further addition of supernates after infection also failed to induce any inhibitory activity.

Because products of O_2^--H_2O_2 interaction (e.g., $OH\cdot$ and $^1O_2$ [15-16]) appear to be key toxoplasmacidal oxygen intermediates (2), we next investigated O_2^- release by in vitro-activated macrophages. A defect in O_2^- production was not anticipated because most or all H_2O_2 arises from the dismutation of O_2^- (17). As illustrated in Fig. 5, lymphokine-treated normal and PP-elicited cells released comparable amounts of H_2O_2 and O_2^-, and threefold more O_2^- than controls after 72 h.

The possibility that ingested toxoplasmas fail to trigger the oxidative burst of in vitro activated macrophages, and thus are able to replicate by evading injurious oxygen metabolites (8), was explored using the qualitative NBT test (8, 18). Normal

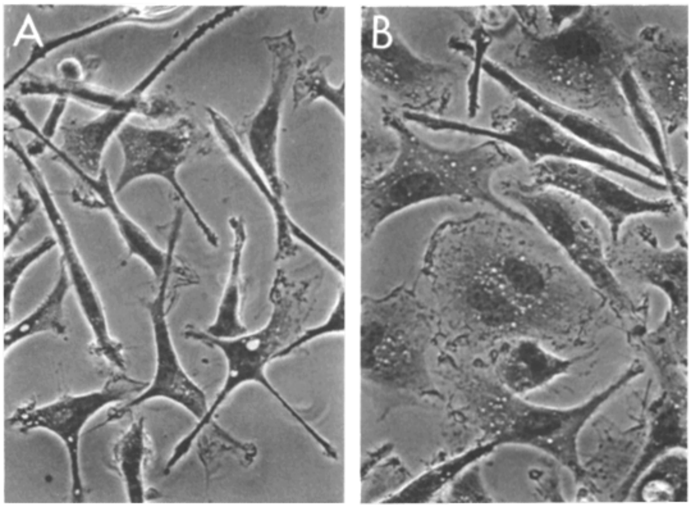

FIG. 4. Phase contrast micrographs of resident macrophages from normal mice cultivated for 72 h in standard medium alone (A) or medium plus 5% toxoplasma lymphokine (B). Media were changed daily. Appearance of resident and PP-elicited cells after 72 h of exposure to active Con A (25%) or BCG (12.5%) supernates was similar to that shown at right. Control supernates failed to morphologically activated normal or PP cells. × 800.

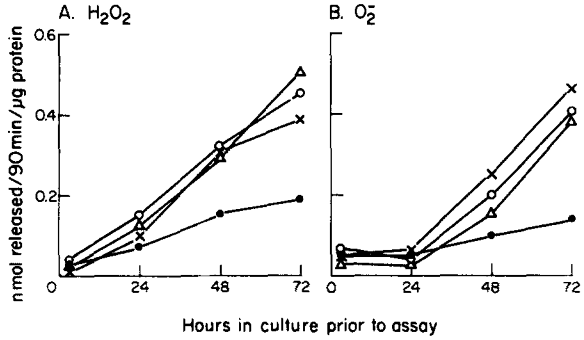

FIG. 5. Enhancement of normal macrophage H_2O_2 (A) and O_2^- (B) release by lymphokines. Resident cells were cultivated for 3-72 h prior to PMA triggering in medium alone (●) or medium plus 5% toxoplasma (x), 12.5% BCG (○), or 25% Con A (△) supernates. Values for lymphokine-stimulated PP-elicited cells were similar to those shown in A and B. Control supernates did not increase H_2O_2 or O_2^- release above that of cells cultivated in standard medium alone. Results are the means of three experiments performed in triplicate.

cells cultivated in standard medium alone or control supernates responded appropriately to zymosan particles, but failed to reduce NBT after phagocytosis of viable toxoplasmas (8) (Table I and Fig. 6A). Lymphokine-treated cells, however, readily reduced NBT in response to parasite ingestion (Table I and Fig. 6B), indicating no apparent defect in recognition of toxoplasmas.

