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Research Article

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ABSTRACT Oxygen products generated by the respiratory burst of mononuclear phagocytes are microbicidal to intracellular pathogens including *Toxoplasma gondii*. The toxicity of one of these products, H_2O_2, is markedly amplified by the granule peroxidase of circulating phagocytes in the presence of a halide. Eosinophil peroxidase (EPO) binds firmly to the surface of *T. gondii* and such organisms remain viable as determined by vital staining, uptake of 2-deoxyglucose, and survival and replication in human fibroblasts. They are, however, rapidly killed by the addition of H_2O_2 and iodide under conditions in which control organisms are unaffected. We have used EPO bound to *T. gondii* to explore the role of peroxidase in the toxoplasmacidal activity of mononuclear phagocytes. Resident mouse peritoneal macrophages lack a granule peroxidase and have a weak respiratory burst; toxoplasma survive and replicate within these cells. However, these cells acquire significant toxoplasmacidal activity, as assessed microscopically and by the inhibition of uracil uptake, when organisms are coated with EPO before ingestion, an effect which is decreased by the hemeprotein inhibitors, aminotriazole and azide. EPO on the surface of Toxoplasma does not increase their ingestion by macrophages or the associated respiratory burst. Monocytes from patients with hereditary myeloperoxidase deficiency have a significant toxoplasmacidal defect that is abolished when EPO-coated organisms are used. In contrast, the toxoplasmacidal defect of monocytes from chronic granulomatous disease patients is unaffected by surface-bound EPO. In these studies, replication of surviving intracellular organisms varied inversely with the magnitude of the respiratory burst: replication was greatest in fibroblasts, slightly less in resident macrophages, and least in monocytes; it was significantly greater in chronic granulomotous disease than in normal or myeloperoxidase-deficient monocytes. These studies support a role for oxygen products and endogenous peroxidase in the optimal killing of *T. gondii* by monocytes and demonstrate that peroxidase-negative phagocytes can utilize peroxidase on the surface of ingested organisms to augment microbicidal activity.

INTRODUCTION

The ability of mononuclear phagocytes (monocytes, macrophages) to kill intracellular pathogens depends in part on the capacity of these cells to generate potentially toxic oxygen metabolites (1–6). The membrane perturbation induced by phagocytosis initiates a burst of respiratory activity and the increased oxygen consumed is converted to the superoxide anion (O_2^-)[1], H_2O_2, hydroxyl radical ($OH^\cdot$), and possibly singlet molecular oxygen ($^1O_2$) (7). The initial product of the

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[1] *Abbreviations used in this paper:* AO, acridine orange; AS, autologous serum; CGD, chronic granulomatous disease; EB, ethidium bromide; EPO, eosinophil peroxidase; FBS, fetal bovine serum; [^{3}H]2DG, [1,2-^{3}H]2-deoxy-D-glucose; M199, Medium 199; MEM, Minimum Essential Medium; MPO, myeloperoxidase; NBT, nitroblue tetrazolium; $^1O_2$, singlet oxygen; O_2^-, superoxide anion; PB, 0.1 M phosphate buffer, pH 7.2; Toxo, control *T. gondii*; Toxo/EPO, *T. gondii* with surface-bound EPO.

respiratory burst, O_2^-, appears to have little direct toxic activity; however its dismutation product, H_2O_2, and the products of the metal-catalyzed interaction of O_2^- and H_2O_2, i.e., $OH^\cdot$ and possibly $^1O_2$, are microbicidal and cytotoxic, and appear to serve this function in mononuclear phagocytes (3, 5). Oxygen-dependent microbicidal activity requires production of these toxic products of oxygen reduction and excitation in amounts sufficient to overcome the protective capacity of the endogenous scavengers present in the pathogen (8, 9). Resident macrophages exhibit a weak respiratory burst and are relatively inactive against intracellular pathogens; in contrast, immunologically activated macrophages and circulating phagocytes have both a more pronounced respiratory burst and enhanced microbicidal activity against these organisms (2, 4–6, 10–14).

Blood phagocytes contain a peroxidase in cytoplasmic granules (myeloperoxidase [MPO] in neutrophils and monocytes, eosinophil peroxidase [EPO] in eosinophils), which greatly potentiates the antimicrobial activity of H_2O_2 in the presence of a halide (15, 16). Macrophages lack a granule peroxidase (1) and thus do not generally utilize this mechanism for the amplification of H_2O_2-dependent antimicrobial activity. It is possible, however, that macrophages can utilize peroxidase released by peroxidase-positive phagocytes at the inflammatory site. EPO, and to a lesser degree MPO, are cationic proteins that bind firmly to the surface of microorganisms with the retention of peroxidatic activity (17, 18). Peroxidase taken up by macrophages, either on the surface of ingested organisms (18), by pinocytosis of fluid-phase peroxidase (19), or by the phagocytosis of leukocytic debris (20–22), might be expected to enhance the microbicidal activity of these cells.

In this study, we used *Toxoplasma gondii* to explore the potential role of acquired peroxidase in the killing of intracellular pathogens by mononuclear phagocytes. *T. gondii* is rich in catalase and superoxide dismutase (23) and is thus resistant to the small amounts of oxygen metabolites produced by resident mouse peritoneal macrophages and human monocyte-derived macrophages (2, 3); Toxoplasma survive and replicate within these cells. We report here that highly purified EPO, bound to the surface of *T. gondii*, augments the toxoplasmacidal activity of resident murine macrophages. Further, monocytes from patients with hereditary MPO deficiency have a defect in toxoplasmacidal activity during the early postphagocytic period, which is reversed by the presence of EPO on the surface of the organism. Our data suggest a mechanism by which acquired peroxidase may augment the killing of intracellular pathogens by mononuclear phagocytes and thus contribute to host defense before the development of macrophage activation.

METHODS

Parasites

T. gondii (RH strain). The parasite was maintained and processed as previously described (24), except that organisms were harvested from the peritoneal cavity of mice in 0.1 M sodium phosphate buffer, pH 7.2 (PB) rather than in phosphate buffer containing sodium chloride (PBS). After filtration through a 3-μm polycarbonate membrane filter (Nuclepore Corp., Pleasanton, CA) to remove leukocytes, organisms were collected by centrifugation at 200 *g* for 10 min and resuspended in PB.

Binding of EPO to Toxoplasma. EPO purified to a 415:280 nm ratio of >0.9 was prepared from equine eosinophils and stored in 0.05 M sodium acetate buffer, pH 4.7–1.0 M sodium chloride at −70°C (25). EPO was thawed immediately before use and assayed by guaiacol oxidation (26). H_2O_2 30%, was purchased fresh monthly from American Scientific and Chemical Co., Portland, OR, and stored at 4°C in an opaque container. The concentration of H_2O_2 was verified by measuring the absorbance at 230 nm using an extinction coefficient (E) = 81 M^{-1} cm^{-1} (27).

