MACROPHAGE OXYGEN-DEPENDENT
ANTIMICROBIAL ACTIVITY

II. The Role of Oxygen Intermediates*

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In the accompanying report (1), we demonstrated that *Toxoplasma gondii*, an obligate intracellular parasite, is susceptible to oxygen intermediates generated by the xanthine oxidase system. The present study examines the contribution of oxidative metabolites to macrophage killing and inhibition of growth of intracellular *T. gondii*. Our observations indicate that an oxygen-dependent antimicrobial system is present in macrophages, and suggest that toxic oxygen intermediates mediate macrophage resistance to intracellular pathogens such as *T. gondii*.

Materials and Methods

Parasites. The virulent RH strain and the nonvirulent Pe strain of *T. gondii* were obtained from Dr. Thomas C. Jones (Cornell University Medical College). Both strains were maintained in female Rockefeller NCS mice by biweekly intraperitoneal passage (RH strain) or monthly intramuscular passage of toxoplasma brain cysts (Pe strain) (2). RH strain toxoplasma trophozoites were prepared as described (1) and were used for infection of cultivated macrophages. Pe strain brain cysts were used to produce chronically infected, toxoplasma immune mice (2).

Cells. Peritoneal cells were harvested according to the methods of Cohn and Benson (3). Normal macrophages were from female NCS mice. Toxoplasma immune (IM)[1] macrophages were obtained from NCS mice infected with brain cysts intramuscularly (thigh) 3–8 wk before (2). 3 wk after infection, these asymptomatic mice are chronically infected and display hepatosplenomegaly and brain cysts (2). There were 4–6 × 10^6 total peritoneal cells per IM mouse, of which 45–55% were macrophages and 0–2% were granulocytes. A third population of peritoneal macrophages was obtained from chronically infected IM mice which were challenged intraperitoneally 3 d before harvest with heat-killed toxoplasmas (5 × 10^6 toxoplasmas in 0.2 ml of phosphate-buffered saline (PBS)). These in vivo immune-boosted (IB) mice yielded 9–10 × 10^6 total peritoneal cells per mouse, of which 50–60% were macrophages and 0–2% were granulocytes.

Peritoneal cells from normal, IM, and IB mice were cultivated on 12-mm round glass coverslips placed in 35-mm plastic tissue culture dishes or in 16-mm wells of Costar tissue culture trays (Costar, Data Packaging, Cambridge, Mass.) in Dulbecco's modified Eagle's

[1] *Abbreviations used in this paper:* AO, acridine orange; DABCO, diazabicyclooctane; D_{20}HIFBS, Dulbecco's modified Eagle's medium containing 20% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin; FBS, fetal bovine serum; GO, glucose oxidase; HBSS, Hanks' balanced salt solution; IB, immune-boosted macrophage; SOD, superoxide dismutase; IM, toxoplasma immune macrophage; PMN, polymorphonuclear neutrophil(s); XO, xanthine oxidase.

medium (Grand Island Biological Co., Grand Island, N. Y.) 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). After 30-60 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 then incubated for varying periods in D_{20}HIFBS.

Oxygen Intermediate Scavengers and Other Special Reagents. These were obtained and prepared as in the accompanying report (1). Before use, scavengers were dissolved or diluted to the desired concentration in D_{20}HIFBS and millipored (0.45 μm, Millex, Millipore Corp., Bedford Mass.).

Infection of Untreated Macrophages. Coverslips on which macrophages had been cultivated were placed in 35-mm tissue culture dishes and exposed to 1-2 × 10^8 toxoplasmas in 1 ml of D_{20}HIFBS for 30 min at 37°C in 5% CO_2. Uningested parasites were removed by washing with HBSS and fresh medium was added. Immediately after (time 0) and at various intervals after the infection period, duplicate coverslips were fixed 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.

Infection of Scavenger-treated or Glucose-deprived Macrophages. After removing nonadherent cells, coverslips on which freshly explanted macrophages had been plated were incubated with oxygen intermediate scavengers diluted in D_{20}HIFBS. After 3 h, this medium was removed and 1-2 × 10^6 toxoplasmas, suspended in 1 ml of scavenger plus D_{20}HIFBS, were added for 30 min at 37°C. Uningested parasites were removed and fresh scavenger in D_{20}HIFBS was readded. Cultures were then incubated for up to 24 h. At the indicated times, duplicate coverslips were fixed and examined. In glucose deprivation experiments, freshly explanted macrophages were plated for 30 min in D_{20}HIFBS, washed, and then incubated with glucose-free galactose (5 mM)-supplemented Eagle's minimum essential medium with Earle's salts containing 20% dialyzed heat-inactivated fetal bovine serum (FBS), penicillin, and streptomycin (4). Cells were cultivated in this medium for 3 h before, during, and 2 h after toxoplasma infection. At this time, the medium was replaced with standard glucose-containing D_{20}HIFBS to preserve the monolayer during the remainder of the 18 to 24 h incubation period.

Acridine Orange (AO) Fluorescence Microscopy. As described in the accompanying report, viable extracellular toxoplasmas consistently displayed a distinctive AO staining pattern. The fluorescent appearance of intracellular toxoplasmas was examined by first exposing uninfected cultivated macrophages to AO (5 μg/ml) in D_{20}HIFBS for 20 min at 37°C, followed by washing and reincubation in dye-free medium for 1 h. Monolayers were then infected with viable or heat-killed toxoplasmas. After 30 min, coverslips were washed, and either immediately inverted on to glass slides and examined by fluorescent microscopy (1) or were reincubated in D_{20}HIFBS for varying intervals before examination.

