FAILURE TO TRIGGER THE OXIDATIVE METABOLIC BURST BY NORMAL MACROPHAGES

Possible Mechanism for Survival of Intracellular Pathogens*

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Reactive oxygen metabolites produced during phagocytosis are important for the microbicidal activity of granulocytes and probably of monocytes as well (1, 2). Although the role of reactive oxygen metabolites in the microbicidal activity of macrophages is not established, recent studies (3–6) suggest that they may be important because activated macrophages that exhibit enhanced antimicrobial activity produce increased amounts of these metabolites in response to various stimuli. Because reactive oxygen metabolites are potentially lethal, organisms that survive within phagocytes must either fail to stimulate production of such substances or must be resistant to or fail to encounter them. One such intracellular organism is *Toxoplasma gondii.* This organism survives and replicates in vitro within human monocyte-derived macrophages and within normal mouse macrophages (7–10). However, other mononuclear phagocytes can kill or inhibit multiplication of Toxoplasma. For example, we recently have observed that Toxoplasma is rapidly destroyed by freshly isolated human blood monocytes (11). In addition, macrophages can be activated to kill or inhibit intracellular replication of Toxoplasma (7–9), and normal macrophages can kill the organism if it is coated with antibody (12, 13). Because the ability of Toxoplasma to survive within mononuclear phagocytes depends upon the stage of differentiation or state of activation of mononuclear phagocytes and also depends upon whether the organism is opsonized, we studied the oxidative metabolic burst by different mononuclear phagocytes during phagocytosis of Toxoplasma. These studies were performed to determine the relationship between survival of this intracellular pathogen and production of toxic oxygen metabolites by these cells. The results revealed that the ability of this organism to survive within certain mononuclear phagocytes is dependent in part upon its being phagocytosed without stimulating production of reactive oxygen metabolites by these cells.

Materials and Methods

Special Reagents. Reagents were obtained from Sigma Chemical Co., St. Louis, Mo., unless

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otherwise indicated. Nitroblue tetrazolium (NBT)[1] was dissolved in Krebs-Ringer phosphate buffer (pH 7.4) with 5.5 mM glucose (KRPG), and superoxide dismutase (SOD) (≃2,900 U/mg) was dissolved in phosphate-buffered saline (pH 7.2) (PBS); each was sterilized by filtration before use. When indicated, SOD was inactivated by autoclaving at 120°C for 20 min; this resulted in the loss of ≃ 90% of activity.

Cell Preparations. Blood was obtained from healthy adult donors and from a child with X-linked chronic granulomatous disease (CGD), his heterozygote mother, and his normal sister; each donor lacked antibodies to Toxoplasma in the Sabin-Feldman dye test (DT). Leukocytes were obtained by separation of blood on Ficoll (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) -Hypaque (Winthrop Laboratories, New York) (FH) gradients (14). The erythrocyte-polymorphonuclear leukocyte (PMN) pellet was resuspended in Tris-buffered 0.83% NH_4Cl for 10 min to lyse erythrocytes, and PMN were collected by centrifugation. PMN and mononuclear cells were washed in KRPG and resuspended in KRPG; these are referred to as human PMN and human mononuclear cell preparations (MCP). For preparation of monolayers of monocytes, mononuclear cells were washed twice in Hanks' balanced salt solution (HBSS) (Grand Island Biological Co., Grand Island, N. Y.) and resuspended in Medium 199 (Grand Island Biological Co.) that contained 100 U/ml penicillin G, 100 μg/ml streptomycin, 2 mM L-glutamine (M199), and 40% autologous serum (M199 + AS) before plating. In some experiments, mononuclear cells were depleted of monocytes by using a magnet to remove cells that phagocytosed carbonyl iron and then by using glass adherence.

Macrophages were derived by in vitro culture of human monocytes for 5–6 d, as previously described (7). Transformation of monocytes into macrophages was confirmed by characteristic morphology (15) and by absence of histochemically detectable peroxidase (16). Human macrophages were obtained in suspension by washing Petri dishes in HBSS, incubating monolayers in Ca^{++}- and Mg^{++}-free HBSS at 4°C for 30 min to facilitate cell detachment, and gently resuspending the cells with a rubber policeman. The macrophages in suspension were then washed twice in KRPG and resuspended in KRPG.

Unelicited peritoneal cells were obtained from 6- to 8-wk-old Swiss Webster female mice (Simonsen Laboratories, Gilroy, Calif.), as previously described (17). Peritoneal cells were resuspended in M199 that contained 20% heat-inactivated fetal calf serum (M199 + FCS) or were washed once in KRPG and resuspended in KRPG, as described for human MCP; when used in suspension, these are referred to as mouse MCP. In some experiments, peritoneal cells were depleted of phagocytic and adherent cells, as described for human mononuclear cells. Peritoneal cell preparations rich in PMN were obtained from mice injected intraperitoneally 16 to 18 h previously with 1 ml of sterile thioglycollate broth. Mouse MCP that contained macrophages that were activated (i.e., that inhibited intracellular replication of Toxoplasma [9, 18]) were obtained from mice chronically infected with *T. gondii* or from mice injected intraperitoneally 7 d earlier with 1,400 μg of formalin-killed *Corynebacterium parvum* (lot no. CA582A; Burroughs Wellcome Co., Research Triangle Park, N. C.). Control MCP were obtained from age-matched mice. To decrease the number of contaminating PMN, cells from *C. parvum*-injected mice and cells from the controls for these experiments were centrifuged on FH gradients before washing in KRPG.

Human and mouse MCP were used within 1 h after they were isolated. The percentage of PMN in cell preparations was determined by differential counts of Giemsa-stained smears; the percentage of monocytes or macrophages was determined by phagocytosis of neutral red particles (14, 19). Human MCP contained 21.0 ± 1.1% monocytes and 1.2 ± 0.3% PMN; human macrophage preparations contained 100% macrophages; MCP from normal mice contained 75.1 ± 2.5% macrophages and 2.8 ± 0.9% PMN; MCP from mice injected with *C. parvum* contained 47.8 ± 7.9% macrophages and 3.0 ± 0.9% PMN; and MCP from mice chronically infected with *T. gondii* contained 45.0 ± 3.8% macrophages and 1.3 ± 0.5% PMN.

[1] *Abbreviations used in this paper:* CGD, chronic granulomatous disease; DT, Sabin-Feldman dye test; FH, Ficoll-Hypaque; HBSS, Hanks' balanced salt solution; HMP, hexose monophosphate; H_2O_2, hydrogen peroxide; KRPG, Krebs-Ringer phosphate buffer (pH 7.4) with 5.5 mM glucose; M199, Medium 199 that contained 100 U/ml penicillin G, 100 mg/ml streptomycin, 2 mM L-glutamine; M199 + AS, M199 with 40% autologous serum; M199 + FCS, M199 that contained 20% heat-inactivated fetal calf serum; MCP, mononuclear cell preparation(s); NBT, nitroblue tetrazolium; O_2^-, superoxide anion; PBS, phosphate-buffered saline (pH 7.2); PMN, polymorphonuclear leukocyte(s); SOD, superoxide dismutase.

Organisms and Latex. Yeast-phase *Candida albicans* were killed by boiling for 30 min and were washed in PBS and stored at 4°C in PBS. Latex particles (0.3- or 2-μm in diameter; Dow Chemical Co., Midland, Mich.) were washed six times in PBS, sonicated to produce a uniform suspension, and diluted to 2% in KRPG before use. The RH strain of *T. gondii* was obtained and processed as previously described (9), except that polycarbonate membrane filters (3-μm pore diameter; Nuclepore Corp., Pleasanton, Calif.) were used to purify Toxoplasma. Such preparations contained < 1 leukocyte/10^4 Toxoplasma.