EPO (2.82–5.12 U in 20 μl) was incubated with 10^8 Toxoplasma in 0.5 ml PB in 12 × 75-mm polystyrene test tubes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) at 37°C for 12 min in a shaking water bath. The dilution of the stock EPO preparation was >25 times so that the final sodium chloride and acetate buffer concentrations, due to their presence in the enzyme preparation, were <0.02 M and 0.002 M, respectively. The organisms (Toxo/EPO) were washed three times in PB at 4°C and collected by centrifugation at 200 *g* for 10 min. No peroxidase activity could be detected in the supernatant of the final wash. Control organisms (Toxo) were handled identically except that incubation was with 20 μl of the suspending buffer without EPO. In some experiments, Toxoplasma were heat-killed (100°C for 5 min) before EPO binding.

Measurement of viability of Toxo and Toxo/EPO in cell-free systems. Toxo or Toxo/EPO suspended in PB were incubated with the components of the reaction mixture (see legends) in a final volume of 0.5 ml in 12 × 75-mm polystyrene test tubes; organisms (2–4 × 10^7/tube) were added last to start the reaction. After incubating for 30 min, tubes were vortexed and 40 μl of the reaction mixture were added to 0.1 ml of PB at 4°C for examination by fluorescence microscopy (see below). 40 μCi of [1,2-^{3}H]2-deoxy-D-glucose ([^{3}H]2DG[1], 40 Ci/mmol; New England Nuclear, Boston, MA) in 40 μl of PB were added to the remaining reaction mixture and the incubation was continued. After an additional 90 min, the reaction mixture was layered over 0.5 ml of silicone oil (Versilube F50; General Electric Co., Waterford, NY) and centrifuged at 10,000 *g* (Eppendorf centrifuge 5412; Brinkmann Instruments, Inc., Westbury, NY) for 2 min. The upper aqueous phase was removed by aspiration, and the bottom of the tube containing the pellet was cut off and placed in a scintillation vial containing 10 ml of Aquasol-2 (New England Nuclear). Vials were sonicated in a bath sonicator (Bransonic 220; Branson Sonic Power Co., Danbury, CT) to facilitate dispersion of the pellet, and counted in a liquid scintillation counter (Packard Instrument Co., Downers Grove, IL). The 30-min samples (see above) were assayed for viability by fluorescence microscopy within 10 min of their collection, using a modification of previously described methods (28). Duplicate 25-μl aliquots were mixed with an equal volume of an acridine orange (Allied Chemical Corp., Morristown, NJ) and ethidium bromide (Sigma Chemical

Co., St. Louis, MO) solution (AO/EB) on a glass microscope slide. The AO/EB solution was prepared by mixing 5 μg/ml AO and 10 μg/ml EB in PB and stored in the dark at 4°C. Cover slips were overlayed, the edges sealed with clear nail polish, and the preparations examined immediately using a Zeiss Photomicroscope III with epiillumination (Carl Zeiss, Inc., New York). At least 200 organisms were counted, and only Toxoplasma exhibiting bright green nuclear fluorescence were scored as viable. Dead organisms fluoresce orange.

Cell preparations

Mouse peritoneal macrophages. Resident peritoneal cells were collected in cold (4°C) Ca^{++}-, Mg^{++}-free Hanks' balanced salt solution (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) containing 10 U/ml heparin (Abbott Diagnostics, Diagnostic Products, North Chicago, IL) by lavage of 6–10-wk-old female BALB/c mice (Fred Hutchinson Cancer Research Center, Seattle, WA). Cells collected by centrifugation at 200 *g* for 12 min were resuspended in Medium 199 (Gibco Laboratories) containing penicillin G (100 U/ml), streptomycin (100 μg/ml), 2 mM L-glutamine and 10% heat-inactivated, Sabin-Feldman dye test negative, fetal bovine serum (Sterile Systems Inc., Logan, UT) (M199-FBS) and counted in a hemocytometer. Preparations contained 44±2% (range 38–59%) macrophages (as determined by phagocytosis of neutral red particles [29] and by staining for nonspecific esterase [30], <3% polymorphonuclear leukocytes (as determined by examination of Giemsa-stained smears), and the remainder, lymphocytes. Cell viability was >96% as determined by trypan blue exclusion.

Human blood monocytes. Blood was collected from two patients with hereditary MPO deficiency, two patients with chronic granulomatous disease (CGD), and normal healthy volunteers. All were negative for antibody to *T. gondii* in the Sabin-Feldman dye test. Mononuclear cells obtained as previously described (31) were suspended in Medium 199 containing penicillin G (100 U/ml), streptomycin (100 μg/ml), 2 mM L-glutamine, and 25% autologous serum (M199-AS). Cell preparations contained 26±3% (range 14–42%) monocytes, <2% polymorphonuclear leukocytes, and the remainder, lymphocytes, determined as described above.

Human fibroblasts. Human embryonic tonsil fibroblasts, obtained from Dr. Lawrence Corey, Virology Laboratory, Children's Orthopedic Hospital, Seattle, WA, were maintained in Eagles Minimum Essential Medium (MEM) (Flow Laboratories, Inc., Rockville, MD) containing 10% heat-inactivated FBS (MEM-FBS), and passed by trypsinization when the cell density approached a confluent monolayer.

Preparation of monolayers. Aliquots containing 5×10^5 mouse macrophages in 0.5 ml M199-FBS, 5×10^5 human monocytes in 0.5 ml M199-AS, or $1–2 \times 10^5$ human fibroblasts in 0.5 ml MEM-FBS were placed on 15-mm round glass cover slips in 16-mm tissue culture wells (24-FB-TC; Linbro Chemical Co., Hamden, CT) or in wells of four-chamber tissue culture slides (Lab-Tek Div. Miles Laboratories Inc., Naperville, IL). After incubation for 1 h at 37°C in 5% CO_2-air, nonadherent cells were removed from macrophage and monocyte cultures by vigorously washing monolayers six times with warm (37°C) Hanks' balanced salt solution. Monocyte monolayers were infected immediately (see below). Macrophage monolayers were either infected immediately or after overnight incubation in fresh medium. Fibroblast monolayers were maintained at 37°C in 5% CO_2-air and infected when approaching confluence (3–5 d). Cellular density was evaluated both by direct visualization using an ocular grid at x40 magnification, and by assaying the total monolayer protein content at different periods following infection. For the latter, monolayers were lysed with 0.1% Triton X-100 (Sigma Chemical Co.) in deionized water, and the total protein content was determined by a modification of the method of Lowry et al. (32) using bovine serum albumin as standard.

Intracellular survival and replication of Toxo and Toxo/EPO. Medium overlying duplicate monolayers was replaced with 1×10^6 Toxo or Toxo/EPO in 0.5 ml M199-FBS for macrophage or monocyte cultures and with 2.5×10^6 Toxo or Toxo/EPO in 0.5 ml MEM-FBS for fibroblast cultures. After 1 h at 37°C in 5% CO_2-air, monolayers were washed six times with warm (37°C) Hanks' balanced salt solution to remove extracellular organisms, and reincubated with fresh medium. Immediately, and 6 and 20 h after the infection period, monolayers were fixed with 0.4% aminoacridine (Sigma Chemical Co.) in 50% ethanol, stained with Giemsa stain, and the percentage of infected cells, the number of organisms per cell, and the number of organisms per vacuole was assessed microscopically (33). All determinations were carried out in duplicate and the means were used for statistical analysis.