Preparation of Spleen Cell Products. 3 × 10^7 cells (5 × 10^6/ml) from spleens of chronically infected, toxoplasma immune mice were incubated for 48 h at 37°C in 5% CO_2 with 300 μg (50 μg/ml) of toxoplasma frozen-thawed lysate antigen (2) in a total vol of 6 ml of Dulbecco's medium containing 2% fresh FBS, 5 × 10^{-5} M mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin. Supernates were collected, centrifuged at 500 g for 20 min, and sterilized by filtration.

Assay for H_2O_2 Release. For freshly harvested peritoneal cells in suspension (3 × 10^6 total cells in a 3-ml reaction volume), the methods of Nathan and Root (5) were used to assay H_2O_2 release after triggering with 100 ng/ml phorbol myristate acetate (PMA) (Consolidated Midland Co., Brewster, N. Y.). The oxidation of 5-10 nmol of reduced scopoletin by H_2O_2, catalyzed by horseradish peroxidase, was followed fluorometrically for 2.5 h at 37°C as fully described elsewhere (5). Scopoletin and horseradish peroxidase (165 purpurogallin U/mg) were obtained from Sigma Chemical Co., St. Louis, Mo. For H_2O_2 release by scavenger-treated or glucose-deprived IB macrophages, the assay was modified as follows. After plating in D_{20}HIFBS for 30-60 min and washing, adherent cells on coverslips placed singly in Costar tray wells were incubated in D_{20}HIFBS, D_{20}HIFBS plus scavenger, or glucose-free medium. After 3 h, the coverslips were extensively washed and 1.5 ml of Krebs-Ringer phosphate buffer, pH 7.4, with (for scavenger-treated cells) or without 5.5 mM glucose (for deprived cells) (4) was added to each well. The 1.5-ml reaction mixture contained scopoletin, 5-10 nmol/ml; horseradish peroxidase, 0.44 purpurogallin U/ml; and PMA, 100 ng/ml (4, 5). After 90 min at 37°C (water
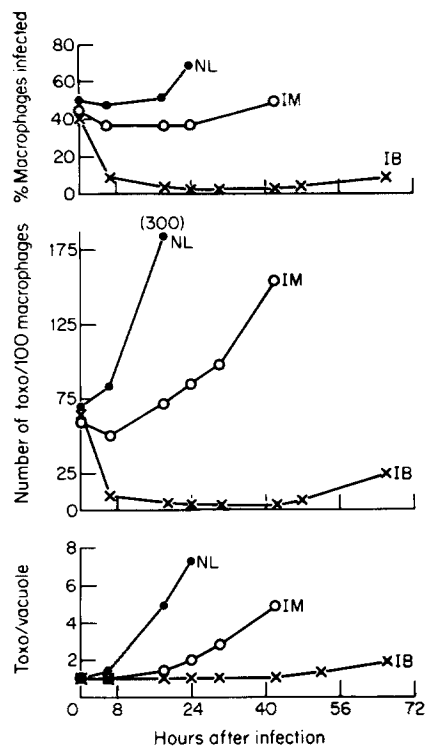
Fig. 1.   Fate of virulent toxoplasmas in freshly explanted normal (NL), IM, and IB macrophages. Killing of parasites by IB macrophages at 6 and 18 h is indicated by a decline in the percentage of macrophages infected and the number of toxoplasmas per 100 macrophages. Medium was changed daily.

bath), the mixture was aspirated and promptly assayed fluorometrically. After this cultivation period (a total of 4.5 h), the proportion of adherent cells which were granulocytes never exceeded 1%. Cell protein was determined by the Lowry method (6) after treatment of the coverslips with 0.5 ml of 0.5 N NaOH. Results are expressed in terms of nanomoles of H_2O_2 released per 90 min per microgram of adherent cell protein.

Results

Fate of Intracellular Toxoplasmas in Three Macrophage Populations.   Toxoplasmas multiply freely in resident peritoneal macrophages from normal mice after a brief lag period (Fig. 1). These cells were incapable of restricting the intracellular growth of viable toxoplasmas. 24 h after infection vacuoles house an average of 7–8 toxoplasmas (Fig. 2 A) and one may occasionally encounter vacuoles with rosettes of 16–32 toxoplasmas. Thereafter cells rupture, parasites are released, and other macrophages become infected. By 36–40 h, the monolayer is destroyed (7). In contrast, peritoneal macrophages obtained from chronically infected mice display clear microbistatic activity (Fig. 1). 18 h after infection there are 1.0–1.5 toxoplasmas per vacuole (Fig. 2 B) and only after 28–32 h does multiplication ensue. These IM cells remain only inhibitory, even if preincubated for 1–2 d with supernates of toxoplasma antigen-spleen cell cultures (lymphokines), and kill few if any of the ingested parasites. In keeping with the observations of Jones and Hirsch (2, 8), the in vitro inhibitory
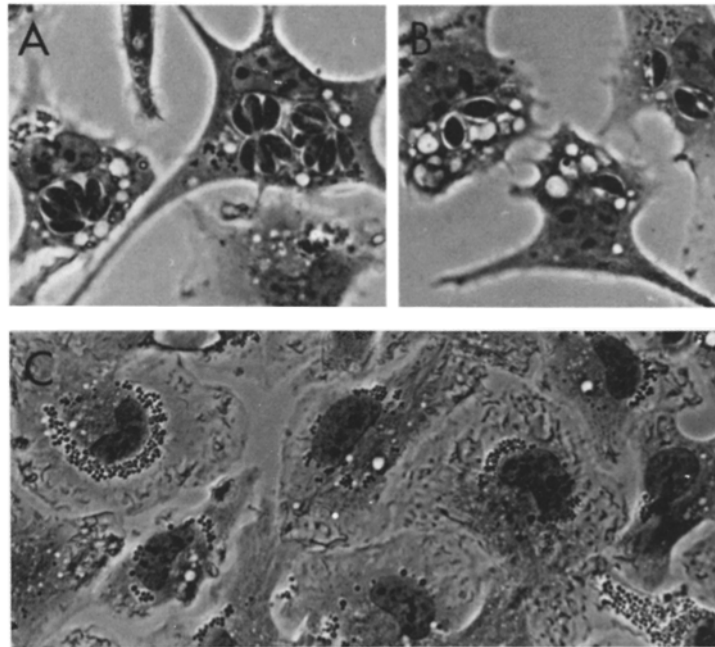
FIG. 2. (A) Normal macrophages 24 h after infection; parasites multiply freely. (B) IM macrophages 24 h after infection demonstrating inhibition of toxoplasma multiplication. (C) IB macrophages 18 h after infection, illustrating eradication of original intracellular inoculum. 18 h previously, 50% of these IB macrophages had ingested one or more toxoplasmas. Phase-contrast microscopy, Giemsa stain, X 800.