ROLE OF OXYGEN INTERMEDIATES IN LYMPHOKINE HIB-INDUCED ANTITOXOPLASMA ACTIVITY. In contrast to the preceding lymphokine experiments, cocultivation with HIB and toxoplasma lymphokine for 18 h before infection renders normal macrophages as effective as IM cells in inhibiting parasite multiplication (13). HIB alone has no effect. Although NBT reduction in response to toxoplasma ingestion was

TABLE I

Qualitative NBT Reduction by Normal Macrophages

Treatment‡	Percent of cells with precipitated formazan 1 h after ingestion of*	
	Toxoplasmas	Zymosan
Medium alone	16 ± 9	85 ± 6
Lymphokines:		
Toxoplasma (5%)	68 ± 7	93 ± 2
BCG (12.5%)	70 ± 10	86 ± 8
Con A (25%)	64 ± 6	82 ± 9

* Percent of macrophages with intracellular toxoplasmas or zymosan stained blue-black by formazan precipitation as in Fig. 6. Results are the means ± SEM of three to four experiments, each in duplicate. Control supernates failed to enhance NBT reduction in response to toxoplasma ingestion. Results for PP-elicited cells were similar to those shown in this table. The inclusion of SOD (1 mg/ml) with the parasites or particles decreased the percent of cells reducing NBT by a mean of 46% in two experiments.

‡ Freshly explanted normal macrophages were cultivated in medium alone or medium plus lymphokine in the indicated concentrations for 72 h (media changed daily), and were then challenged for 30 min with either 2 × 10^6 toxoplasmas or 2 × 10^6 opsonized zymosan particles suspended in medium containing NBT, 0.5 mg/ml. Uningested parasites or particles were removed by washing, and cultures were reincubated for an additional 30 min in medium alone.

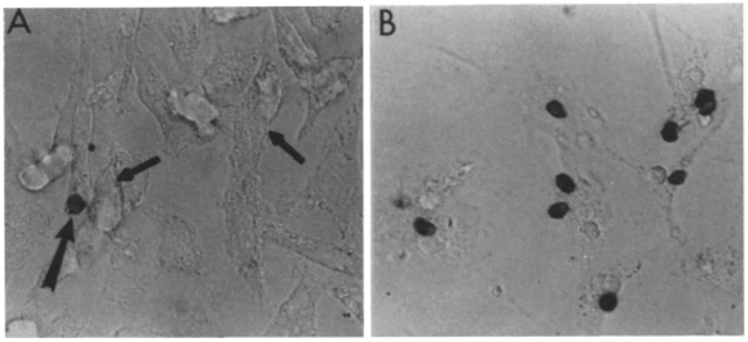

FIG. 6. Bright field micrographs demonstrating qualitative NBT reduction by normal macrophages 1 h after ingestion of toxoplasmas. Cells were cultivated for 72 h in medium alone (A) or medium plus 5% toxoplasma lymphokine (B), and then challenged with toxoplasmas suspended in 0.5 mg/ml NBT as described. Approximately one-third of the control cells in this field (A) have ingested parasites (short arrows), but only one shows a formazan-stained organism (long arrow). In contrast, most toxoplasmas ingested by lymphokine-stimulated cells (B) have provoked NBT reduction with formazan precipitation. Extracellular toxoplasmas did not reduce NBT. × 800. Also see Table I.

clearly enhanced in treated cells (Table II), there was only a 30% increase in O_2^- release and no increase in extracellular H_2O_2 release after 18–24 h of exposure to HIB-lymphokine (data not shown). However, HIB was found to effectively scavenge H_2O_2 in both a cell-free H_2O_2-generating system (Fig. 7 A) and in PMA-triggered IB macrophages (Fig. 7 B). PP and THIO also quenched H_2O_2 production. In contrast, HIB and THIO were less effective scavengers of enzymatically generated O_2^- (Fig. 7 A), and a 4-h preincubation with 10 mg/ml HIB or THIO reduced IB O_2^- release by less than 10% (data not shown).