Intracellular survival and replication of organisms was also assessed using [5,6-^{3}H]uracil (sp act 40 Ci/mmol, New England Nuclear), as previously described (14). Briefly, 2×10^5 Toxo or Toxo/EPO were added to wells of microtiter trays (IS-FB-96-TC; Linbro Chemical Co.) containing either peritoneal cells (10^5 macrophages) or fibroblast monolayers; control wells contained cells or organisms alone in an equivalent total volume (0.2 ml). [^{3}H]Uracil (1.25 μCi in 25 μl M199) was then added to each well. After 20 h at 37°C in 5% CO_2-air, the well contents were deposited on glass fiber filter papers using a multiple automated sample harvester (MASH II; Microbiological Associates, Bethesda, MD). Filters were dried, placed in scintillation vials containing 10 ml of Liquifluor (New England Nuclear), and counted in a liquid scintillation counter. All determinations were carried out in quadruplicate and the means were used for statistical analysis.

Measurement of the respiratory burst

O_2^- release. The components of the reaction mixture (see legend to Table IV) were incubated in 12 × 75-mm polystyrene test tubes at 37°C in a shaking water bath. Each reaction tube was run in parallel with an identical tube except for the addition of superoxide dismutase (2,500 U/mg, Sigma Chemical Co.) at a final concentration of 25 μg/ml. Aliquots, removed when the reaction was initiated and 1 h later, were placed in iced microfuge tubes, cleared by centrifugation (8,000 *g* for 2 min) and the concentration of ferrocytochrome *c* was determined spectrophotometrically at 550 nm using $E = 2.1 \times 10^4\ M^{-1}\ cm^{-1}$. Superoxide dismutase-inhibitable ferricytochrome *c* reduction was used as a measure of O_2^- production (34).

Nitroblue tetrazolium (NBT) reduction. Quantitative and qualitative NBT reduction was determined as previously described (2).

Electron microscopic studies

Peritoneal cell suspensions containing 3×10^6 macrophages in 3 ml M199-FBS were incubated with 6×10^6 Toxo

or Toxo/EPO in 12 × 75-mm polystyrene test tubes for 1 h at 37°C in 5% CO_2-air. Cells and organisms were collected by centrifugation (200 *g* for 10 min), the supernatants discarded, and the pellets fixed in 1.5% glutaraldehyde (Polysciences, Inc., Warrington, PA) in 0.1 M sodium cacodylate buffer, pH 7.0, for 10 min at 4°C. The pellets were washed twice in 0.1 M sodium cacodylate buffer, and then reacted with a mixture containing 0.5 mg/ml diaminobenzidine (Scientific Chemical Co., Huntington Beach, CA), 2 mM glucose and 25 μg/ml glucose oxidase (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA) in 0.05 M Tris buffer, pH 7.6, for 2 h at 22°C. Cells and organisms were then washed and postfixed in osmium tetroxide (Scientific Chemical Co.), dehydrated with ethanol, and collected by low-speed centrifugation before embedding in Epon 812 (Scientific Chemical Co.) for examination with a JEOL 100B electron microscope (Japan Electron Optics Laboratory, Tokyo, Japan) at 60 kV. Unless otherwise indicated, preparations were stained in the block with uranyl acetate and on the section with lead citrate.

Statistical analysis

Data are reported as the mean±standard error. Differences were analyzed for significance using the Student's two-tailed *t* test for independent means (35).

RESULTS

Binding of EPO to Toxoplasma preparations. *T. gondii*, preincubated with EPO (Methods) and washed extensively to remove unbound peroxidase, retained a portion of the EPO bound to the surface of the organism. Thus, when these preparations of *T. gondii* (Toxo/EPO) were examined by peroxidase cytochemistry, diaminobenzidine-positive material was evident as a thin electron-dense rim uniformly coating all organisms (Fig. 1A). This electron-dense material was