Quantitative Reduction of NBT. Quantitative reduction of NBT was determined by a modification of the method of Baehner and Nathan (20). Candida were opsonized at 37°C for 30 min in 2 ml of KRPG and 2 ml of human type AB serum, washed, and resuspended in KRPG. Toxoplasma were washed once and resuspended in KRPG. Both Candida and Toxoplasma were then counted in a hemacytometer and adjusted to the desired concentration. In some experiments, Toxoplasma were incubated at 37°C for 30 min in 80% immune serum and 20% KRPG, centrifuged, washed twice in KRPG, and resuspended in KRPG; such organisms were antibody coated (12). Immune sera were from two human subjects (DT titers of 1:32 and 1:256) or from mice chronically infected with Toxoplasma (DT titer of 1:4096; this was diluted 1:10 in normal serum before use). To insure that organisms would remain viable, immune sera were heat inactivated (12) at 53°C for 90 min before use (21). DT-negative human and mouse sera were used as controls. Results of experiments in which Toxoplasma were incubated in heat-inactivated or noninactivated DT-negative serum were identical with those in which they were not incubated in serum.

Components of the reaction mixtures, as indicated in the appropriate table, were added to siliconized glass tubes, and tubes were placed on a rotater in a 37°C incubator and rotated at 100 cycle/min for 30 min. This time period was found in preliminary experiments to result in maximum increments in NBT reduction between resting and phagocytosing cell preparations. Thereafter, reaction mixtures were processed and assayed as described by Baehner and Nathan (20), except that the reduced dye was extracted in 3 ml of pyridine. The amount of NBT reduced was calculated with the extinction coefficient E = 2.8 mM^{-1} cm^{-1} (22).

Intracellular Location of Reduced NBT. Monolayers of each cell type were prepared in Lab-Tek tissue culture chamber/slides (Lab-Tek Div., Miles Laboratories Inc., Naperville, Ill.) (23). After medium was aspirated from the monolayers, 0.5 ml that contained 1×10^6 Candida or 2×10^6 Toxoplasma and 0.1 ml of NBT (1 mg/ml in M199) was added. Identical results were observed with monolayers of each cell type whether organisms were suspended in M199 + AS, in M199 with 20% human type AB serum, or in M199 + FCS (all sera were negative in the DT). Slides were incubated at 37°C for varying periods of time, rinsed in warmed saline, fixed in methanol, and counterstained with 0.2% safranin. The percentage of cells that contained formazan-stained organisms within phagocytic vacuoles was determined microscopically by counting 100–200 cells that contained organisms (24). Only cells that contained organisms that were stained deeply blue were scored as formazan positive. Cells that contained only diffuse, fine deposits of formazan were present in similar numbers in challenged and unchallenged monolayers; they were therefore scored as negative.

Intracellular survival and replication of Toxoplasma within monocytes and macrophages was evaluated as previously described (9).

Chemiluminescence. Chemiluminescence was determined as described by Rosen and Klebanoff (25). Components of the reaction mixtures (experiments performed in duplicate) are indicated in the appropriate figure. The reaction was initiated by addition of organisms. Vials were placed in the scintillation counter, counted for 24 s, and then placed in a 37°C shaking water bath at 20 cycle/min. At intervals, vials were blotted dry and returned to the scintillation counter, counted for 24 s, and then placed back in the water bath. Background activity of empty vials was subtracted from test values; results are expressed as counts per minute above background.

$^{14}CO_2$ Production from [1-^{14}C]glucose. Oxidation of [1-^{14}C]glucose was determined by a modification of the method of Keusch et al. (26). Components of the reaction mixtures, as indicated in the appropriate table, were added to center-well flasks, and flasks were placed on a rotator in a 37°C incubator and rotated at 100 cycle/min for 60 min. $^{14}CO_2$ was trapped in a cup that contained hyamine hydroxide, transferred to a glass scintillation vial that contained 10 ml of

Aquasol (New England Nuclear Co.), and counted in a liquid scintillation counter.

Evaluation of Phagocytosis. At the end of the reaction period in each of the quantitative assays, a replicate reaction mixture of each type or a portion of each reaction mixture was diluted with an equal volume of ice-cold 10 mM disodium ethylenediaminetetraacetate to stop phagocytosis and deposited on glass slides with a cytocentrifuge. In a few experiments, replicate reaction mixtures were not available because of an inadequate number of cells. Cytocentrifuge preparations were fixed in methanol, stained with Giemsa stain, examined microscopically, and scored for phagocytosis by determining the percentage of cells that contained organisms and the total number of organisms ingested per 100 cells. Results are expressed as a phagocytic index, which is the mean number of organisms ingested per cell.

The ratios of organisms to phagocytes used in each of the quantitative assays were found in preliminary experiments to produce maximum increments in oxidative metabolic activity above base-line values and maximum phagocytic indices at the end of the reaction period. In some assays, a higher Candida:cell ratio resulted in a greater initial rate of metabolic activity but $<10\%$ difference at the end of the reaction period. We found, as have others (27), that accurate determination of phagocytosis of latex particles by cell suspensions was not possible by microscopic techniques. However, the ratios employed produced maximum increments in metabolic activity, and $> 90\%$ of monocytes or macrophages ingested one or more latex particles by the end of the reaction period.

Phagosome-Lysosome Fusion. Fusion of lysosomes with phagosomes that contained Toxoplasma was assessed by the acridine-orange method of D'Arcy Hart and Young (28). Monolayers of human monocytes and mouse macrophages were cultured on glass cover slips in wells of Linbro trays (FB-16-24-TC; Linbro Chemical Co., Hamden, Conn.) and challenged with Toxoplasma as described above, except that, before preparation of monolayers, MCP were incubated in HBSS that contained 20 μg/ml of acridine orange for 15 min, washed in HBSS, resuspended in medium, and added to wells. At various intervals after the addition of Toxoplasma, monolayers were examined in a fresh state with a Zeiss incident light fluorescence microscope (Carl Zeiss, Inc., New York) equipped with an acridine-orange filter set. Phagosome-lysosome fusion was considered to have occurred when Toxoplasma, which were initially unstained, became diffusely orange.

Data Presentation and Statistical Analysis. For values presented in the text, quantitative NBT reduction and $^{14}CO_2$ production stimulated by phagocytosis were determined by subtracting counts per minute for resting cells (and, when appropriate, by also subtracting counts per minute for Toxoplasma preparations alone) from counts per minute for phagocytosing cells. Corrected and uncorrected values and values for preparations of resting cells and Toxoplasma are given in the tables. Mean ± standard error was employed unless otherwise indicated. Statistical differences between means were determined using the Student's t test. Linear regression lines were fitted to data points by the least squares method, and the significance of the correlation coefficient (r) was determined by Student's t test.

Results

Quantitative Reduction of NBT. NBT reduction increased significantly ($P < 0.001$) during phagocytosis of Candida by each type of cell preparation (Table I). The increase in NBT reduction by human MCP and human macrophages was significantly greater ($P < 0.001$) than that by normal mouse MCP. Phagocytosis of latex particles also effectively stimulated NBT reduction by each type of cell preparation studied.