activity of IM macrophages gradually wanes as the duration between immunization and macrophage harvest increases. 8 wk after immunization the average number of toxoplasmas per vacuole at 18 h was 1.5–2.0.

Macrophages from IM mice boosted intraperitoneally with heat-killed toxoplasmas, however, appeared strikingly different and displayed microbicidal behavior (Fig. 1). These IB macrophages spread promptly on glass and exhibited plasma membrane ruffing and numerous pinocytic vesicles and lysosomes (Fig. 2 C). In contrast to IM cells, IB macrophages rapidly killed and degraded $76 \pm 2.1\%$ (mean $\pm$ SE) of ingested virulent toxoplasmas by 6 h and $90 \pm 1.4\%$ by 18 h (Fig. 1). The few parasites remaining at 18 h multiplied slowly after 40 h. If toxoplasma lymphokine was added to IB cells multiplication of the few surviving parasites was completely inhibited for up to 72 h (data not shown). In contrast to IM macrophages, there was no decline in the microbicidal capacity of IB macrophages as the time from original immunization lengthened. Neither boosting of IM mice with PBS alone nor normal mice with heat-killed toxoplasmas resulted in enhancement of macrophage anti-toxoplasma activity. By light microscopy, freshly explanted IM and IB macrophages showed no peroxidase activity as judged by examination of multiple diaminobenzi-dine-stained preparations (9).

More detailed intracellular killing assays were conducted in IB macrophages using the AO technique (1). Replicating, viable parasites in normal resident cells showed distinctly stained lysosomes embedded in a homogenous dark green cytoplasm. In
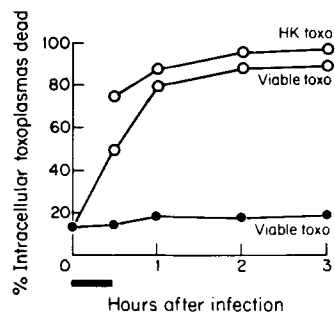
Fig. 3.   IB macrophage killing of intracellular toxoplasmas as indicated by diffuse AO cytoplasmic fluorescence and absence of lysosomal staining of parasites (1). Results are the mean of three experiments. Normal (·) or IB (O) macrophages were preincubated with AO and infected for 30 min (bar) with viable or heat-killed (HK) toxoplasmas. Coverslips were sequentially examined for parasite AO staining patterns indicating dead organisms (Fig. 4). 10–20% of the original toxoplasma inoculum was not viable by AO criteria (1).
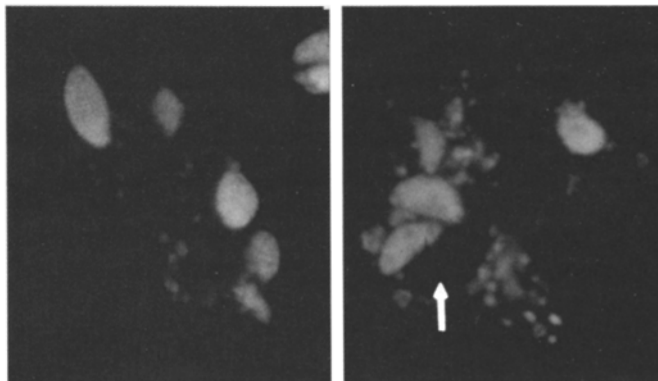

Fig. 4.   AO fluorescent staining of toxoplasmas killed within IB macrophages. (Left) Four oval-shaped, diffusely stained (orange-red) dead toxoplasmas, 1 h after ingestion. Punctate macrophage lysosomes are also stained X 2,000. (Right) Four crescent shaped, diffusely stained (dead) intracellular parasites and one unstained toxoplasma (viable) within crescent-shaped vacuole (arrow) surrounded by macrophage lysosomes X 2,000.

contrast, 60 min after uptake by IB macrophages, 80% of toxoplasmas were scored nonviable by AO criteria (1) and $\cong$ 90% were dead by 2 h (Figs. 3 and 4). Thus, IB cells were able to kill the majority of organisms by 1 h, whereas morphological disintegration as indicated by Giemsa staining required up to 6 h.

Both IM and IB macrophages maintained their respective activities for 48 h in culture (Fig. 5), but thereafter, parasites multiplied freely in both populations. By this time, most IB cells had also lost their activated appearance.

We had therefore established three clearly distinguishable macrophage populations which either (a) permitted toxoplasma multiplication (normal), (b) inhibited growth (IM), or (c) promptly killed the majority of parasites (IB). These cells were then employed to establish the role of oxygen intermediates.