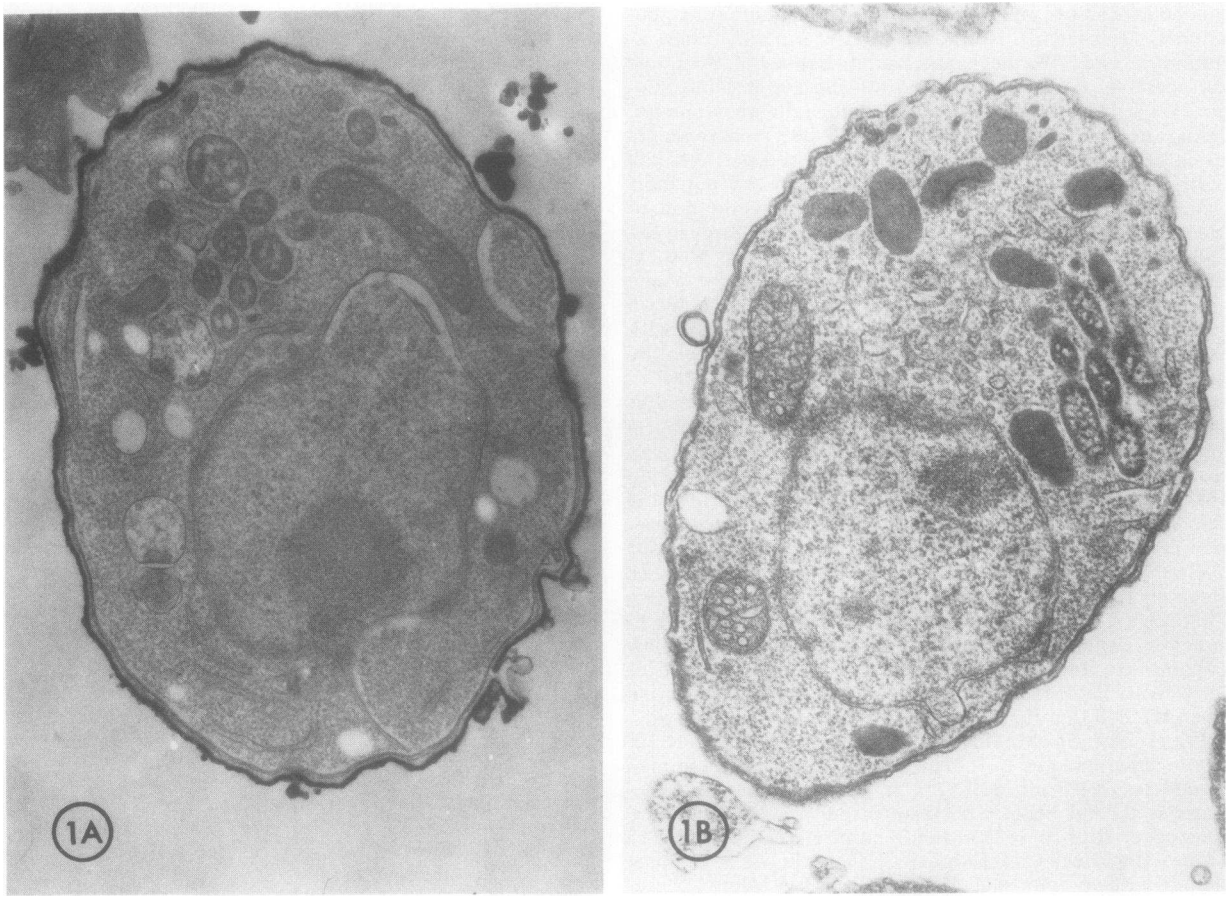

FIGURE 1 Electron microscopic localization of peroxidase in Toxoplasma preparations. A. Toxo/EPO were stained for peroxidase by the diaminobenzidine method. The preparations were not counterstained with either uranyl acetate or lead in order to highlight the peroxidase-positive reaction. EPO is evident as an electron-dense rim that uniformly coated all organisms. The cellular detail is undisturbed (×33,000). B. Control Toxo. Note the absence of diaminobenzidine-positive material (×33,000).

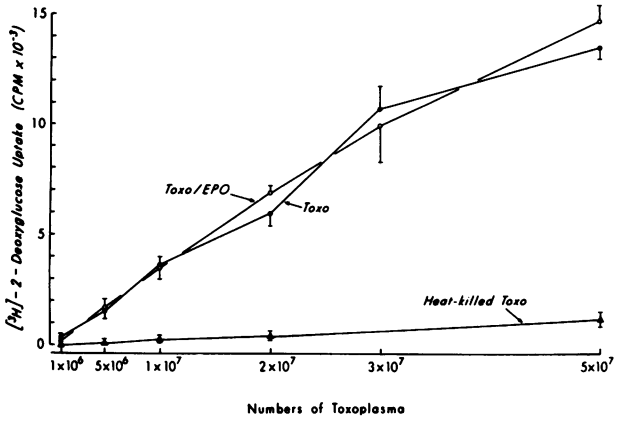

FIGURE 2 Uptake of [^{3}H]2DG by Toxoplasma preparations. The reaction mixture contained 0.1 M phosphate buffer, pH 7.2, 2 μM [^{3}H]2 DG (40 μCi), and either control Toxo, Toxo/EPO, or heat-killed Toxo in the numbers indicated. The final volume was 0.5 ml. Incubation was for 90 min at 37°C in a shaking water bath. Each point is the mean±SE of four to eight experiments with duplicate determinations.

absent from similarly treated control organisms that had not been preincubated with EPO (Toxo; Fig. 1B).

The coating of organisms with peroxidase was not toxic under these experimental conditions; thus the cellular detail of the Toxo and Toxo/EPO preparations was equivalent by electron microscopic morphologic criteria. The viability of Toxo and Toxo/EPO suspended in PB was 89±2 and 89±3%, as indicated by their bright green fluorescence upon vital staining with AO/EB. In contrast, heat-treated organisms (100°C for 5 min) were 100% nonviable, as indicated by their uniform orange fluorescence. The uptake of [^{3}H]2DG by Toxoplasma preparations was used as an additional measure of viability. Uptake was comparable with either Toxo or Toxo/EPO, and with each, was related in a linear fashion to the numbers of organisms used over the range illustrated in Fig. 2. Uptake of [^{3}H]2DG was markedly reduced when heat-treated organisms were used; the residual uptake was felt to represent nonspecific trapping.

Toxoplasmacidal activity of bound EPO in cell-free systems. Toxo/EPO were rapidly killed on the addition of H_2O_2 (0.1 mM) and iodide (10 μM) (Table I). The percentage of viable organisms fell from 89 to 2% as assessed by AO/EB fluorescence microscopy ($P < 0.001$); similarly, the uptake of [^{3}H]2DG decreased from 9,025 to 1,997 cpm ($P < 0.005$). This loss of viability was not seen when either H_2O_2 or iodide was omitted or when both were added to control Toxo. The toxic effect of H_2O_2 and iodide on Toxo/EPO was greatly decreased by the hemeprotein inhibitors aminotriazole, azide, and cyanide, with cyanide the least effective of the inhibitors.

Survival and replication of Toxo and Toxo/EPO in mouse peritoneal macrophages. The clearance of Toxo and Toxo/EPO by resident mouse peritoneal macrophages was investigated to determine if organism-bound peroxidase could enhance the killing of

TABLE I
*Toxoplasmacidal Activity of the EPO-H_2O_2-Iodide System**

Supplements	AO/EB fluorescence		[^{3}H]2DG uptake	
	% viable		cpm	
Toxo/EPO	89±3(7)‡		9,025±1,805(6)	
Toxo/EPO + H_2O_2 + I	2±1(7)	$P < 0.001$§	1,997±376 (7)	$P < 0.005$
H_2O_2 omitted	86±6(3)		10,023±1,701(3)	
I omitted	84±5(5)		9,627±2,045(5)	
Aminotriazole added	80±3(3)		8,804±475 (3)	
Azide added	90±5(3)		8,293±1,227(3)	
Cyanide added	75±5(3)		7,414±708 (3)	
Toxo	89±2(7)		9,610±1,559(7)	
Toxo + H_2O_2 + I	89±1(6)		8,119±2,825(4)	

* The reaction mixture contained PB and the following supplements: Toxo or Toxo/EPO, 2–4 × 10^7 organisms; H_2O_2, 0.1 mM; iodide, (I) 10 μM; aminotriazole, 10 mM; azide, 1 mM; cyanide, 1 mM. The final volume was 0.5 ml.

‡ Mean±SE of (n) experiments.

§ The probability values are shown where significantly different from Toxo or Toxo/EPO alone ($P < 0.05$).

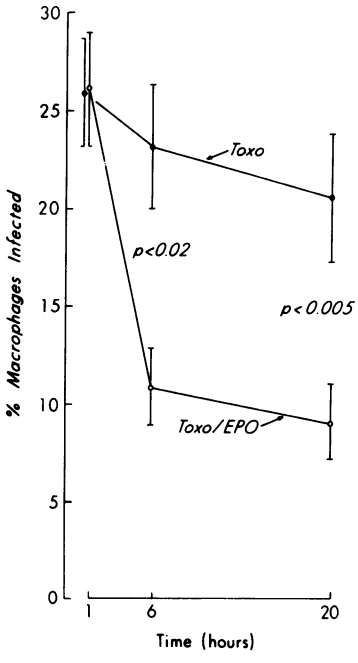

FIGURE 3 Survival and replication of Toxo and Toxo/EPO in resident mouse peritoneal macrophages. Toxo or Toxo/EPO were incubated with macrophage monolayers as described in Methods, extracellular organisms were removed by vigorous washing after 1 h, and the infected monolayers incubated for the periods indicated. The percentage of infected macrophages was determined by light microscopy after fixation and staining. Results represent the mean±SE of 15 (1 and 20 h time points) and 6 (6 h time points) experiments.