The increase in NBT reduction by human MCP during phagocytosis of Toxoplasma was significantly greater than NBT reduction by resting cells ($P < 0.001$) although less than that observed during phagocytosis of Candida or latex particles. In contrast, the increase in NBT reduction by human macrophages and by normal mouse MCP during phagocytosis of Toxoplasma was not significantly greater than NBT reduction by resting cells. Relative amounts of NBT reduction were similar when the reaction time was prolonged from 30 to 60 or 90 min in three experiments with human MCP

TABLE I

*Quantitative Reduction of NBT**

Cell preparation	Phagocytic stimulus				Δ Toxoplasma/Δ Candida‖
	None	Candida	Latex particles	Toxoplasma	
	NBT red.‡	Δ NBT red.§	Δ NBT red.	Δ NBT red.	
Human mononuclear (11)¶	2.9 ± 1.0‖‖	43.6 ± 4.5	39.5 ± 8.3	11.2 ± 1.4	0.25 ± 0.04
Human macrophage (4)	2.1 ± 1.7	40.0 ± 8.3	72.1 ± 13.6	1.9 ± 0.7¶¶	0.04 ± 0.01¶¶
Mouse mononuclear					
Normal** (16)	1.9 ± 0.2	17.9 ± 1.7***	9.5 ± 2.9	1.9 ± 0.5‡‡‡	0.08 ± 0.02‡‡‡
C. parvum‡‡ (4)	4.3 ± 0.7	20.7 ± 5.2	ND§§§	6.7 ± 2.1‖‖‖	0.31 ± 0.04‖‖‖
Chronic Toxoplasma§§ (5)	3.8 ± 0.5	21.4 ± 1.9	ND§§§	7.1 ± 2.4¶¶¶	0.31 ± 0.09¶¶¶

* Reaction mixtures (in duplicate) contained 0.1 ml of NBT (2 mg/ml); 0.25 ml of the suspension of Candida, 2 μm latex particles, or Toxoplasma (or, in controls, 0.25 ml KRPG); 0.5 ml of cell preparations that contained 1.5×10^6 monocytes or macrophages; and sufficient KRPG to effect a final volume of 1 ml in siliconized glass tubes. The final ratio of organisms or particles to cells was 10:1 for Candida, ≈600:1 for 2 μm latex particles, and 25:1–30:1 for Toxoplasma.

‡ Nanomoles of NBT reduced (red.) per 10^6 monocytes or macrophages per 30 min.

§ Increase in nanomoles of NBT reduced per 10^6 monocytes or macrophages per 30 min after subtraction of values for resting leukocytes and for organisms alone. Candida and latex particles alone did not reduce NBT. NBT reduction by Toxoplasma preparations alone was 2.4 ± 0.5, 0.3 ± 0.3, and 1.2 ± 0.5 nmol/30 min for experiments with human MCP, human macrophage cell preparations, and mouse MCP, respectively.

‖ Increase in NBT reduction during phagocytosis of Toxoplasma/increase in NBT reduction during phagocytosis of Candida.

¶ Numbers in parentheses are the number of experiments performed with controls, Candida, and Toxoplasma; the number of experiments with latex particles was 4, 2, and 2 for human MCP, human macrophage cell preparations, and normal mouse MCP, respectively.

** MCP from normal mice.

‡‡ MCP from mice given 1,400 μg formalin-killed *C. parvum* intraperitoneally 7 d previously.

§§ MCP from mice chronically infected with C56 strain of Toxoplasma.

‖‖ Mean ± SEM.

¶¶ $P < 0.005$ compared with human MCP.

*** $P < 0.001$ compared with human MCP and human macrophage cell preparations.

‡‡‡ $P < 0.001$ compared with human MCP.

§§§ ND, not determined.

‖‖‖ $P < 0.02$ compared with mouse MCP from age-matched normal controls that were studied in parallel.

¶¶¶ $P < 0.05$ compared with mouse MCP from age-matched normal controls that were studied in parallel.

(Candida 38.6 ± 6.7 nmol; Toxoplasma 11.7 ± 1.4 nmol), in three experiments with normal mouse MCP (Candida 15.5 ± 4.2 nmol; Toxoplasma 0.7 ± 0.5 nmol), and in two experiments with human macrophages (Exp. 1: Candida 43.8 nmol, Toxoplasma 6.4 nmol; Exp. 2: Candida 18.1 nmol, Toxoplasma 1.2 nmol).

MCP from mice chronically infected with Toxoplasma or from mice injected with *C. parvum* (MCP that contained activated macrophages) reduced significantly more NBT during phagocytosis of Toxoplasma than did normal mouse MCP (Table I). Mouse MCP that contained activated macrophages also reduced more NBT during phagocytosis of Candida than did normal mouse MCP; however, these differences were not significant.

To determine whether phagocytosis of Toxoplasma failed to stimulate or inhibited NBT reduction by normal mouse or human MCP, two experiments were performed in which these cell preparations were challenged with Toxoplasma (or, as a control, an equal volume of KRPG) and after 15 min also challenged with Candida. The reaction was then allowed to proceed for 30 min. NBT reduction during phagocytosis of Toxoplasma and Candida (12.6 ± 1.9 nmol) by normal mouse MCP did not differ significantly from NBT reduction during phagocytosis of Candida alone (10.7 ± 0.2 nmol). Results in the experiments with human MCP were comparable. Thus, phagocytosis of Toxoplasma did not inhibit NBT reduction.

Because Toxoplasma were obtained from the peritoneal cavities of mice, something in the Toxoplasma preparations other than organisms might have been responsible

TABLE II

Contribution of Different Types of Leukocytes to Reduction of NBT

Source of cells	Cell preparation							
	Mononuclear*		PMN‡		Monocyte depleted§		Monocyte enriched‖	
	Candida¶	Toxoplasma¶	Candida	Toxoplasma	Candida	Toxoplasma	Candida	Toxoplasma
	Δ NBT red.**	Δ NBT red.	Δ NBT red.	Δ NBT red.	Δ NBT red.	Δ NBT red.	Δ NBT red.	Δ NBT red.
Human (2)‡‡	74.7 ± 2.9§§	21.5 ± 4.7			10.0 ± 3.9	1.8	43.9 ± 0.0	13.9 ± 1.8
Human (3)	39.3 ± 17.9	8.6 ± 3.9	3.2 ± 3.2	0.0 ± 0.0				
Mouse (2)	34.6 ± 13.6	ND‖‖			4.6 ± 1.1	ND	ND	ND
Mouse (1)	32.1	ND	2.9	ND				

* Unseparated MCP adjusted to contain 1.5×10^6 monocytes (human) or normal macrophages (mouse).

‡ Human PMN cell preparations contained >95% PMN, and PMN reaction mixtures contained 1.5×10^5 PMN; in the same experiments, MCP contained $0–1.4 \times 10^5$ PMN. The mouse PMN-enriched cell preparation contained 53% PMN, and PMN reaction mixture contained 7.5×10^4 PMN; the corresponding MCP contained 3×10^4 PMN.

§ MCP depleted of monocytes (human) or macrophages (mouse). Reaction mixtures contained lymphocytes in a number equal to that in the initial MCP and contained ≃25% the number of human monocytes or ≃15% the number of normal mouse macrophages in the initial MCP. Viability was 98–99% by trypan blue exclusion.

‖ Freshly isolated mononuclear cells were allowed to adhere to glass for 30 min, washed to remove nonadherent cells, and resuspended as described for human macrophages (see text). The reaction mixtures contained an equal number of monocytes but 10% as many lymphocytes as the initial MCP. Viability was 100% by trypan blue exclusion.

¶ Phagocytic stimulus.

** Increase in nanomoles of NBT reduced (red.) per reaction mixture per 30 min after subtraction of values for resting leukocytes and for organisms alone.

‡‡ Numbers in parentheses are the number of experiments performed.

§§ Mean ± SEM. Single experiment where no SEM is shown.

‖‖ ND, not determined.

for stimulation of NBT reduction by human MCP. However, addition of supernate from a Toxoplasma preparation did not stimulate NBT reduction by these cell preparations.

The procedure used to prepare suspensions of human macrophages did not result in their inability to reduce NBT during phagocytosis of Toxoplasma. NBT reduction during phagocytosis of Toxoplasma by human adherent mononuclear cells (75–80% monocytes) that were resuspended 45 min after addition to glass Petri dishes by the same procedure used to resuspend human macrophages was similar to that observed with the initial MCP (Table II). Also, the ratios of NBT reduction (90-min reaction period) during phagocytosis of Toxoplasma to those during phagocytosis of Candida by monolayers of human monocytes (Toxoplasma 13.6 nmol/Candida 35.0 nmol = 0.39) and by monolayers of human macrophages (Toxoplasma 2.7 nmol/Candida 40.7 nmol = 0.07) were similar to ratios obtained with the corrresponding cell preparations in suspension.