H_2O_2 Release by Macrophages.   Fig. 6 demonstrates the striking differences between microbicidal (IB), microbistatic (IM), and normal macrophages in terms of the rate and magnitude of extracellular H_2O_2 release after pharmacologic triggering with
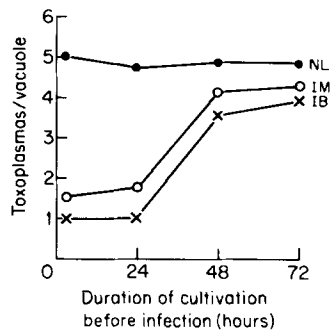
FIG. 5. Loss of macrophage in vitro anti-toxoplasma activity. Normal (NL), IM, and IB macrophages were cultivated for 3–72 h before infection. Medium was changed daily. Results of this representative experiment are expressed in terms of the average number of toxoplasma per vacuole 18 h after infection.
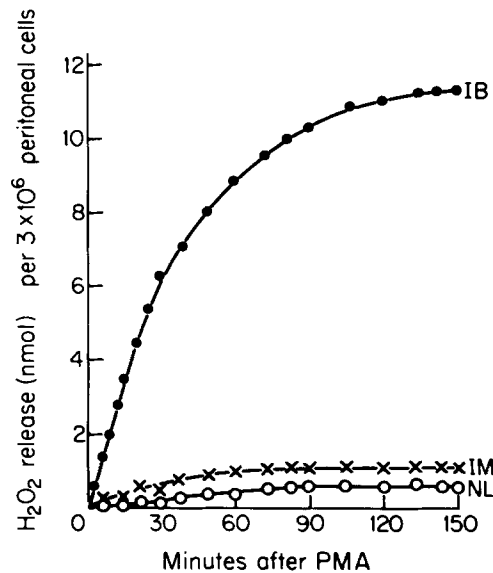

FIG. 6. H_2O_2 release by peritoneal cells in suspension. Freshly-harvested peritoneal cells from normal (NL), IM, and IB mice were prepared as described (5) and triggered with PMA 100 ng/ml. Extracellular H_2O_2 release was followed fluorometrically at 37°C (5). There was no detectable H_2O_2 release in the absence of PMA. IM and IB cells were from the same batch of mice. Differential counts of the peritoneal cells are summarized in Table I.

PMA (5). In the experiment illustrated, IB macrophages released 11 times more H_2O_2 than their IM counterparts, which in turn, released 2 times more than peritoneal cells from normal unimmunized mice. Table I summarizes the H_2O_2 values, composition, and properties of the three cell populations. No clear decline in H_2O_2 release by IB or IM cells was noted as the duration after immunization increased.

Effect of Oxygen Intermediate Scavengers on Macrophage Anti-toxoplasma Activity. Exposure of toxoplasmas for 30–60 min to the scavengers employed did not alter viability as indicated by AO staining or subsequent parasite multiplication in normal macrophages. The concentrations of scavengers used were the highest tolerated by macro-

TABLE I

H_2O_2 Release by Peritoneal Cells *

Mice‡	Macrophages	Granulocytes	Amount of H_2O_2 released in 2 h per 3 × 10^6 peritoneal cells
	%	%	nmol
Immune-boosted (6)§	56 ± 3.8	1.7 ± 0.4	7.8 ± 0.8
Immune (3)	49 ± 2.6	1.4 ± 0.3	1.1 ± 0.07
Normal (3)	52 ± 2.9	0.6 ± 0.3	0.3 ± 0.06

* Freshly harvested peritoneal cells in suspension were prepared as previously described (5) and triggered with PMA (100 ng/ml). There was no detectable H_2O_2 release in the absence of PMA. Cell viability (trypan blue exclusion): immune-boosted 83 ± 2.2%, immune 83 ± 6.9%, normal 87 ± 4.9%.

‡ Immune-boosted mice: immunized 25–155 d before boosting and harvest; immune mice: immunized 35–55 d before harvest.

§ Number of experiments; results expressed as means ± SE.

phage monolayers after 24 h of exposure. The battery of scavengers included superoxide dismutase (SOD), catalase, mannitol and benzoate, and diazabicyclooctane (DABCO) and histidine (1, 10). Preliminary experiments showed that adding scavengers only after toxoplasma ingestion resulted in little or no effect. Moreover, only a slightly greater effect on macrophage antitoxoplasma activity was observed upon adding scavengers and parasites to macrophages simultaneously. For these reasons and those already outlined (1), we elected to maximize the opportunity for scavengers to affect macrophage anti-toxoplasma activity by exposing monolayers to scavengers 3 h before, during, and 18–24 h after parasite ingestion.

The intracellular multiplication of toxoplasmas in scavenger-treated normal macrophages was not influenced. 18 h after infection there was an average of four to five toxoplasmas per vacuole, and, at 24 h, seven to eight toxoplasmas per vacuole. In contrast, addition of each of the six scavengers to microbistatic IM macrophages resulted in a consistent increase in toxoplasma multiplication (Figs. 7 and 8). Scavenger effectiveness appeared to depend upon the number of weeks elapsed from immunization, and became more pronounced as IM macrophage anti-toxoplasma activity began to decline (Fig. 7). In separate experiments, there was a clear correlation between the number of toxoplasmas per vacuole at 24 h and the concentrations of SOD and catalase (0.5–2.5 mg/ml) to which IM macrophages were exposed. Heated catalase (2.5 mg/ml) and autoclaved SOD (2.5 mg/ml) did not reverse inhibition. 10-fold less DABCO (0.1 mM) and histidine (1 mM) were also ineffective, as was sucrose (50 mM) which produced cell vacuolization similar to mannitol.