Toxoplasma by macrophages. Under the conditions used in Fig. 3, Toxo and Toxo/EPO infected equivalent numbers of macrophages after 1 h exposure as assessed microscopically (27 and 28% of cells infected, respectively). The number of ingested Toxo (1.4±0.03) and Toxo/EPO (1.3±0.02) per infected macrophage also was similar. However, 6 h after the initiation of the infection period, 23% of macrophages remained infected with Toxo whereas only 11% of macrophages contained Toxo/EPO ($P < 0.02$). Similarly, at 20 h, 21% of macrophages were infected with Toxo and 9% with Toxo/EPO ($P < 0.005$). This difference was not due to a greater loss of cells from the monolayer infected with Toxo/EPO. Thus in eight experiments, Toxo- and Toxo/EPO-infected monolayers contained 77±11 and 79±10 macrophages/grid field at 1 h, and 59±12 and 57±14 macrophages/grid field at 20 h after infection; furthermore, in five experiments, the total monolayer protein content at 20 h was comparable (29±1.1 and 27.2±5.5 μg protein/well, respectively). The enhanced rate of disappearance of Toxo/EPO from macrophages as compared with Toxo could be due to more efficient killing or to more efficient digestion of organisms. The latter appears unlikely since the percentage of macrophages containing recognizable Toxoplasma fell at the same rate when monolayers were challenged for 1 h with heat-killed organisms with and without bound EPO and subsequently incubated for periods up to 20 h. At the completion of the 20-h incubation, only 1% of macrophages contained recognizable organisms. Thus, the digestion of heat-killed organisms was unaffected by EPO, suggesting that EPO enhances the killing of Toxoplasma by macrophages.

The enhanced killing of Toxo/EPO by macrophages during the initial 6 h after infection was supported by electron microscopic studies (Fig. 4). The majority of Toxo within macrophages showed no loss of cellular detail 1 h after infection. In contrast, many Toxo/EPO, with peroxidase still evident on their surfaces, showed morphological changes consistent with death of the organism. Toxo/EPO that survived the early postphagocytic period replicated as well as did control Toxo. Thus at 20 h, infected macrophages contained 6.1±0.2 and 6.3±0.2 organisms/vacuole when infected with Toxo and Toxo/EPO.

Uptake of [^{3}H]uracil by macrophages infected with *T. gondii* is dependent on the survival and intracellular replication of the organisms (36); inhibition of uptake has been used as an index of macrophage toxoplasmacidal activity (37). As shown in Table II, macrophages, Toxo, or Toxo/EPO alone took up little [^{3}H]uracil during the 20-h culture period. However, macrophages infected with Toxo incorporated uracil in an amount (11,988 cpm) significantly greater than that taken up by macrophages infected with equivalent numbers of Toxo/EPO (5,524 cpm; $P < 0.01$). The 54% decrease in [^{3}H]uracil incorporation supports the decreased survival of Toxo/EPO within macrophages, as demonstrated microscopically.

The effect of the hemeprotein inhibitors aminotriazole and azide on the survival and replication of Toxo and Toxo/EPO in macrophages is shown in Table III. Aminotriazole markedly enhanced the survival of Toxo/EPO as assessed both by microscopic evaluation and by [^{3}H]uracil incorporation. Azide also was protective, although less so than aminotriazole at the concentrations used. Neither aminotriazole nor azide altered the survival of control Toxo, although fewer macrophages were infected after a 1-h challenge in the presence of azide. Cyanide could not be used in these experiments because it was toxic to the monolayers.

The enhanced killing of Toxo/EPO by macrophages was not associated with an increased respiratory burst as measured by increased O_2^- production or NBT reduction (Table IV). Indeed, a respiratory burst was not detected by these parameters with either Toxoplasma