As reported by others (22, 29–31), SOD or SOD adsorbed to latex particles (but not inactivated SOD) partially inhibited NBT reduction in a concentration-dependent fashion (data not shown). These data indicate the role of superoxide anion (O_2^-) in NBT reduction by intact phagocytes.

Phagocytosis of Organisms. Phagocytosis of Candida and Toxoplasma by the different types of mononuclear phagocytes is shown in Table III. Because results of phagocytosis in experiments on quantitative NBT reduction and $^{14}CO_2$ production were similar, they have been pooled. The data show that human monocytes ingested fewer Toxoplasma than did human or mouse macrophages. To allow comparison with the amount of NBT reduced, we calculated the ratio of the mean phagocytic index for

TABLE III

*Phagocytosis of Organisms**

Cell preparation	Organism	
	Candida	Toxoplasma
Human mononuclear (11)‡	2.8 ± 0.3**	0.7 ± 0.1
Human macrophage (4)	4.5 ± 0.6	2.4 ± 0.9
Mouse mononuclear		
Normal§ (16)	3.8 ± 0.3	1.3 ± 0.1
C. parvum‖ (4)	4.2 ± 1.2	1.5 ± 0.2
Chronic Toxoplasma¶ (5)	4.4 ± 0.5	1.2 ± 0.4

* Results in experiments on quantitative NBT reduction and $^{14}CO_2$ production have been pooled because the values were similar.

‡ Numbers in parentheses are the number of determinations.

§ MCP from normal mice.

‖ MCP from mice given 1,400 μg formalin-killed *C. parvum* intraperitoneally 7 d previously.

¶ MCP from mice chronically infected with C56 strain of Toxoplasma.

** Mean ± SEM of phagocytic indices.

Toxoplasma to the mean phagocytic index for Candida; the ratio was 0.25 for human monocytes, 0.53 for human macrophages, and 0.34 for normal mouse macrophages. The ratio of NBT reduction by human MCP during phagocytosis of Toxoplasma to that during phagocytosis of Candida (0.25 ± 0.04) correlated with the ratio of the phagocytic indices for these two organisms. The ratios of NBT reduction during phagocytosis of Toxoplasma to that during phagocytosis of Candida by human macrophages ($P < 0.005$) and normal mouse MCP ($P < 0.001$) (Table I) were significantly less than that by human MCP and were far below what would be predicted on the basis of the phagocytic indices for these two organisms. In contrast, the ratios of NBT reduction during phagocytosis of Toxoplasma to that during phagocytosis of Candida by mouse MCP that contained activated macrophages (Table I) were similar to those observed with human MCP. As noted, these experiments were performed with ratios of organisms to phagocytes that were found in preliminary experiments to produce maximum increments in metabolic activity and maximum phagocytic indices at the end of the reaction period. In three experiments with normal mouse macrophages and one with human macrophages, lowering the ratio of Toxoplasma to phagocytes so that the phagocytic indices approached those observed with human monocytes was found to accentuate the discrepancies in NBT reduction during phagocytosis of Toxoplasma by these three cell types (data not shown).

Contribution of Different Types of Leukocytes to Reduction of NBT. Although human macrophage cell preparations contained only macrophages, human and mouse MCP contained both mononuclear phagocytes and lymphocytes, and some preparations contained a small number of PMN. To determine whether PMN or lymphocytes significantly contributed to NBT reduction by MCP, human PMN- or mouse PMN-enriched cell preparations and human and normal mouse MCP that were intentionally depleted of monocytes or macrophages were studied in parallel with the appropriate MCP. PMN could account for <10% of phagocytosis-stimulated NBT reduction by human and mouse MCP (Table II). Phagocytosis-stimulated NBT reduction by

TABLE IV
*Intracellular Location of Reduced NBT**

Cell preparation	Cells that contain organisms / total cells × 100			Cells that contain formazan-stained organisms / Cells that contain organisms × 100		
	Candida	Toxoplasma	Toxoplasma	Candida	Toxoplasma	Toxoplasma
	0.5 h‡	1 h	2 h	0.5 h	1 h	2 h
Human monocyte	84 ± 4§(6)‖	27 ± 6 (6)	12 ± 6 (3)	82 ± 5 (6)	71 ± 7 (6)	87 ± 9 (3)
Human macrophage	97 ± 1 (2)	37 ± 6 (3)	10 (1)	85 ± 5 (2)	4 ± 1 (3)¶	10 (1)
Mouse macrophage	70 ± 9 (5)	48 ± 12 (5)	28 ± 20 (2)	88 ± 3 (5)**	2 ± 1 (5)¶	7 ± 4 (2)

* Monolayers were prepared by adding MCP that contained 1×10^6 monocytes (human) or normal macrophages (mouse) to glass slide tissue culture chambers. Nonadherent cells were washed off after 1 h. Human monocyte and normal mouse macrophage monolayers were challenged immediately with organisms. Human macrophage monolayers were challenged after 5–6 d of in vitro culture (see text).
‡ Time after addition of organisms to monolayers.
§ Mean ± SEM. Single experiment where no SEM is shown.
‖ Numbers in parentheses are the number of experiments performed.
¶ $P < 0.001$ compared with human monocytes.
** $P < 0.05$ compared with human monocytes.

human MCP was ~7–10 times greater than that by monocyte-depleted preparations that contained equal numbers of lymphocytes but only ≃ 25% as many monocytes. Similar results were obtained with mouse macrophage-depleted cell preparations. Additional experiments were performed with cell preparations that had been depleted of lymphocytes: Results with monolayers of mouse macrophages and with monolayers of human monocytes (>90% macrophages or monocytes; <10% lymphocytes) were similar to those with cell suspensions (data not shown); results with human monocyte-enriched cell preparations in suspension (75–80% monocytes; Table II) were similar to those with the initial MCP. Also, in the experiments in Table I, in which the number of monocytes or macrophages in reaction mixtures was constant (1.5×10^6) and the number of lymphocytes varied, there was no significant correlation between the number of lymphocytes and the amount of NBT reduced by human ($r = -0.26$; $P > 0.2$) or mouse ($r = -0.4$; $P > 0.1$) MCP. We conclude, therefore, that NBT reduction by human and mouse MCP was a result of monocytes and macrophages, respectively.

Intracellular Location of Reduced NBT. 30 min after challenge with Candida, one or more formazan-stained organisms was present in ≃ 80% of each cell type that had ingested the organism (Table IV). 1 h after challenge with Toxoplasma, one or more formazan-stained organisms was present in 71 ± 7% of monocytes but only in 4 ± 1% of human macrophages and 2 ± 1% of normal mouse macrophages that had ingested Toxoplasma. The difference between human monocytes and both types of macrophages is significant ($P < 0.001$). Similar results were obtained with monolayers that were incubated for 2 h (Table IV) and 3 h (data not shown) after challenge with Toxoplasma. These differences are illustrated in Fig. 1. As noted in the quantitative assays, Toxoplasma did not inhibit NBT reduction because macrophages that ingested