The ability of oxygen intermediate scavengers to slow or prevent toxoplasma killing by IB macrophages was assessed by comparing the percentage of macrophages infected and the number of toxoplasmas per 100 macrophages at time 0, 6, and 18 h. These IB macrophages consistently killed and removed 75% of the original intracellular inoculum by 6 h and at least 90% by 18 h (Fig. 9, controls). In the presence of the scavengers, parasite killing was clearly inhibited (Fig. 9). Although IB macrophages exhibited no decline in microbicidal activity as the time between immunization and harvest lengthened, scavenger effectiveness was enhanced as time progressed. The only instances in which scavenger treatment permitted actual multiplication of surviving parasites at 18 h in IB macrophages was from mice immunized >7 wk
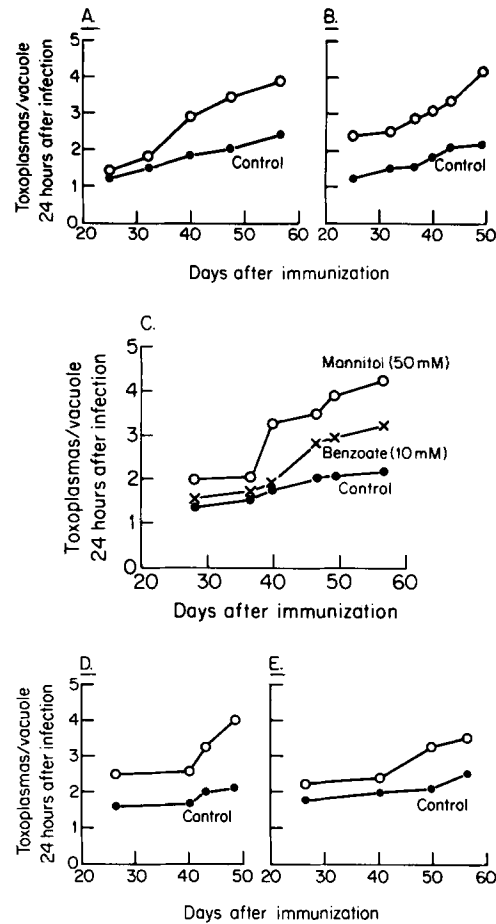
Fig. 7. Reversal of IM macrophage inhibition of toxoplasma multiplication by scavengers of O_2^-, H_2O_2, $OH\cdot$, and $^1O_2$. Freshly-explanted macrophages from mice immunized 25–56 d before harvest were exposed 3 h before, during, and 24 h after infection to either medium alone (controls) or to medium plus scavengers in the indicated final concentrations. (A) SOD 2.5 mg/ml, (B) catalase 2.5 mg/ml, (C) mannitol 50 mM and benzoate 10 mM, (D) histidine 10 mM, and (E) DABCO 1 mM. Also see Fig. 8.

before boosting and harvest (Figs. 9 and 10). Benzoate (10 mM) was not as effective as mannitol, although histidine was as efficacious as DABCO in reducing toxoplasma killing (data not shown). As with IM cells, there was a dose-response relationship between the inhibition of killing and the concentrations of SOD and catalase (0.5–2.5 mg/ml) to which IB macrophages were exposed. Heated catalase, SOD, and mannitol, and 0.1 mM DABCO and 1 mM histidine did not reverse killing. Oxygen intermediate scavengers, therefore, influence both the microbistatic activity of IM macrophages and the microbicidal properties of IB macrophages.

Effect of Glucose Deprivation on Macrophage Anti-toxoplasma Activity. Depriving Bacille Calmette-Gúerin (BCG)-activated mouse peritoneal macrophages of exogenous glucose for 2 h has been shown to ablate their ability to release H_2O_2 after PMA stimulation (4). Toxoplasma growth in normal macrophages deprived by glucose 3 h before, during, and 2 h after infection proceeded unaltered. In contrast, glucose
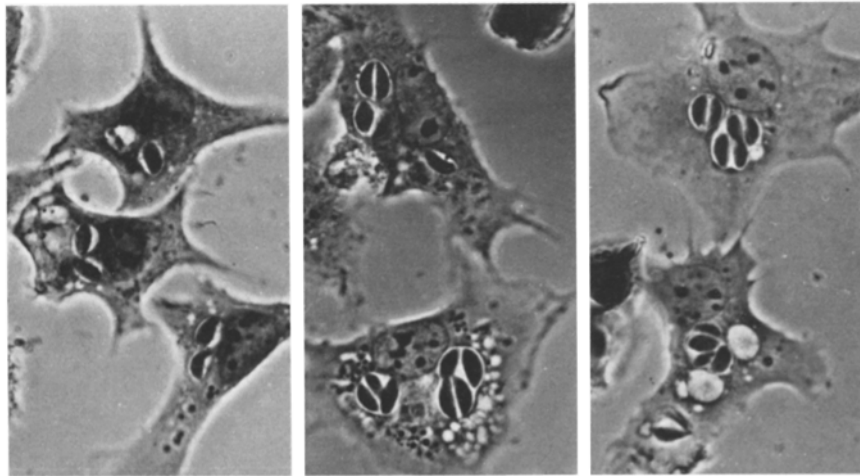
Fig. 8.   Inhibition of IM macrophage anti-toxoplasma activity by mannitol and DABCO. Control (untreated) IM macrophage 24 h after infection (left). Same macrophages treated as described with 50 mM mannitol (center) or 1 mM DABCO (right), 24 h after infection. Phase-contrast microscopy, Giemsa stain, X 800.
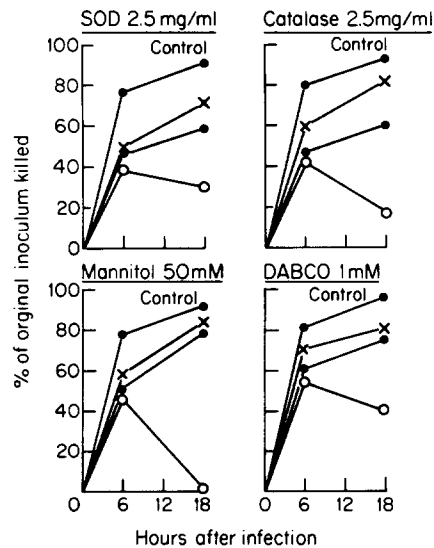