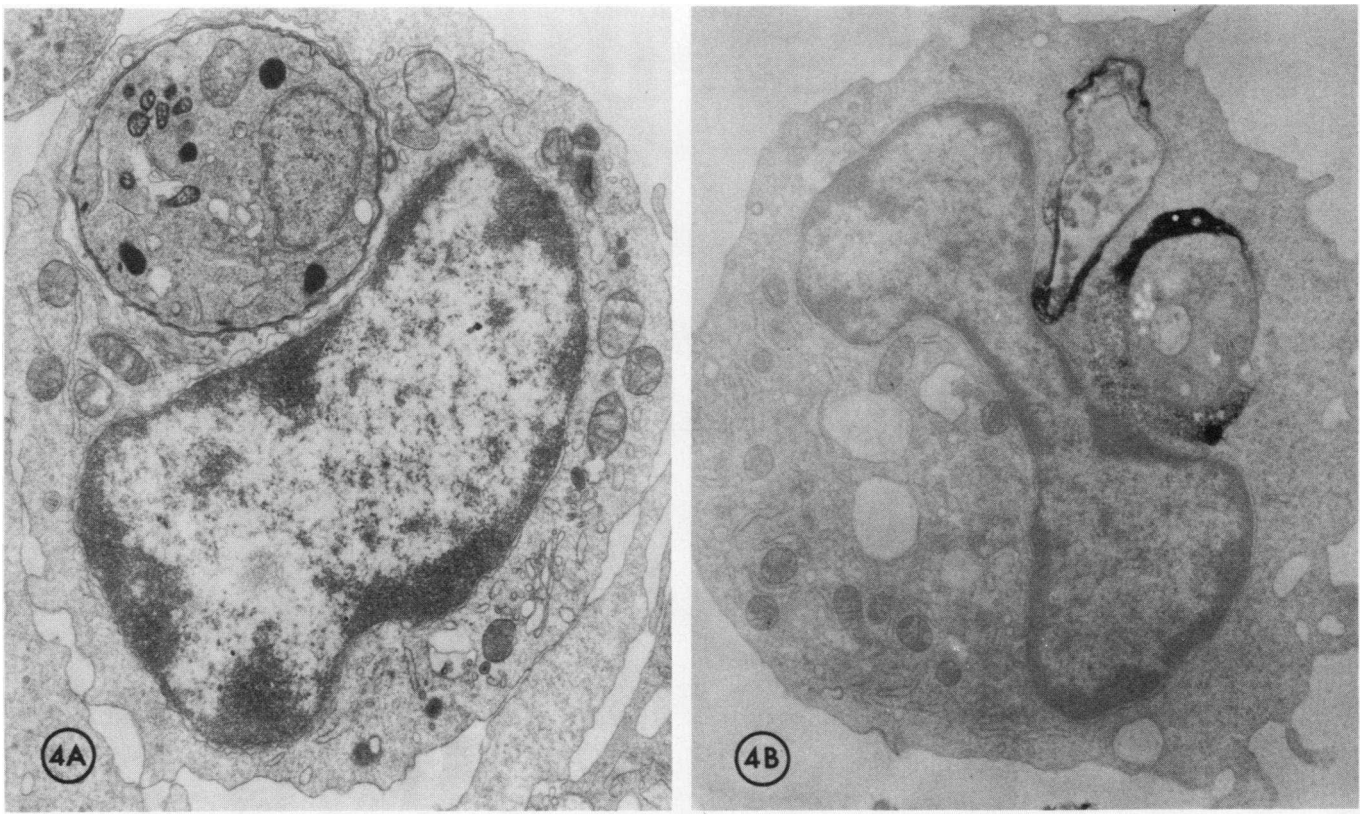

FIGURE 4 Ultrastructure of mouse peritoneal macrophages infected with Toxoplasma preparations. A. Macrophage fixed 1 h after incubation with Toxo. Note the absence of peroxidase-positive material and the preservation of cellular detail of the organism within the phagocytic vacuole (×14,200). B. Macrophage fixed 1 h after incubation with Toxo/EPO. Diaminobenzidine-positive EPO is evident in the phagolysosomes on the surface of the ingested organisms. Note the degeneration of intravacuolar organisms. This preparation was not stained with uranyl acetate or lead (×11,100).

preparation, in contrast to that seen with opsonized zymosan. This confirms earlier studies (2).

Survival and replication of Toxo and Toxo/EPO in normal and MPO-deficient human monocytes. To further elucidate the role of peroxidase in the enhanced destruction of Toxo/EPO by mononuclear phagocytes, monocytes from normal volunteers and from two individuals with hereditary MPO deficiency were infected with preparations of Toxo and Toxo/EPO (Fig. 5). When Toxo was used, comparable percentages of normal and MPO-deficient monocytes were infected following the 1 h challenge (12.6 and 14.5%, respectively), and the number of organisms per infected cell was similar (1.2±0.3 and 1.1±0.2, respectively). However, with continued incubation, Toxo was cleared more rapidly from normal than from MPO-deficient cells. Thus at 6 h only 4% of normal monocytes remained infected in contrast with 11% of MPO-deficient monocytes ($P < 0.05$). At 20 h the difference between the two cell populations was no longer significant. In contrast, when Toxo/EPO was used, organisms were cleared comparably by both cell populations. After a 1-h challenge, the percentages of infected normal and MPO-deficient monocytes were 17.6 and 19.2 and phagocytosis was identical (1.1±0.2 organisms/infected cell for both monocyte populations). However, Toxo-EPO was cleared as well by MPO-deficient monocytes as by normal cells at both 6 (3.8 and 4.9% monocytes infected) and 20 h (3.3 and 4.2% monocytes infected). Thus the toxoplasmacidal defect observed in MPO-deficient monocytes was abolished by coating the organisms with EPO. In contrast to macrophages, the Toxo and Toxo/EPO that survived the early postphagocytic period replicated poorly during the 20-h incubation, both in normal monocytes (1.2±0.2 and 1.1±0.1 organisms/vacuole), and in MPO-deficient monocytes (1.6±0.2 and 1.1±0.1 organisms/vacuole). The cell density of the normal and MPO-deficient monocyte monolayers was comparable at the time of infection (84±18 and 76±12

TABLE II

*Uptake of [^{3}H]Uracil by Resident Mouse Peritoneal Macrophages Incubated with Toxoplasma Preparations**

Preparation	[^{3}H]Uracil uptake	
	cpm	
Macrophages	737±116 (15)‡	
Toxo	847±103 (9)	
Toxo/EPO	803±88 (9)	
Macrophages + Toxo	11,988±1,819(15)	$P < 0.01$
Macrophages + Toxo/EPO	5,524±1,106(15)	

* To microtiter wells were added 10^5 macrophages and/or 2×10^5 Toxo or Toxo/EPO in M199 with 10% FBS in a final volume of 0.2 ml. [^{3}H]Uracil (1.25 μCi) was then added and its uptake was determined after incubation for 20 h at 37°C.
‡ Mean±SE of (*n*) experiments.

cells/grid field) and at 6 and 20 h after infection with either Toxo or Toxo/EPO, as assessed both microscopically and by protein content.

In two experiments, monocytes were challenged with heat-killed organisms with and without bound EPO. No difference in the percentage of normal and MPO-deficient cells containing organisms was seen following 1, 3, 6, and 20 h of incubation; at 20 h no Toxoplasma could be identified. Thus digestion of dead Toxoplasma by both types of monocytes was unaffected by EPO.

Survival and replication of Toxo and Toxo/EPO in CGD monocytes. CGD monocytes were used to investigate the role of the respiratory burst in the enhanced destruction of Toxo/EPO by mononuclear phagocytes. Monocytes from normal volunteers and from two subjects with CGD were incubated with Toxo and Toxo/EPO for 1 h. As shown in Fig. 6, CGD cells, like MPO-deficient monocytes, had a significant toxoplasmacidal defect at 6 h; however, in contrast to the results with MPO-deficient cells, this toxoplasmacidal defect was not abolished when Toxo/EPO was used. The difference in the percentages of infected normal and CGD monocytes was no longer significant at 20 h. However, the surviving control and EPO-coated organisms replicated better during the 20-h incubation in CGD monocytes (3.3±0.5 and 3.1±0.4 organisms/vacuole) than in normal monocytes (1.2±0.2 and 1.1±0.3 organisms/vacuole; $P < 0.05$, CGD vs. normal monocytes for both Toxo and Toxo/EPO). The cell density of the normal and CGD monocyte monolayers was comparable at the time of infection (79±14 and 64±18 cells/grid field) and, for both cell types, the monolayer cell densities and protein contents were comparable at 6 and 20 h following infection with either Toxo or Toxo/EPO.

In two experiments, the clearance of heat-killed Toxoplasma by normal and CGD monocytes at 1, 3, 6, and 20 h was comparable, suggesting that the respiratory burst is not required for digestion under these conditions. The binding of EPO to the dead Toxo-

TABLE III

*Effect of Hemeprotein Inhibitors on Survival of Toxoplasma Preparations in Resident Mouse Peritoneal Macrophages**

Hemeprotein inhibitor	Toxoplasma preparations	Cells Infected			[^{3}H]Uracil uptake at 20 h	
		1 h	20 h			
		%			*cpm*	
None	Toxo	28±6	21±5	$P < 0.05$	9,249±2,790	$P < 0.05$
	Toxo/EPO	29±6	10±1		3,329±969	
Aminotriazole	Toxo	23±4	16±1		8,995±2,414	
	Toxo/EPO	28±6	17±2		7,630±1,913	
Azide	Toxo	18±2	14±1		10,476±3,065	
	Toxo/EPO	17±1	11±1		7,123±1,616	

* Toxo and Toxo/EPO were incubated with macrophages as described in Methods except that aminotriazole (5 mM) and azide (0.5 mM) were added where indicated. For determination of percentage of infected cells, the hemeprotein inhibitors were present in the medium only during the 1-h infection period, whereas for measurement of [^{3}H]uracil uptake, the inhibitors were present throughout the 20-h incubation period. Uninfected macrophages took up 449±28 cpm in the absence of hemeprotein inhibitors, 412±62 cpm in the presence of aminotriazole, and 348±49 cpm in the presence of azide. The results are the mean±SE of five experiments.

plasma did not affect their rate of clearance by either cell type.