TABLE II

Qualitative NBT Reduction by Normal Macrophages

Treatment*	Percent of cells with precipitated formazan 1 h after ingestion of:*	
	Toxoplasmas	Zymosan
Medium alone	14 ± 3	76 ± 4
HIB	15 ± 6	68 ± 9
Toxoplasma lymphokine	21 ± 6	80 ± 6
HIB + toxoplasma lymphokine	56 ± 8	78 ± 4
HIB + control toxoplasma lymphokine	16 ± 2	60 ± 8

* Cells were scored as in the legend to Table I. Results are the means ± SEM of four experiments, each performed in duplicate.

‡ Freshly explanted normal macrophages were cultivated for 18 h in medium alone, medium plus HIB (8 mg/ml), or toxoplasma lymphokine (5%), or both agents together. Cells were then challenged and processed as in Table I legend.

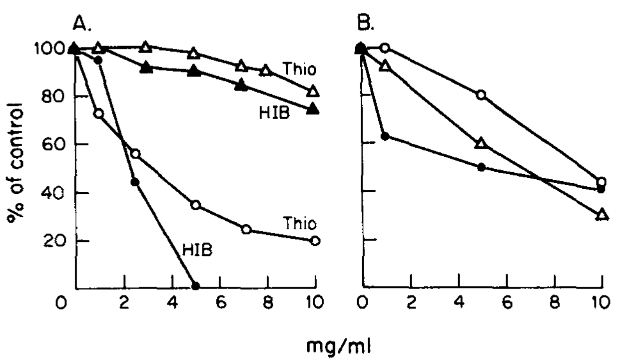

FIG. 7. Differential scavenging of H_2O_2 and O_2^- by inflammatory agents. A. THIO or HIB were added in the indicated concentrations to either a O_2^--generating (△) xanthine oxidase system (1) or an H_2O_2-generating (○) glucose oxidase system (1). O_2^- production was assayed spectrophotometrically (39), and H_2O_2 was assayed fluorometrically (3). Controls for this representative experiment were O_2^-, 1.9 nmol/min, and H_2O_2 2.4 nmol/min, at 22°C. B. 1-d cultures of toxoplasma IB macrophages were preincubated for 4 h with THIO (●), PP (△), or HIB (○) in the indicated concentrations, washed thoroughly, and then assayed for H_2O_2 release after PMA triggering. Results are the means of three experiments performed in triplicate. Control = 0.81 ± 0.11 nmol H_2O_2/90 min per microgram of protein. In two additional experiments, overnight preincubation with 8 mg/ml (0.8%) of THIO or HIB reduced IB macrophage H_2O_2 release to <10% of control values.

Data derived from exposing HIB-toxoplasma lymphokine-activated cells to a battery of oxygen intermediate scavengers, or derived from depriving them of exogenous glucose, are illustrated in Table III. The latter procedure reduces macrophage H_2O_2 release (19). Both SOD (for O_2^-) and catalase (for H_2O_2) effectively reversed inhibition of toxoplasma multiplication (Fig. 8), suggesting a role for products of their interaction such as OH· and $^1O_2$ (15, 16, 20). OH· scavengers (mannitol, benzoate) and $^1O_2$ quenchers (DABCO, histidine) (20) were also effective in inhibiting toxoplasmastatic activity. Thus, similar to our previous observations with in vivo activated macrophages (2), these findings indicate a role for oxidative metabolites in the antimicrobial activity induced by lymphokine.

TABLE III

Effect of Oxygen Intermediate Scavengers and Glucose Deprivation

	Number of toxoplasmas/vacuole 24 h after infection
None (control)	1.5 ± 0.2 (6)
Catalase 2 mg/ml	4.4 ± 0.8 (4)
SOD 2 mg/ml	2.9 ± 0.4 (4)
Mannitol 50 mM	3.2 ± 0.5 (3)
Benzoate 10 mM	2.7 ± 0.6 (3)
DABCO 1 mM	3.1 ± 0.2 (3)
Histidine 10 mM	3.4 ± 0.3 (3)
No glucose	3.5 ± 0.8 (3)

Freshly explanted normal macrophages were first cultivated for 18 h with HIB (8 mg/ml) and toxoplasma lymphokine (5%), and were then exposed to glucose-free medium or scavengers in the indicated concentrations 3 h before, during, and 24 h after infection (2). 5 mM glucose was added back to glucose-deprived cells 2 h after infection (2). Heated catalase and SOD did not reverse parasite inhibition (2). Results are the means ± SEM of (*n*) experiments each performed in duplicate.