Fig. 9.   Reversal of IB macrophage toxoplasma killing by scavengers of O_2^-, H_2O_2, $OH\cdot$, and $^1O_2$. Freshly explanted macrophages from mice immunized 26-30 (x), 40-48 (·), and 50-56 (O) d before boosting were exposed as in Fig. 7 legend to medium alone (controls) or to medium plus scavengers in the indicated final concentrations. Data is expressed as the proportion (percentage) of the original intracellular inoculum killed (removed) by IB macrophages in terms of the number of toxoplasmas/ 100 macrophages. A decline in the percentage of original inoculum killed at 18 h indicates multiplication of surviving parasites. Control preparations for each scavenger represent the mean of four to six experiments.

deprivation resulted in reversal of both IM and IB macrophage activity (Fig. 11). As with the oxygen-intermediate scavengers, the effectiveness of glucose deprivation was more pronounced as macrophage anti-toxoplasma activity began to wane. Depriving IB cells of glucose for 3-h before stimulation with PMA markedly reduced their capacity to release H_2O_2 (see below).
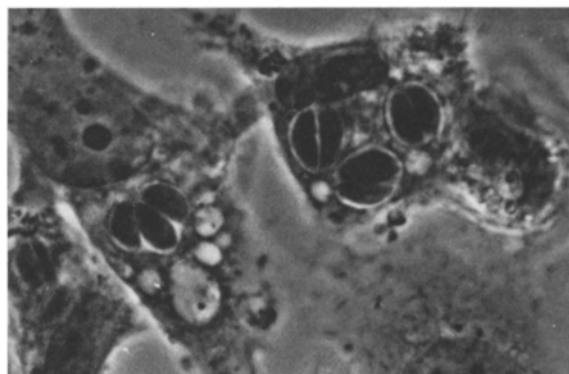
FIG. 10. Survival and multiplication of toxoplasmas in 10 mM histidine-treated IB macrophages 18 h after infection. Untreated control IB macrophages eradicated 90% of the original inoculum by 18 h and inhibited multiplication of surviving parasites for up to 40 h. Phase-contrast microscopy, X 1,200.
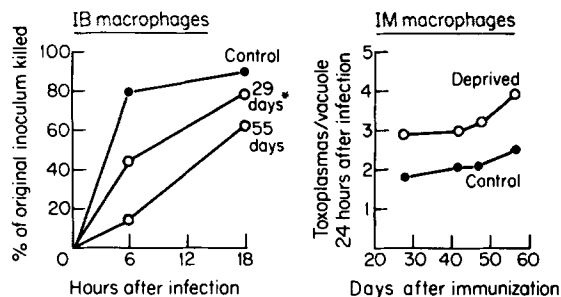

FIG. 11. Effect of glucose deprivation on IB and IM macrophage anti-toxoplasma activity. Freshly explanted macrophages from mice immunized 25–56 d (*) before harvest were cultivated in glucose-free medium (O) 3 h before, during, and 2 h after toxoplasma infection. Controls (·) were incubated in standard glucose-containing medium. (Left) Inhibition of IB macrophage toxoplasma killing (control, mean of four experiments; data for days 32 and 45 not shown). (Right) Reversal of IM macrophage inhibition of toxoplasma multiplication.

Effect of Scavengers and Glucose Deprivation on Other Macrophage Functions. Scavenger-treated macrophages ingested toxoplasmas normally, whereas glucose-deprived cells exhibited at 15–20% decrease in parasite uptake. No abnormalities in the rate at which dead organisms were digested intracellularly were noted in either circumstance. As shown in Table II, preincubation with catalase or glucose-free medium markedly depressed H_2O_2 release by IB macrophages. In the presence of scavengers of O_2^-, OH·, and $^1O_2$, however, H_2O_2 release in response to PMA was normal.

Effect of Exogenous Oxygen Intermediates on Toxoplasma Multiplication in Normal Macrophages. Certain oxidative metabolites traverse cell membranes (11) and appear to enter phagocytic vacuoles after exogenous administration (12). After infecting normal macrophage monolayers and removing uningested parasites, glucose oxidase (GO) was added to culture dishes containing glucose ($D_{20}HIFBS$) in amounts that generated 7–15 nmol H_2O_2/min at 37°C (4). No effect on the subsequent multiplication of intracellular toxoplasmas was observed. Multiplication also proceeded normally if GO was added at the time of parasite ingestion. However, the addition of xanthine-xanthine oxidase (XO) after infection resulted in consistent inhibition of intracellular toxoplasma multiplication (Fig. 12). Substituting acetaldehyde (10^{-3} M) for xanthine

TABLE II

Effect of Scavengers or Glucose-free Medium on Macrophage H_2O_2 Release *

Scavenger/condition	Macrophage H_2O_2 release‡	
	20 d§	53 d§
	nmol H_2O_2/µg cell protein	
None (control)	0.67	0.25
Catalase, 2.5 mg/ml	0.22	0.09
SOD, 2.5 mg/ml	0.68	0.33
Mannitol, 50 mM	0.86	0.22
Benzoate, 10 mM	0.84	0.32
Histidine, 10 mM	0.74	0.30
DABCO, 1 mM	1.10	0.38
No glucose	0.11	0.00

* Scavengers in D_{20} HIFBS or glucose-free medium alone was added to freshly explanted immune-boosted macrophage monolayers for 3 h before PMA (100 ng/ml) stimulation.