Survival and replication of Toxo and Toxo/EPO in human fibroblasts. Survival and replication of Toxo and Toxo/EPO were investigated in human embryonic tonsil fibroblasts to determine whether bound EPO would alter the survival of organisms within the intracellular environment of a nonprofessional phagocytic cell. In four experiments, Toxo and Toxo/EPO survived and replicated comparably in fibroblasts during the 20-h postinfection period as assessed both by microscopic evaluation and by [^{3}H]uracil uptake (Table V).

DISCUSSION

Earlier studies indicated that the toxic effects of macrophages on *T. gondii* correlate directly with the ability of these phagocytes to generate O_2^- and H_2O_2 and presumably their more distal reduction ($OH^\cdot$) and excitation ($^1O_2$) products (3, 11). When the xanthine oxidase system was used as a model of the oxygen-dependent antimicrobial systems of phagocytes, the toxicity of these products of oxygen metabolism (O_2^-, H_2O_2, $OH^\cdot$, $^1O_2$) was found to be considerably increased by the addition of peroxidase and a halide (38).

TABLE IV
O_2^- Generation by Resident Mouse Peritoneal Macrophages Ingesting Toxo, Toxo/EPO, or Zymosan

Preparation	O_2^- produced*	Quantitative NBT reduction‡	Qualitative NBT reduction§
	nmol/10^6 cells/h		*%*
Macrophages	0.4±0.3	2.4±0.1	1.3±0.1%
+ Toxo	0.4±0.1	3.8±1.1	3.8±0.4%
+ Toxo/EPO	0.3±0.4	4.2±0.5	4.8±0.4%
+ Zymosan	7.2±1.4‖	26.1±3.2‖	86.5±4.2%‖

* The reaction mixtures contained 10^6 macrophages, 80 μM ferricytochrome *c* and, where indicated 3×10^7 Toxo, 3×10^7 Toxo/EPO, or 7.5 mg opsonized zymosan in a final volume of 1.5 ml of Krebs-Ringer phosphate buffer pH 7.4 containing 5.5 mM glucose (KRPG).

‡ The reaction mixtures contained 0.2 mg NBT, 10^6 macrophages and, where indicated, 3×10^7 Toxo, 3×10^7 Toxo/EPO, or 5 mg opsonized zymosan in a final volume of 1 ml of KRPG. Incubation was for 1 h at 37°.

§ Macrophage monolayers were incubated alone or with 2×10^6 Toxo, 2×10^6 Toxo/EPO, or 2.5 mg opsonized zymosan in 0.5 ml M199-FBS containing 0.1 mg NBT for 1 h at 37°C. The percentage of cells containing formazan-stained organisms within phagocytic vacuoles was determined microscopically.

‖ Significantly different from macrophages alone ($P < 0.05$). All others NS.

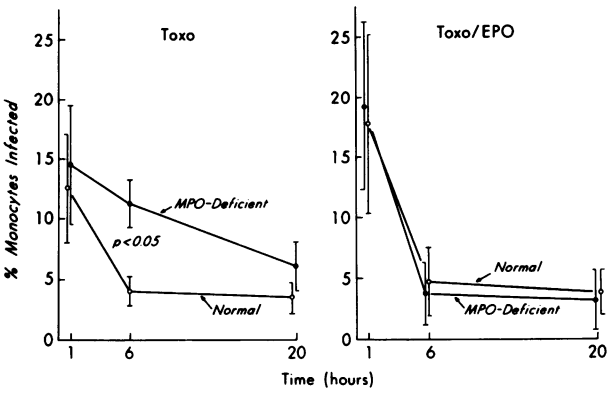

FIGURE 5 Survival and replication of Toxo and Toxo/EPO in normal and myeloperoxidase-deficient human monocytes. Toxo or Toxo/EPO were incubated with monocyte monolayers (Methods) and, at 1 h, extracellular organisms were removed and the infected monolayers incubated for the periods indicated. The percentage of infected monocytes was determined by light microscopy after fixation and staining. Results represent mean±SE of four experiments.

This amplification of toxicity by peroxidase also has been observed using Toxoplasma as the target (8). Since resident peritoneal macrophages (*a*) lack a granule peroxidase that can be used for microbicidal activity (1), (*b*) respond to stimulation with a weak respiratory burst (10), and (*c*) do not kill or inhibit the replication of intracellular Toxoplasma (2, 3), an attempt was made to potentiate the toxoplasmacidal activity of these cells by the introduction of peroxidase. EPO was used for this purpose.

EPO was found to bind firmly to the surface of *T. gondii* with no apparent toxicity to the organism as assessed by vital staining, [^{3}H]2DG uptake, and electron microscopic morphologic evaluation. The predominant membrane proteins of *T. gondii* are acidic (24), providing a basis for the firm binding of the strongly cationic EPO to the cell surface. Cationic

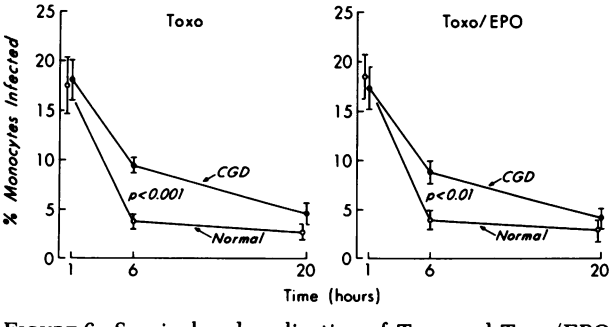

FIGURE 6 Survival and replication of Toxo and Toxo/EPO in normal and CGD monocytes. Monolayers were prepared and infected as in Fig. 5. Results represent mean±SE of four experiments.

TABLE V

Survival and Replication of Toxo and Toxo/EPO in Human Fibroblasts

Preparation	Cells infected*		Mean No. organisms per vacuole at 20 h‡	[^{3}H]Uracil uptake at 20 h§
	1 h	20 h		
	%			*cpm*
Fibroblasts + Toxo	26±4	49±15	7.4±0.6	29,137±8,791
Fibroblasts + Toxo/EPO	26±5	47±16	7.1±0.4	28,422±7,528

* The percentage of infected cells was determined microscopically at the indicated time after addition of Toxoplasma preparations to the monolayers. Results are the mean±SE of four experiments, each with duplicate determinations.

‡ Replication of Toxoplasma expressed as the mean number of organisms per vacuole as determined microscopically at 20 h. Results are the mean±SE of four experiments.