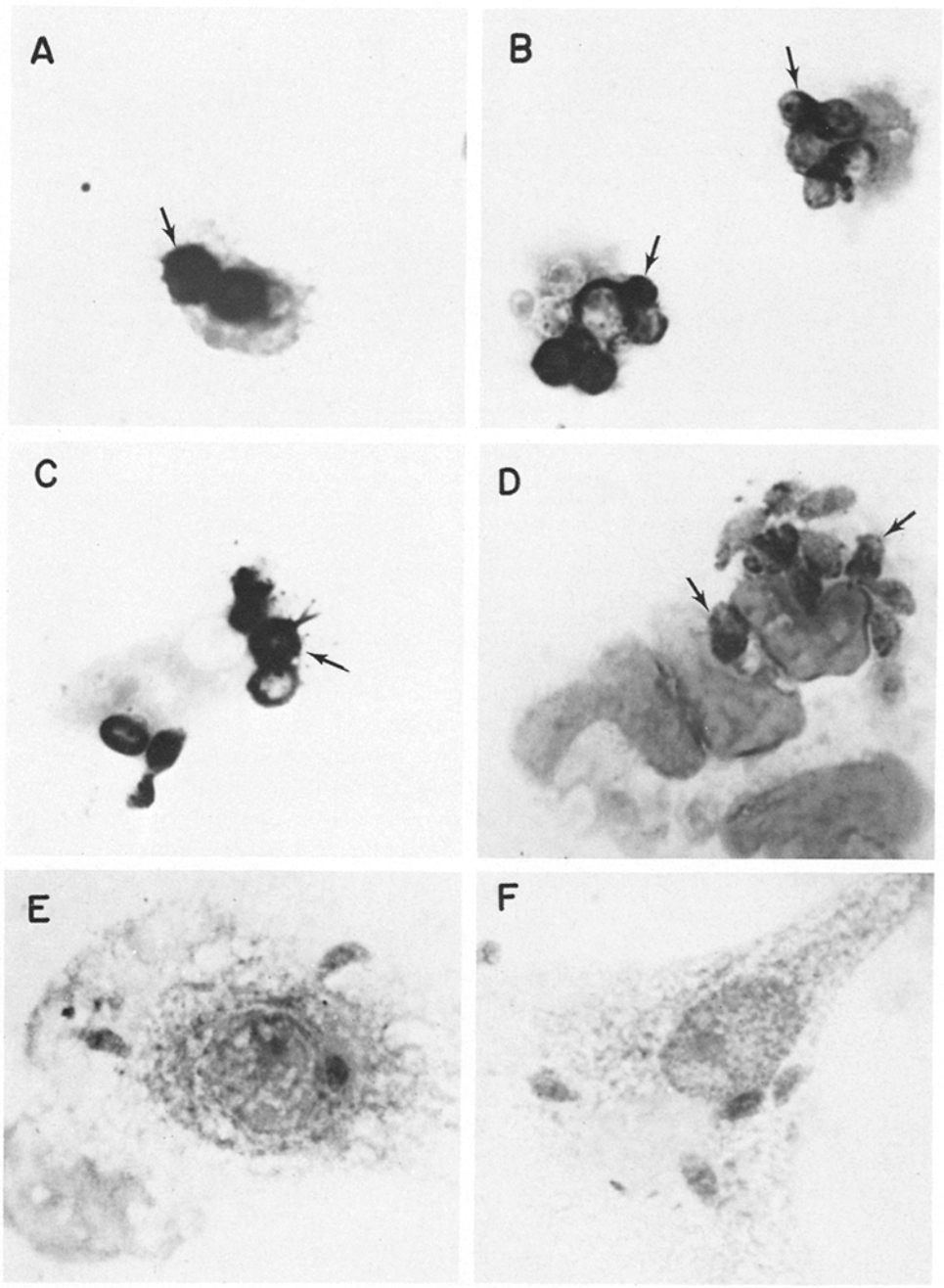

FIG. 1. Reduction of NBT within phagocytic vacuoles. (A) Human monocyte, (B) normal mouse macrophage, and (C) human macrophage that have ingested Candida (× 1,500); the majority of Candida are stained by formazan precipitate, which appears darker black (arrow), within phagocytic vacuoles in each of these cell types. (D) Human monocytes that have ingested Toxoplasma (× 1,800); the majority of Toxoplasma are completely or partially stained by formazan precipitate (arrow) within phagocytic vacuoles. (E) Normal mouse macrophage and (F) human macrophage that have ingested Toxoplasma (× 1,800); the Toxoplasma within phagocytic vacuoles of these cell types are not stained by formazan precipitate.

TABLE V

*Quantitative Reduction of NBT during Phagocytosis of Antibody-coated Toxoplasma**

Cell preparation	Phagocytic stimulus			
	None	Candida	Toxoplasma in non-immune serum‖	Toxoplasma in immune serum‖
	NBT red.‡	Δ NBT red.§	Δ NBT red.	Δ NBT red.
Human macrophage (2)¶	3.3 ± 3.3**	54.3 ± 0.7	2.9 ± 0.2 [2.0]‡‡	21.4 ± 2.9 [3.1]§§
Mouse mononuclear (4)	2.6 ± 0.2	17.4 ± 2.4	1.4 ± 0.0 [1.6 ± 0.1]	12.6 ± 1.2 [2.4 ± 0.1]‖‖

* Reaction mixtures (in duplicate) contained 0.1 ml of NBT (2 mg/ml); 0.25 ml of the suspension of Candida or Toxoplasma (or, in controls, 0.25 ml KRPG); 0.5 ml of cell preparations that contained 1.5 × 10^6 human macrophages or normal mouse macrophages; and sufficient KRPG to effect a final volume of 1 ml in siliconized glass tubes.

‡ Nanomoles of NBT reduced per 10^6 macrophages per 30 min.

§ Increase in nanomoles of NBT reduced per 10^6 macrophages per 30 min after subtraction of values for resting leukocytes and for organisms alone. Candida did not reduce NBT. NBT reduction by Toxoplasma preparations alone was 0.7 ± 0.3 nmol/30 min for all experiments.

‖ See text.

¶ Numbers in parentheses are the number of experiments performed.

** Mean ± SEM.

‡‡ Numbers in brackets are the mean ± SEM of phagocytic indices; where no SEM is shown, results were determined in a single experiment.

§§ $P < 0.05$ compared with human macrophages phagocytosing Toxoplasma preincubated in nonimmune serum.

‖‖ $P < 0.001$ compared with normal mouse MCP phagocytosing Toxoplasma preincubated in nonimmune serum.

both Candida and Toxoplasma contained formazan-stained Candida as frequently as did macrophages that ingested only Candida.

Quantitative Reduction of NBT During Phagocytosis of Antibody-coated Toxoplasma. Incubation of Toxoplasma in heat-inactivated serum that contained Toxoplasma antibody allows normal human and mouse macrophages to kill this organism (7, 13). We considered that specific antibody might act as a trigger for the respiratory burst of human and mouse macrophages during phagocytosis of Toxoplasma. NBT reduction by human macrophages and by normal mouse MCP during phagocytosis of Toxoplasma preincubated in either homologous or heterologous immune serum was significantly greater than that which occurred during phagocytosis of Toxoplasma preincubated in nonimmune serum and was much greater than the increase in number of organisms ingested (Table V). In two experiments with human monocytes (data not shown), there was a twofold increase in NBT reduction during phagocytosis of Toxoplasma preincubated in immune serum compared with that during phagocytosis of Toxoplasma preincubated in nonimmune serum and a similar twofold increase in number of organisms ingested.