‡ Total H_2O_2 released 90 min after addition PMA. H_2O_2 release was not detected in the absence of PMA. Results are the means of closely agreeing duplicate coverslips.

§ Macrophages from mice immunized with toxoplasma brain cysts 20 and 53 d before boosting and harvest. Granulocytes comprised <1% of adherent cells in both experiments illustrated.
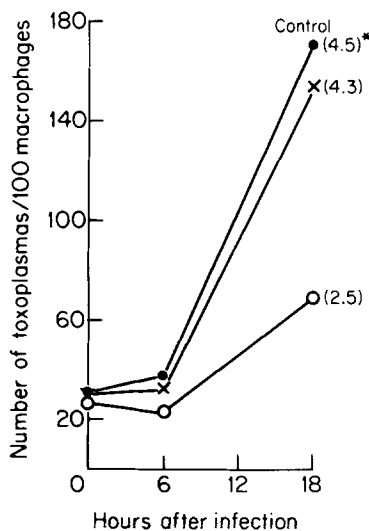

FIG. 12.   Inhibitory effect of exogenous oxygen intermediates on toxoplasma multiplication in normal macrophages. Uningested parasites were removed by extensive washing and cultures were reincubated for 18 h in medium alone (·), medium plus 1.5 X 10^{-4} M xanthine (x), or medium plus xanthine plus 25 µg xanthine oxidase (O). Addition of xanthine oxidase alone after infection had no effect. Decline at 6 h in the number of toxoplasmas/100 macrophages in xanthine-XO-treated macrophages (O) indicates modest parasite killing. (*) indicates the number of toxoplasmas per vacuole 18 h after infection. Using higher concentrations of xanthine or >25 µg xanthine oxidase was toxic to macrophages. The figure illustrates one of four similar experiments.

(10) did not enhance parasite inhibition. Addition of scavengers of O_2^- (SOD, 100 µg/ml), H_2O_2 (catalase, 200 µg/ml), OH· (mannitol, 50 mM), or $^1O_2$ (DABCO, 1 mM; histidine 10 mM) in the presence of xanthine-XO, reversed the inhibition and permitted toxoplasmas to multiply normally.

Discussion

In this study, the spectrum of mouse peritoneal macrophage in vitro anti-toxoplasma capacity ranged from no activity (normal cells) to brief inhibition of parasite multiplication (immune [IM] cells) to striking eradication of the bulk of the ingested inoculum (immune-boosted [IB] cells). These widely disparate cellular responses appeared to correlate with the capacity to generate oxidative metabolites as judged by extracellular H_2O_2 release. IB (microbicidal) macrophages released seven times more H_2O_2 than IM (microbistatic) cells and >25 times that released by normal macrophages, whereas IM cells released 4 times more H_2O_2 than normal cells. Although these findings give no indication of intracellular or intravacuolar H_2O_2 levels and shed no light on production of other key oxygen intermediates, they do suggest that one of the differences between the absence of anti-toxoplasma activity and the presence of microbistatic or microbicidal effects may be a quantitative one as expressed by the levels of toxic oxidative metabolites.

Scavengers of O_2^-, H_2O_2, OH·, and $^1O_2$ (10, 13) all reversed the anti-toxoplasma activity of IM and IB macrophages. Optimal conditions for inhibiting macrophage activity included high concentrations of scavengers and using IM cells whose induced anti-toxoplasma activity had begun to wane. Although the microbicidal capacity of IB macrophages remained undiminished up to 8 wk after immunization, scavengers were consistently more efficacious in cells from the oldest mice examined. These findings may indicate a threshold phenomenon in terms of the amount or concentration of oxidative metabolites necessary to produce inhibition or killing. Thus, the generation of oxygen intermediates may decline with time after immunization to levels which are still sufficient to result in toxoplasma inhibition or killing but are also more readily overcome by exogenous scavengers.

The role of oxygen intermediates in human phagocyte antimicrobial activity has been examined by similar methods. SOD, catalase, mannitol, and benzoate all impair polymorphonuclear neutrophil (PMN) bactericidal activity indicating roles for O_2^-, H_2O_2, and OH· (13). Incubating human monocytes with SOD during phagocytosis also inhibits the killing of *S. aureus* (14). In the former study (13), it was necessary to provide PMN with certain scavengers (SOD, catalase) on cophagocytized latex particles, whereas in the latter (14), co-incubating monocytes with SOD and *Staphylococcus aureus* was sufficient to demonstrate impairment of phagocyte killing. In our studies, optimal results were achieved by exposing macrophages to scavengers before, during, and after toxoplasma infection. Administering scavengers only at the time of parasite ingestion was ineffective, presumably reflecting the minute volume of medium enclosed within single phagocytic vacuoles (15). Alternatively, scavengers may have been present in considerable amounts, but failed to gain access to a crucial intravacuolar site where concentrations of oxidative metabolites may be the greatest. Previously interiorized large molecular weight scavengers such as catalase would not be expected to enter phagocytic vacuoles harboring viable toxoplasmas because phagolysosomal fusion fails to occur (16). Preincubation of macrophages with scavengers may, however, be effective by providing sufficiently high intracellular levels to act as extravacuolar sinks for oxidative metabolites which traverse vacuolar membranes and enter the cytosol (17). Moreover, preinfection interiorization of scavengers might also partially quench preformed oxidative metabolites or those possibly generated from an extravacuolar source.