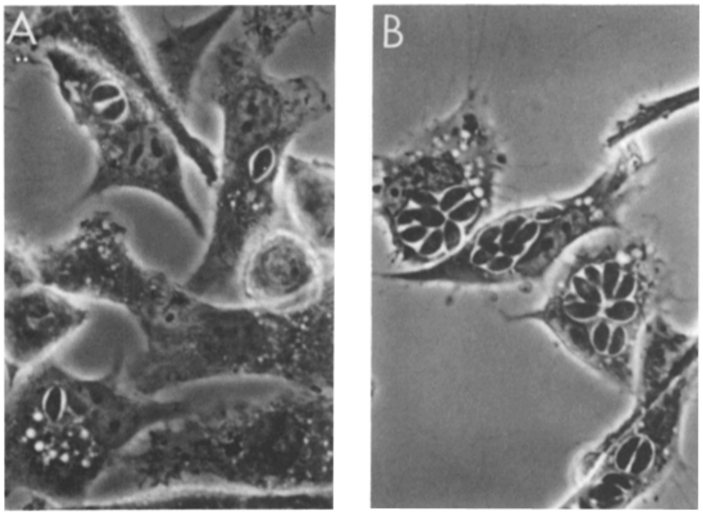

FIG. 8. Phase contrast micrographs of macrophages 24 h after infection. A. Inhibition of toxoplasma replication by normal macrophages cultivated 18 h before infection with HIB (8 mg/ml) and 5% toxoplasma lymphokine. B. Same macrophages after exposure to exogenous catalase (2 mg/ml) as described in Table III legend. × 1,200.

Discussion

By quantitatively characterizing the oxidative capacity of a wide spectrum of in vivo- and in vitro-stimulated macrophage populations, we have provided firm evidence that the enhanced capacity to generate oxygen intermediates is a consistent marker that distinguishes activated macrophages from their unstimulated or inflammatory counterparts. Moreover, the close relationship between H_2O_2 release and the ability to act against *T. gondii*, an intracellular organism susceptible to products of O_2^--H_2O_2 interaction (1, 2), illustrates a clear biologic correlation for augmented oxidative metabolism in the antimicrobial activity of in vivo immunologically activated macrophages. This latter finding, however, does not necessarily apply to cells activated in vitro by lymphokine (see below).

Although macrophages elicited by inflammatory agents, such as THIO, PP, or starch, spread quickly on glass, phagocytize avidly, and demonstrate certain increased metabolic and secretory activities (21), they, like normal cells, fail to display the key protective properties of enhanced microbicidal or tumoricidal activity (5, 22–25). Our current findings indicate that augmented oxidative metabolism, which has been implicated in both cytocidal processes (2, 8, 17, 19), is consistently not a feature of normal or inflammatory macrophages when compared with cells obtained after stimuli such as systemic BCG or toxoplasma infection or immunization with dead BCG or *C. parvum*. Thus, the ability to kill or inhibit intracellular toxoplasmas may be a useful in vitro biologic assay system by which both a heightened state of activation and an augmented oxidative capacity of in vivo-stimulated macrophages can be identified. Indeed, we have yet to encounter an in vivo activated, high H_2O_2-releasing macrophage that does not exert some antitoxoplasma activity. In addition to H_2O_2 and O_2^- release (6), mononuclear phagocyte oxidative activity has also been assessed recently by $OH\cdot$ formation (26–28). The latter procedure would be particularly appropriate for studies investigating O_2^-- and H_2O_2-resistant, but $OH\cdot$-susceptible targets such as *T. gondii* (1, 2).