Chemiluminescence. Chemiluminescence, like NBT reduction, is associated with the phagocytosis-associated burst of oxidative metabolic activity by leukocytes (25). Each type of cell preparation that was studied showed significant chemiluminescence during phagocytosis of Candida (Fig. 2). In contrast, only human MCP showed a definite increase in chemiluminescence during phagocytosis of Toxoplasma. As observed in the other assays, the differences in chemiluminescence during phagocytosis of Toxoplasma contrasted with the observations on phagocytosis; human monocytes ingested fewer Toxoplasma than did human or normal mouse macrophages, and the

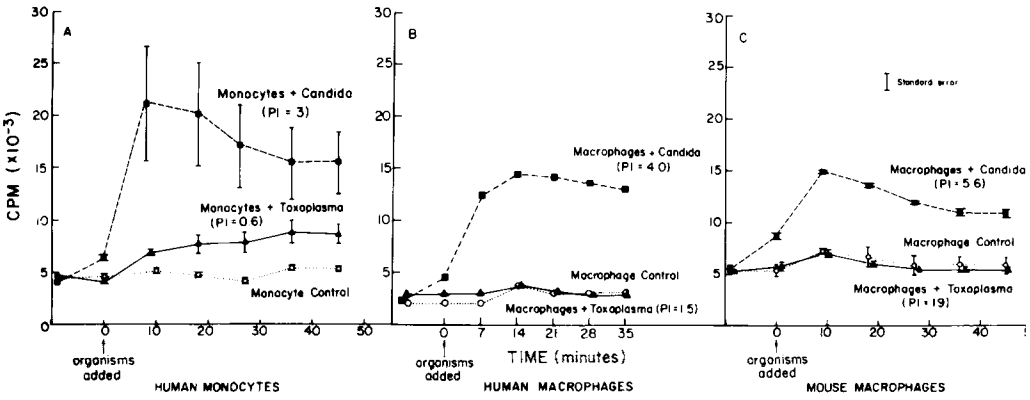

FIG. 2. Chemiluminescence during phagocytosis of Candida and Toxoplasma. Reaction mixtures in 15-ml siliconized glass scintillation vials (which had been stored in the dark > 24 h before use) consisted of cell preparations that contained 4×10^6 monocytes or macrophages; 10% human type AB serum (DT negative); 4×10^7 Candida or 1×10^8 Toxoplasma; and KRPG to effect a final volume of 2 ml. Controls contained no organisms. Results are expressed as mean ± SEM of counts per minute from a representative experiment of four with human MCP (A), two with human macrophage cell preparations (B), and four with normal mouse MCP (C).

ratio of Toxoplasma:Candida ingested by human monocytes was less than that by human or normal mouse macrophages. Chemiluminescence was dependent upon O_2^-, as shown in two experiments in which SOD (250 μg/ml) inhibited 83 ± 6% of maximum phagocytosis-associated chemiluminescence both by human and mouse MCP. Human and mouse PMN in the numbers present in vials that contained human or mouse MCP did not emit detectable chemiluminescence during phagocytosis of Candida or Toxoplasma. Because lymphocytes do not emit or inhibit chemiluminescence (32, 33), we conclude that chemiluminescence by human and mouse MCP was a result of monocytes and macrophages, respectively.

$^{14}CO_2$ Production from [1-^{14}C]glucose. $^{14}CO_2$ production from [1-^{14}C]glucose by each type of cell preparation that was studied increased significantly during phagocytosis of Candida (Table VI). The increase in $^{14}CO_2$ production by human MCP was significantly greater ($P < 0.02$) than that by normal mouse MCP during phagocytosis of Toxoplasma; there was no increase in $^{14}CO_2$ production by human macrophages during phagocytosis of Toxoplasma. This contrasted with results of phagocytosis (Table III).

Lymphocytes and PMN present in human and mouse MCP did not account for the phagocytosis-associated increase in glucose oxidation. Maximum counts per minute for phagocytosing human or mouse PMN in numbers equal to or greater than the numbers present in corresponding MCP were 309 and 85 ± 42, respectively, which is < 3% of cpm for phagocytosing human and mouse MCP. Similar experiments with monocyte- (human) or macrophage- (mouse) depleted MCP indicated that lymphocytes could account for < 3% of the increase in $^{14}CO_2$ produced by human and mouse MCP during phagocytosis.

Although Toxoplasma alone produced substantial amounts of $^{14}CO_2$, the increase in glucose oxidation during phagocytosis of this organism did not reflect increased glucose oxidation by the organism itself. Phagocytosis of formalin-killed (1% formalin in PBS for 1 h) organisms, compared with viable organisms, was associated with a

TABLE VI

*$^{14}CO_2$ Production from [1-^{14}C]glucose**

Cell preparation	Phagocytic stimulus					
	None	Candida		Toxoplasma		Δ cpm Toxoplasma/Δ cpm Candida‖
	cpm‡	cpm	Δ cpm§	cpm	Δ cpm	
Human mononuclear (5)¶	404 ± 88**	4,479 ± 642	4,096 ± 588	2,739 ± 549	1,720 ± 377	0.41 ± 0.06
Human macrophage (2)	568 ± 152	3,305 ± 887	2,737 ± 735	804 ± 50	0 ± 0‡‡	0.00 ± 0.00§§
Mouse mononuclear (6)	268 ± 34	2,259 ± 272	1,991 ± 300§§	1,307 ± 227	541 ± 153‖‖	0.29 ± 0.10

* Reaction mixtures consisted of cell preparations that contained 4 × 10^6 monocytes or normal mouse macrophages or 2 × 10^6 human macrophages; 4 × 10^7 Candida or 1 × 10^8 Toxoplasma; 0.25 μCi of [1-^{14}C]glucose (56.8 mCi/mmol sp act; Amersham Corp., Arlington Heights, Ill.); and KRPG to effect a final volume of 1.1 ml in duplicate center-well Erlenmeyer flasks. For determination of resting activity, organisms were omitted.

‡ Counts per minute of $^{14}CO_2$ released per 4 × 10^6 monocytes or normal mouse macrophages per 60 min or 2 × 10^6 human macrophages per 60 min.

§ Increase in counts per minute of $^{14}CO_2$ released after subtraction of values for resting leukocytes and for organisms alone. Candida alone did not release $^{14}CO_2$. $^{14}CO_2$ release by Toxoplasma preparations alone was 771 ± 131, 775 ± 31, and 527 ± 125 cpm for experiments with human MCP, human macrophage cell preparations, and normal mouse MCP, respectively.

‖ Increase in counts per minute during phagocytosis of Toxoplasma/increase in counts per minute during phagocytosis of Candida.

¶ Numbers in parentheses are the number of experiments performed.

** Mean ± SEM.

‡‡ $P < 0.05$ compared with human MCP.

§§ $P < 0.01$ compared with human MCP.

‖‖ $P < 0.02$ compared with human MCP.

somewhat greater increase in glucose oxidation by human MCP (Δ cpm live Toxoplasma = 1,170; Δ cpm dead Toxoplasma = 2,553) and by mouse MCP (Δ cpm live Toxoplasma = 738; Δ cpm dead Toxoplasma = 876); data are representative of two experiments. In addition, aerobic glycolysis was shown to be the primary pathway by which Toxoplasma oxidized glucose because Toxoplasma organisms produced $^{14}CO_2$ from [6-^{14}C]glucose (cpm = 679) and [1-^{14}C]glucose (cpm = 538) in comparable amounts; in contrast, the hexose monophosphate (HMP) shunt was the primary pathway by which glucose oxidation increased during phagocytosis because the increase in phagocytosis-associated $^{14}CO_2$ production from [6-^{14}C]glucose was < 10% of that from [1-^{14}C]glucose.

Intracellular Survival and Replication of Toxoplasma. Each cell type was studied to determine the fate of ingested Toxoplasma. As shown in Fig. 3, there was a marked decrease in the percentage of human monocytes infected 20 h after challenge in comparison to the percentage infected at 1 h after challenge; the decrease in the percentage of infected human macrophages and normal mouse macrophages was significantly less ($P < 0.01$ and $P < 0.001$, respectively). Also, intracellular replication of the organism was less in human monocytes than in human macrophages ($P < 0.01$) or in normal mouse macrophages ($P < 0.001$). Examination of cytocentrifuge preparations of culture supernates from each type of monolayer never revealed more than two infected nonadherent cells; thus, loss of infected cells from monolayers did not account for the differences observed. We have obtained similar results in previous experiments using a radiolabel technique that measures selective uptake of [^{3}H]uracil by Toxoplasma replicating intracellularly and which is not dependent upon adherence of cells to the monolayer or on microscopic observations (11).