In neither the cell-free xanthine-XO system (1) nor this macrophage model could

O_2^- or H_2O_2 alone be implicated as toxoplasmacidal agents. Parasites withstood exposure to reagent H_2O_2, glucose-GO, and H_2O_2 formed by xanthine-XO in the presence of SOD (10). Moreover, after exposure to H_2O_2-sparing scavengers, macrophage anti-toxoplasma activity was consistently reduced. Finally, when normal macrophages were provided with an exogenous source of H_2O_2, intracellular parasite multiplication proceeded unchecked. Endogenous parasite catalase may explain resistance to H_2O_2 (1). It also appeared that O_2^- alone was not toxic to either extracellular or intracellular toxoplasmas. Xanthine-XO induced parasite killing as well as macrophage inhibition and killing were reduced by scavengers of H_2O_2, $OH\cdot$, and $^1O_2$ which should not affect O_2^- production. The requirement for both O_2^- and H_2O_2 for expression of anti-toxoplasma activity, however, as well as the inhibitory effects of scavengers of $OH\cdot$ and $^1O_2$ suggest key roles for $OH\cdot$ and $^1O_2$ in the cell-free XO system (1) and the macrophage model. Because the specificity of the inhibition produced by $OH\cdot$ scavengers and $^1O_2$ quenchers has not been fully established (10), the nature of the active intermediate remains in question. Given the specificity of SOD and catalase, however, O_2^- and H_2O_2 appear to function as precursors of the toxoplasmacidal agents in the systems we employed. Further, providing normal macrophages with an exogenous source of oxygen intermediates including $OH\cdot$ and probably $^1O_2$ (10) resulted in inhibition of intracellular toxoplasma multiplication.

Additional evidence implicating oxidative mechanisms in macrophage antimicrobial activity was derived from glucose-deprivation experiments. Most H_2O_2 appears to arise from the dismutation of O_2^- formed by the partial reduction of molecular oxygen by NADPH (10, 18). Depriving macrophages of exogenous glucose may, therefore, retard H_2O_2 production by limiting substrate for the hexose-monophosphate shunt (4). Moreover, PMN from patients with profound glucose-6-phosphate dehydrogenase deficiency also fail to elaborate H_2O_2 (19), and may exhibit bactericidal defects similar to PMN from patients with chronic granulomatous disease (19, 20). In our study, H_2O_2 release by glucose-deprived IB macrophages was either ablated or markedly reduced, and glucose deprivation inhibited the anti-toxoplasma activity of both IM and IB macrophages.

Experiments in which infected macrophages were deprived of oxygen were not carried out because toxoplasmas ingested by normal macrophages failed to multiply appropriately under anaerobic conditions. Thus, we have not excluded the possibility that oxygen-independent mechanisms contribute in some fashion to IM and IB macrophage anti-toxoplasma activity. For instance, the pH of normal mouse peritoneal macrophage lysosomes is 4.5–4.8 (21), a level at which toxoplasmas in suspension are readily killed (1). It is not known, however, whether the pH of phagosomes harboring viable toxoplasmas also decreases to low levels in the absence of phagolysosomal fusion (16). Other oxygen-independent mechanisms potentially important in mononuclear phagocyte antimicrobial activity have been reviewed elsewhere (22).

Our studies confirm and extend the recent observation that macrophage release of H_2O_2 correlates well with enhanced microbicidal activity (23). In addition to the IM and IB cells described above, the capacity of other immunologically activated (BCG, *Corynebacterium parvum*) or chemically elicited macrophages to release H_2O_2 also correlates closely with their ability to inhibit toxoplasma multiplication (H. Murray, unpublished observations). However, these studies have demonstrated important

differences in the intrinsic susceptibilities of intracellular pathogens to oxidative metabolites. In comparison to *Trypanosoma cruzi* (23), *T. gondii* is resistant to H_2O_2, perhaps because the latter contains catalase (1). Thus, whereas H_2O_2 release may serve as a marker for enhanced production of reactive oxygen metabolities by activated macrophages, some pathogens may only be killed or inhibited in the presence of additional oxidative species such as $OH\cdot$ or $^1O_2$. Moreover, certain macrophages sufficiently activated in vitro to release increased H_2O_2 and eradicate intracellular *T. cruzi* display no toxoplasmacidal activity (H. Murray, unpublished observations). It is possible, then that some macrophages either do not generate or properly deliver the full complement of toxic oxygen intermediates required to kill H_2O_2-resistant pathogens such as *T. gondii*, or that these cells utilize other currently unidentified microbicidal mechanisms.

Summary

The capacity of three populations of mouse peritoneal macrophages to generate oxidative metabolites (as judged by extracellular release of H_2O_2) was compared to their ability to influence the intracellular fate of virulent *Toxoplasma gondii*. Macrophages from normal mice released little H_2O_2 and allowed unrestricted multiplication of intracellular toxoplasmas. Cells from chronically infected, immune (IM) mice released 4 times more H_2O_2 and displayed microbistatic activity. In contrast, macrophages from immune-boosted (IB) mice released 25 times more H_2O_2 than normal cells and rapidly killed the bulk of ingested toxoplasmas within 1 h. When macrophage monolayers were exposed to scavengers of O_2^-, H_2O_2, $OH\cdot$, and $^1O_2$, both the inhibition of intracellular toxoplasma multiplication by IM macrophages and the killing of toxoplasmas by IB macrophages were reversed. Depriving cells of glucose, which markedly reduced H_2O_2 release, resulted in similar reversal of IM and IB macrophage anti-toxoplasma activity. As judged by the effect of the individual oxygen intermediate scavengers, O_2^- and H_2O_2 appeared to serve as precursors for the key toxic agents which may include $OH\cdot$ and $^1O_2$. Providing normal macrophages with an exogenous source of oxidative metabolites generated by xanthine and xanthine oxidase, but not glucose and glucose oxidase, resulted in inhibition of intracellular toxoplasma growth. These findings suggest the presence of an oxygen-dependent antimicrobial system in mononuclear phagocytes beyond the production of O_2^- and H_2O_2, and indicate an important role for oxygen intermediates in macrophage resistance to the intracellular pathogen *T. gondii*.

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