After 48 h of cultivation in standard medium, IM and IB macrophages spontaneously lose their respective antitoxoplasma activities and fail to restrict parasite replication (2). Because oxygen intermediates appear to mediate this activity (2), it was important to observe that macrophage oxidative capacity appropriately declined in a parallel fashion. Nathan et al. (29) have reported similar results with *T. cruzi*-immune cells. Daily addition of fresh lymphokine, however, preserved both of these markers of IM and IB macrophage activation. Previous work has also indicated that soluble products of sensitized T lymphocytes (lymphokines) stimulate a variety of macrophage processes (30, 31), including induction or enhancement of tumoricidal or antimicrobial activity (5, 10–14, 24, 32–34) and augmented oxidative metabolism (29). In the latter study, macrophage release of H_2O_2 correlated closely with the capacity to kill intracellular trypanosomes (29). It should be pointed out, however, that although lymphokine maintained both the ability of IM and IB cells to release increased O_2^- and H_2O_2, and to inhibit parasite replication (toxoplasmastatic activity), it did not prevent the eventual loss of IB cells' toxoplasmacidal activity. This finding may reflect biologic deficiencies inherent with experimentally prepared lymphokines, loss of macrophage responsiveness to lymphokine, alteration in the generation or delivery of the key toxoplasmacidal oxygen intermediates ($OH\cdot$ and $^1O_2$) (2), or perhaps atrophy of a synergistic but lymphokine-unresponsive microbicidal mechanism.

To explore in vitro activation of macrophages, we attempted to induce resident and inflammatory (PP) cells to display antitoxoplasma activity by exposing them to mitogen- and antigen-stimulated supernates both before and after infection. In similar models, resident and elicited macrophages from normal mice can be induced to eradicate the intracellular parasites, *L. enrietti* (14) and *T. cruzi* (5). As reported by others (5, 29), we too found that lymphokine exposure morphologically activates resident and PP cells and, in parallel, augments O_2^- and H_2O_2 release. However, in contrast to the other parasite-macrophage models, lymphokine-activated cells failed to kill or inhibit the replication of intracellular toxoplasmas. This dissociation of enhanced oxidative capacity from antimicrobial activity was further emphasized by the finding that in vitro-activated cells released as much O_2^- and H_2O_2 as in vivo-activated macrophages. The latter readily inhibited parasite multiplication (Fig. 1). As judged by enhanced intracellular NBT reduction (8, 9), there was no failure on the part of lymphokine-treated cells to generate O_2^- in response to toxoplasma ingestion. This was important to investigate because *T. gondii* avoids triggering the oxidative respiratory burst of macrophages that permit toxoplasma replication (8).

The formation of OH· and $^1O_2$, both of which appear to be toxoplasmacidal (1, 2), seems to depend upon the initial interaction of O_2^- and H_2O_2 in the presence of trace metal (iron) ions (15, 16, 20). Thus, OH· generation and resultant antitoxoplasma activity would be anticipated by O_2^-- and H_2O_2-releasing in vitro-activated cells. However, as shown in the accompanying report (35), these macrophages contain high activity of endogenous O_2^- and H_2O_2 scavenging mechanisms (SOD, catalase, glutathione peroxidase) that may interfere with OH· or $^1O_2$ production within or delivery to the phagocytic vacuole. Such mechanisms might theoretically not affect the microbicidal activity of lymphokine-activated, H_2O_2-releasing cells toward an H_2O_2-susceptible parasite such as *T. cruzi* (29).

The HIB-lymphokine system, which sufficiently activates normal cells in vitro to inhibit toxoplasma replication, permitted assessment of the role of oxygen intermediates in lymphokine-mediated enhancement of antiprotozoal activity. The molecular basis underlying the synergistic action of HIB and lymphokines has not been investigated, but may be related to the presence of endotoxin (36). Thus, in this system, HIB can be replaced by endotoxin (13), as well as THIO (13) or PP, and BCG and Con A supernates can substitute for toxoplasma lymphokine (H. Murray. Unpublished observations). Although there was only a small increase in extracellular O_2^- release and no increase in H_2O_2 release after HIB-lymphokine exposure (presumably related to the broad scavenging effects of HIB), intracellular oxidative activity was clearly enhanced as judged by NBT reduction in response to toxoplasma ingestion (8). Furthermore, exogenous scavengers and quenchers of O_2^-, H_2O_2, OH·, and $^1O_2$ and glucose deprivation all inhibited the toxoplasmastatic activity of HIB-lymphokine-treated macrophages. These results, which are similar to our previous findings with IM and IB cells (2), clearly demonstrate the presence and activity of an oxygen-dependent system beyond the production of O_2^- and H_2O_2 in macrophages activated in vitro by lymphokines.