The data noted above showed a correlation between intracellular killing of Toxoplasma by mononuclear phagocytes and activation of the oxidative metabolic burst during phagocytosis of this organism. We therefore studied the killing of Toxoplasma by monocytes from a child with X-linked CGD, his mother, and his sister in parallel

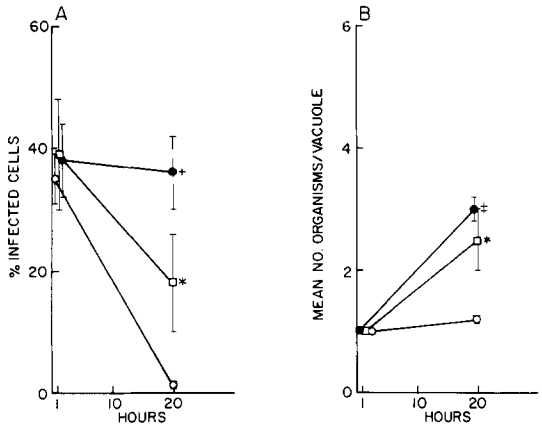

FIG. 3. Fate of Toxoplasma within mononuclear phagocytes. Results are mean ± SEM from 11 experiments with human monocytes, 5 experiments with human macrophages, and 11 experiments with normal mouse macrophages. In all experiments with human macrophages, monocytes from the same subject were studied in parallel. (○) Human monocytes, (□) human macrophages, and (●) normal mouse macrophages. The abscissa shows the time after addition of Toxoplasma to monolayers. (A) Percentage of cells that contained Toxoplasma. (B) Replication of Toxoplasma expressed as mean number of organisms per vacuole. $*P < 0.01$ compared with human monocytes; $\dagger P < 0.001$ compared with human monocytes, $P < 0.01$ compared with human macrophages; $\ddagger P < 0.001$ compared with human monocytes.

with monocytes from a normal adult control; values for NBT reduction and hydrogen peroxide (H_2O_2) release by leukocytes from these subjects are shown in Fig. 4. Killing of Toxoplasma by monocytes from the affected child and his mother was markedly less 6 h after challenge and somewhat less 20 h after challenge than that by monocytes from his sister and the control (Fig. 4). However, Toxoplasma did not replicate in monocytes from any of these subjects. Our attempts in these and other experiments to block the anti-Toxoplasma activity of monocytes by inhibitors of some oxygen-independent mechanisms have been unsuccessful. Addition of heparin (100 U/ml; an inhibitor of lysosomal cationic proteins) or α-1 anti-trypsin (2 mg/ml, sufficient to inhibit 2,000 *N*-Benzoyl-Arginine Ethyl Ester units of trypsin; an inhibitor of lysosomal neutral proteases) to monolayers of monocytes from the child with CGD and his mother and from normal subjects or addition of sodium azide (10^{-3} M; an inhibitor of myeloperoxidase) to monolayers of monocytes from normal subjects did not affect survival and replication of Toxoplasma.

Phagosome-Lysosome Fusion. Fusion of lysosomes with phagosomes that contained Toxoplasma paralleled destruction of organisms. In three experiments, fusion had occurred by 4 h after the addition of Toxoplasma to monolayers in 66 ± 6% of human monocytes, 43 ± 8% of macrophages from mice injected with *C. parvum*, 61 ± 16% of macrophages from mice chronically infected with Toxoplasma, and 20 ± 6% of macrophages from normal mice. Subsequently (by 20 h after challenge), Toxoplasma were eliminated from 97 ± 1% of human monocytes, 50 ± 10% of macrophages from mice injected with *C. parvum*, 68 ± 2% of macrophages from mice chronically infected with Toxoplasma, and 18 ± 7% of macrophages from normal mice. Although the parasite replicated within nonfused phagosomes of normal mouse macrophages, it did not appear to replicate within nonfused phagosomes of human monocytes or activated mouse macrophages.

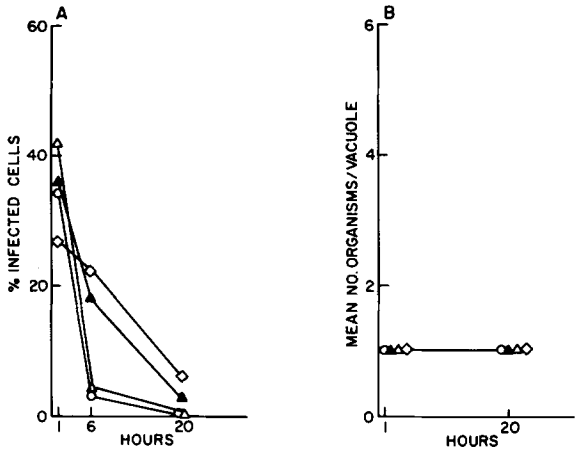

FIG. 4. Fate of Toxoplasma within monocytes from a child with X-linked CGD (▲), his heterozygote mother (◇), his normal sister (△), and an unrelated normal control (○). The abscissa shows the time after addition of Toxoplasma to monolayers. (A) Percentage of cells that contained Toxoplasma. (B) Replication of Toxoplasma, expressed as mean number of organisms per vacuole. Results of phagocytosis-associated (Candida) and phorbol myristate acetate stimulated NBT reduction and H_2O_2 release by leukocytes. H_2O_2 release was determined by the method of Root et al. (27). Means: child, 8% of control; mother, 31% of control; sister, 124% of control.

Discussion

Our results reveal that mononuclear phagocytes that generate reactive oxygen metabolites during phagocytosis of Toxoplasma are able to kill this organism and to inhibit intracellular replication of those few organisms that are not killed. In contrast, mononuclear phagocytes that generate little or no reactive oxygen metabolites during phagocytosis of Toxoplasma are unable to kill this organism and do not inhibit its intracellular replication.

In each of the assays we employed, phagocytosis of Toxoplasma significantly stimulated oxidative metabolic activity by human monocytes, whereas in none of the assays did phagocytosis of Toxoplasma significantly stimulate oxidative metabolic activity by human macrophages. Results with normal mouse macrophages were similar to those with human macrophages, except that phagocytosis of Toxoplasma by mouse macrophages did stimulate increased HMP shunt activity; however, this increase was significantly less than that observed with human monocytes. Previous studies have also found that HMP shunt activity does not necessarily correlate with production of O_2^- and H_2O_2 (34, 35).

Differences between mononuclear phagocytes in generation of reactive oxygen metabolites during phagocytosis of Toxoplasma could not be explained by differences in phagocytosis. Human monocytes actually ingested fewer Toxoplasma, either in absolute terms or relative to the numbers of Candida ingested, than did human or mouse macrophages. Microscopic observations confirmed the differences noted in the quantitative assays of oxidative metabolic activity; O_2^- (as indicated by the presence of formazan precipitate) was present within phagocytic vacuoles that contained Toxoplasma in nearly all human monocytes but only in rare normal human and mouse macrophages.

Previous studies of the role of oxidative metabolism in the microbicidal activity of macrophages have shown that activated macrophages, which have increased antimi-

crobial activity, generate increased amounts of reactive oxygen metabolites when triggered by particulate and chemical agents such as zymosan and phorbol myristate acetate (3–5, 36). This suggests that differences in maximum oxidative metabolic response between mononuclear phagocytes of different types or of different degrees of activation to such general triggering agents may be causally related to differences in antimicrobial activity. We observed only a partial correlation between generation of reactive oxygen metabolites by different types of mononuclear phagocytes when triggered by phagocytosis of Candida or latex particles and the ability of these cells to kill Toxoplasma. However, consistent correlation was observed between killing of Toxoplasma and generation of reactive oxygen metabolites when triggered by phagocytosis of Toxoplasma. Thus, whereas human macrophages and normal mouse macrophages generated little or no H_2O_2 and reduced little or no NBT when phagocytosing nonopsonized Toxoplasma, human monocytes and activated mouse macrophages did. Furthermore, human macrophages and normal mouse macrophages, which kill opsonized Toxoplasma, did reduce significant amounts of NBT when phagocytosing opsonized Toxoplasma. These data suggest that differences in the ability of mononuclear phagocytes to generate increased amounts of reactive oxygen metabolites during phagocytosis of specific organisms, rather than or in addition to differences in their oxidative metabolic activity in response to unrelated particulate or chemical-triggering agents, may account for differences in their antimicrobial activity.