§ The reaction mixture and assay conditions were the same as used in Table II except that fibroblasts were maintained in monolayers overlayed with 0.1 ml MEM with 10% FBS and challenged with 5×10^5 Toxo or Toxo/EPO in MEM with 10% FBS. The results are the mean±SE of four experiments, each with quadruplicate determinations. Fibroblasts alone took up 175±43 cpm.

proteins, including those from eosinophils (39–42), can be directly toxic to cells; if this is the case with EPO, the amount bound, under the conditions used here, is inadequate for this effect. Toxoplasma with surface-bound EPO are, however, rapidly killed by the addition of H_2O_2 and iodide. H_2O_2 was effective under these conditions at a concentration (0.1 mM), which was considerably less than that required to kill organisms without EPO on their surface (50 mM; data not shown). Killing of EPO-coated Toxoplasma was largely prevented by the hemeprotein inhibitors aminotriazole, azide, and cyanide, with cyanide the least effective as noted previously in other EPO-dependent cytotoxic systems (26, 43).

In contrast to control Toxoplasma, resident mouse peritoneal macrophages were able to kill Toxoplasma with surface-bound EPO, as assessed both microscopically and by [^{3}H]uracil uptake. EPO did not affect the rate of digestion of heat-killed organism, suggesting that its effect on viable organisms is to potentiate killing rather than digestion. Why all EPO-coated organisms are not killed by the macrophages is unclear. This might reflect either heterogeneity in the macrophage population or the ability of Toxoplasma to cap and shed surface-bound molecules (44).

There are a number of lines of evidence that suggest that the enhanced toxoplasmacidal activity observed when Toxo/EPO is ingested by resident peritoneal macrophages is due to the interaction of macrophage-derived H_2O_2 with the EPO on the organism surface.

(*a*) Peroxidase was detected in the phagosome on the surface of the ingested organism by electron microscopic cytochemical studies.

(*b*) The enhanced toxoplasmacidal activity was inhibited by aminotriazole and azide. These hemeprotein inhibitors are commonly used in concentrations employed here as evidence of peroxidatic mechanisms in phagocytes (15). Hemeprotein inhibitors also may affect other parasite or macrophage heme-containing enzymes; however, control Toxoplasma incubated with macrophages in the presence of the hemeprotein inhibitors survived and replicated normally, suggesting the absence of a significant effect on organisms or macrophages in the absence of EPO under the conditions used. Azide is also a scavenger of singlet oxygen (45) and hydroxyl radicals (46); however, higher concentrations than those used here are generally required for azide to compete effectively with cellular scavengers of these agents.

(*c*) Peroxidase appears to be required for the optimum killing of Toxoplasma by monocytes. Thus monocytes from patients with hereditary MPO deficiency had a toxoplasmacidal defect evident during the initial 6 h postinfection. The toxoplasmacidal defect of MPO-deficient monocytes was completely reversed by EPO bound to the surface of the ingested organisms, emphasizing the role of peroxidase in early postphagocytic toxoplasmicidal events.

(*d*) A respiratory burst (and presumably H_2O_2 production) appears to be required for the enhanced destruction of EPO-coated organisms by mononuclear phagocytes. Thus the toxoplasmacidal defect seen in monocytes that lack a respiratory burst, i.e., from patients with CGD, is unaffected by surface-bound EPO. Furthermore, EPO-coated Toxoplasma survive normally in human fibroblasts, which do not respond to

phagocytosis with a respiratory burst. Our data also suggest a role for the respiratory burst in the inhibition of replication of ingested Toxoplasma, as suggested by others (11). Thus replication of intracellular organisms at 20 h was greatest in the fibroblasts (7.4 organisms/vacuole), slightly less in the macrophages (6.1 organisms/vacuole), and significantly lower in the monocytes (1.2 organisms/vacuole). Of the various monocyte populations studied here, replication was greatest in CGD monocytes (3.3 organisms/vacuole), cells which do not respond to phagocytosis with a respiratory burst. We were unable to measure enhanced O_2^- generation or NBT reduction by resident macrophages ingesting Toxo or Toxo/EPO and conclude that toxicity to peroxidase-coated organisms is a more sensitive measure of the small respiratory burst in these cells.

While the studies described here, and those of others (2, 3), emphasize the role of oxygen metabolites in the killing of Toxoplasma by phagocytes and the amplification of this effect by peroxidase, it should be stressed that oxygen-independent antimicrobial systems also appear to be effective against Toxoplasma in monocytes. Thus the toxoplasmacidal activity of CGD monocytes, while depressed, is not abolished, indicating the presence of toxic systems in these cells which are not dependent on the respiratory burst. EPO-coated organisms were not cleared more efficiently by CGD monocytes than were uncoated organisms, suggesting that bound EPO does not render Toxoplasma more susceptible to nonoxidative killing mechanisms. The contrast between the toxoplasmacidal activity of human blood monocytes and resident mouse macrophages indicates that the macrophages in the absence of peroxidase lack effective oxygen-independent and oxygen-dependent mechanisms.

Can the toxicity of resident macrophages be amplified by acquired peroxidase in vivo? This question cannot be answered definitively at the present time; however, several lines of evidence suggest that the mechanism described here may have an in vivo counterpart. Moderate numbers of eosinophils are found in foci of inflammation in human toxoplasmosis (47, 48). Further, intraperitoneal infection of mice with Toxoplasma (2×10^3) causes an increase in peritoneal fluid eosinophils from 0.1% of total cells (1.6×10^3 eosinophils) to 9% (7.5×10^5 eosinophils) at 5 d postinfection. An influx of neutrophils also occurs while macrophages are the predominant cell type throughout the infection period (unpublished data). Macrophages in inflammatory foci may acquire phagosomal peroxidase by ingesting cellular debris, including the peroxidase-positive granules of neutrophils and eosinophils (21). Such macrophages are able to iodinate protein (22), a characteristic of phagocytes with active granule peroxidase (49), but not of normal resident macrophages (50). As shown here, peroxidase, particularly EPO, released at an inflammatory site also may coat adjacent organisms and be ingested by macrophages in this way. Another mechanism for the coating of protozoan parasites by eosinophil granule material was suggested by the studies of Sanderson and de Souza (51). They found that trypomastigotes of *Trypanosoma cruzi* ingested by esoinophils were transformed in the phagosome into amastigotes. Degranulation occurred and the amastigotes were released from the cell by an exocytotic process, with eosinophil granule material coating their surface. Such organisms would presumably be available for ingestion by macrophages. We have found that mice infected intraperitoneally with Toxo/EPO (2×10^3) survive significantly longer (11.9±0.3 d) than mice infected with control Toxoplasma (7.8±0.7d; $P < 0.002$) (unpublished data) indicating that protection is afforded to the host by EPO on the surface of the organism.

Our findings suggest a mechanism by which peroxidase-positive phagocytes and peroxidase-negative macrophages may interact early in infection and afford some protection to the host before the development of macrophage activation.

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