In the course of this study, we also investigated the conflicting data surrounding the oxidative capacity of THIO-elicited macrophages that, like normal cells, fail to inhibit toxoplasma replication. Johnston et al. (6) reported that, after PMA triggering, cultivated THIO macrophages release O_2^- in amounts comparable with those from BCG-infected and -boosted mice, and 10-fold more than normal resident macrophages. In contrast, others have found little O_2^- (37) or H_2O_2 (3) release from freshly harvested THIO cells in suspension. In our hands, after overnight cultivation these macrophages showed intermediate results with a twofold increase in O_2^- release (0.12 ± 0.02 nmol/μg protein), but no increase in H_2O_2 release compared with normal cells. THIO macrophages also generated fourfold less O_2^- than cells from IB mice. Interestingly, THIO was found to be a much more effective scavenger of H_2O_2 than O_2^-, which may explain in part these disparate results. Nathan and Cohn (38) have also recently shown that THIO both inhibits activated macrophages' H_2O_2 release and antitumor cell activity, an H_2O_2-dependent process. In addition, although the intracellular catalase levels of THIO, resident, and IB are comparable after 24 h of cultivation, THIO cells contain 50% less SOD and 1.5- to 2.5-fold more glutathione peroxidase (35), thus raising the possibilities of both diminished formation of H_2O_2 from O_2^- and/or enhanced H_2O_2 decomposition by the reduced glutathione pathway. The potentially important role played by the enzymatic oxygen intermediates scavengers, SOD, catalase, and glutathione peroxidase, which are present within both *T. gondii* and activated macrophages, is examined in the accompanying report.

Summary

The capacity of 15 separate populations of mouse peritoneal macrophages to generate and release H_2O_2 (an index of oxidative metabolism) was compared with their ability to inhibit the intracellular replication of virulent *Toxoplasma gondii*. Resident macrophages and those elicited by inflammatory agents readily supported toxoplasma multiplication and released 4–20× less H_2O_2 than macrophages activated in vivo by systemic infection with Bacille Calmette-Guérin or *T. gondii*, or by immunization with *Corynebacterium parvum*. Immunologically activated cells consistently displayed both enhanced H_2O_2 production and antitoxoplasma activity. Exposure to lymphokines generated from cultures of spleen cells from *T. gondii* immune mice and toxoplasma antigen preserved both the antitoxoplasma activity and the heightened H_2O_2 release of toxoplasma immune and immune-boosted macrophages, which otherwise were lost after 48–72 h of cultivation.

In vitro activation of resident and chemically-elicited cells by 72 h of exposure to mitogen- and antigen-prepared lymphokines, conditions that induce trypanocidal (5) and leishmanicidal activity (14), stimulated O_2^- and H_2O_2 release, and enhanced nitroblue tetrazolium reduction in response to toxoplasma ingestion. Such treatment, however, failed to confer any antitoxoplasma activity, indicating that intracellular pathogens may vary in their susceptibility to macrophage microbicidal mechanisms, including specific oxygen intermediates. In contrast, cocultivating normal macrophages with lymphokine plus heart infusion broth for 18 h rendered these cells toxoplasmastatic. This in vitro-acquired activity was inhibited by scavengers of O_2^-, H_2O_2, $OH\cdot$, and $^1O_2$, demonstrating a role for oxidative metabolites in lymphokine-induced enhancement of macrophage antimicrobial activity.

These findings indicate that augmented oxidative metabolism is a consistent marker of macrophage activation, and that oxygen intermediates participate in the resistance of both in vivo- and in vitro-activated macrophages toward the intracellular parasite, *T. gondii*.

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