The results of our studies with monocytes from a patient with X-linked CGD and his heterozygote mother indicate that oxygen-dependent mechanisms contribute significantly to early and efficient killing of Toxoplasma by these cells but that other less efficient nonoxidative reserve mechanisms are capable of killing this organism. Murray et al. (37), studying activated mouse macrophages, also concluded that reactive oxygen metabolites play a role in the ability of these cells to kill Toxoplasma. We observed both increased oxidative metabolic activity and increased phagosome-lysosome fusion during phagocytosis of Toxoplasma by those cells that killed the organism (human monocytes and activated mouse macrophages). Thus, lysosomal contents may normally act in concert with reactive oxygen metabolites to kill Toxoplasma. In addition, lysosomal contents may be a less efficient mechanism whereby Toxoplasma is killed by cells that fail to generate reactive oxygen metabolites during phagocytosis of this organism. Although it is likely that reactive oxygen metabolites and perhaps lysosomal contents as well are first-line mechanisms whereby mononuclear phagocytes kill Toxoplasma, both reactive oxygen metabolites and other mechanisms (as yet undefined and apparently nonlysosomal) may participate in the ability of these cells to inhibit replication of surviving organisms. Even though lysosomes did not fuse with phagosomes that contained those rare Toxoplasma that were not killed by human monocytes, these organisms replicated more slowly than did Toxoplasma within normal human or mouse macrophages. As Jones (38) and we observed, this was also true for those organisms that were not killed by activated mouse macrophages. Therefore, nonlysosomal mechanisms must account for inhibition of replication of Toxoplasma within mononuclear phagocytes. Additional evidence that supports this conclusion is our observation that inhibitors of lysosomal cationic proteins, neutral proteases, or myeloperoxidase did not alter the ability of monocytes from the child with CGD and his mother or those from normal subjects to

kill or inhibit replication of Toxoplasma. Others have found that reactive oxygen metabolites contribute to inhibition of Toxoplasma replication within activated mouse macrophages (37). Although we observed that Toxoplasma failed to replicate within monocytes from a patient with X-linked CGD and his mother, this does not exclude participation of reactive oxygen metabolites in the inhibition of Toxoplasma replication by monocytes from normal subjects. Thus, the ability of some intracellular pathogens to be phagocytosed without triggering the oxidative metabolic burst may be necessary, although not always sufficient, for their survival. That such a mechanism for the survival of intracellular pathogens is not unique to Toxoplasma is suggested by the ability of virulent *Salmonella typhi* (39) and *Brucella abortus* (40) to be phagocytosed by PMN without stimulating increased HMP shunt activity and to survive within these cells.

A number of possible mechanisms were examined in an attempt to explain the differences that we observed in the oxidative metabolism of the different types of mononuclear phagocytes during phagocytosis of Toxoplasma. Toxoplasma did not inhibit the oxidative metabolic response because monocytes and macrophages that phagocytosed both Candida and Toxoplasma were able to reduce NBT as well as did those that phagocytosed Candida alone. The differences in oxidative metabolic response were also not dependent upon Toxoplasma viability; results of HMP shunt activity (as noted above) and NBT reduction (C. B. Wilson, V. Tsai, and J. S. Remington. Unpublished observations.) during phagocytosis of viable Toxoplasma by monocytes and normal mouse macrophages were similar to results during phagocytosis of formalin-killed organisms. Interestingly, phagocytosis of boiled organisms was a poor stimulus for NBT reduction and H_2O_2 production by these cell types (C. B. Wilson, V. Tsai, and J. S. Remington. Unpublished observations.). We found that the differences in oxidative metabolic response could be overcome by coating the organisms with antibody; attachment of antibody-coated Toxoplasma, presumably by IgG-Fc receptors, triggered both phagocytosis and the respiratory burst by each type of mononuclear phagocyte. Thus, these data suggest that both structural (presumably membrane) integrity of the organism, which is altered by specific antibody or by boiling but not by formalin, and differences between mononuclear phagocytes in their mechanisms for activation of the respiratory burst dictate whether attachment of nonopsonized Toxoplasma will trigger the respiratory burst as well as phagocytosis.

Both maturation and activation of mononuclear phagocytes are associated with marked alterations of plasma membrane proteins and with alterations in membrane receptors involved in C3b-coated particle ingestion (41). It is possible that similar changes in mononuclear phagocyte membrane receptors are responsible for the differences we observed in activation of the respiratory burst during ingestion of Toxoplasma. Certain alterations of phagocyte membranes have previously been shown to affect triggering of the respiratory burst without affecting phagocytosis (42, 43). Desialylation of leukocyte membranes blocks stimulation of O_2^- and H_2O_2 production but not HMP shunt activity by latex phagocytosis or by concanavalin A (42); inhibition of membrane sulfhydryl groups blocks stimulation of HMP shunt activity but not O_2^- or H_2O_2 production by the same agents (43). Similarly, a naturally occurring defect, thought to be membrane related, in activation of the respiratory burst by nonantibody-coated particles has been observed in two children with a

variant of CGD (44); phagocytosis of zymosan and latex particles did not trigger the respiratory burst by their leukocytes, whereas phagocytosis of IgG-coated latex did. The differences we observed between mononuclear phagocytes were selective and were not a result of the inability of normal macrophages to generate reactive oxygen metabolites during phagocytosis of non-IgG-coated particles other than Toxoplasma because normal macrophages reduced NBT and generated H_2O_2 (C. B. Wilson, V. Tsai, and J. S. Remington. Unpublished observations.) during phagocytosis of latex particles. Although electron microscopy has revealed that attachment and ingestion of nonopsonized Toxoplasma is similar to that of antibody-coated particles (13), the nature of phagocyte membrane receptors for Toxoplasma is unknown. Further studies to elucidate the nature of those receptors in different types of phagocytes are indicated. Such studies may reveal the basis for the differences between mononuclear phagocytes in the activation of the respiratory burst during phagocytosis of Toxoplasma and increase our understanding of the mechanism by which phagocyte membranes control activation of the respiratory burst.

Summary

As previously reported, normal human monocytes (11) and activated mouse macrophages (9) are able to kill or inhibit intracellular replication of Toxoplasma that are not antibody coated, whereas normal human and mouse macrophages are not (7, 9). Each of these types of mononuclear phagocytes is able to kill antibody-coated Toxoplasma. In our studies, phagocytosis of antibody-coated Toxoplasma stimulated the respiratory burst by each of these types of mononuclear phagocytes, whereas phagocytosis of organisms that were not antibody coated stimulated the respiratory burst only by human monocytes and by activated mouse macrophages. Phagocytosis of Toxoplasma did not inhibit production of reactive oxygen metabolites by normal macrophages; rather, it failed to stimulate their production. Killing of Toxoplasma by monocytes from a child with X-linked chronic granulomatous disease and his heterozygote mother was impaired. Thus, reactive oxygen metabolites, perhaps in conjunction with lysosomal contents, appear to be first-line mechanisms whereby mononuclear phagocytes kill this organism. We were not able to determine the exact mechanisms whereby mononuclear phagocytes inhibit the replication of those Toxoplasma that were not killed, although both oxygen-dependent and other nonlysosomal mechanisms may be involved. The differences we observed in oxidative response to phagocytosis of Toxoplasma appear to be one determinant of the antimicrobial activity of these cells and may account for the ability of some intracellular pathogens to survive within phagocytes. These differences may be membrane related. Further studies of Toxoplasma membranes, phagocyte membrane receptors for Toxoplasma, and membrane-related mechanisms for activation of the respiratory burst are needed to define their true basis.

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