OXYGEN-INDEPENDENT KILLING BY ALVEOLAR MACROPHAGES

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Alveolar macrophages play a key role in defense of the host against pulmonary infection (1–6). Their clinical importance is emphasized by the high incidence of life-threatening pneumonia (7, 8) in patients with abnormal macrophage function (6) or impaired cell-mediated immunity (6–9). However, the mechanisms by which alveolar macrophages kill microorganisms are poorly understood.

We recently examined the mechanism by which human alveolar macrophages kill the intracellular parasite, *Toxoplasma gondii* (10). We chose this organism because it causes pneumonia in immunosuppressed patients (11–14) but not in healthy individuals (11), and because macrophages play an important part in the host's resistance to this organism (5, 15–17). Our studies showed that killing of *T. gondii* by human alveolar macrophages occurred without involvement of toxic metabolites of oxygen (Catterall, J. R., and J. S. Remington, manuscript in preparation). This suggested that previous studies of intracellular killing by normal macrophages might have limitations as models for the human alveolar macrophage, since most of them (18–20, and reviewed in 21), including all those that employed *T. gondii* (18–23), have emphasized the overriding importance of oxidative killing mechanisms. Nonoxidative antimicrobial activity has been shown in subcellular macrophage fragments (21, 24) and in oxidatively deficient cells (19, 21–23, 25), but in none of the previously described models using normal intact macrophages has the killing of intracellular parasites been reported as nonoxidative.

To facilitate the study of nonoxidative killing by alveolar macrophages, we have sought a laboratory animal model relevant to the human alveolar macrophage. Since rats more closely resemble human subjects in their resistance to *T. gondii* than many other animals (26, 27), we have examined the interaction between *T. gondii* and rat alveolar macrophages in vitro. The studies reported here indicate that rat alveolar macrophages, like those from human subjects, also kill *T. gondii*, and by nonoxidative mechanisms. They suggest that nonoxidative antimicrobial mechanisms may be important in alveolar macrophages, and they provide a convenient model for the study of such mechanisms.

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Materials and Methods

Animals. Lewis rats, 6–8 wk old and weighing 180–220 g each (Harlan Sprague Dawley Inc., Indianapolis, IN) were used for most experiments. Sprague-Dawley rats of similar age and weight (Simonsen Laboratories, Gilroy, CA) were used as noted. Mice were outbred Swiss-Webster females, 6–8 wk old, and weighed 20–22 g each (Simonsen Laboratories).

Macrophages. Animals were killed by asphyxiation with carbon dioxide, and resident peritoneal macrophages harvested by lavaging the peritoneal cavity once with calcium- and magnesium-free PBS (Gibco Laboratories, Grand Island, NY) containing EDTA 0.1 g/100 ml (PBS-EDTA). We obtained resident alveolar macrophages by repeatedly lavaging the lung in situ (28) with PBS-EDTA until 100 ml (rat) or 10 ml (mouse) of lavage fluid had been collected from each animal. For most experiments, cells from 2–6 rats, or 10–12 mice, were pooled.

The lavage fluid was centrifuged at 225 *g* for 10 min at 4°C, and the pellet of cells washed with PBS, recentrifuged, and resuspended in serum-free Iscove's modified Dulbecco's medium (IMDM[1]; Gibco Laboratories) containing gentamicin 40 mg/liter (hereafter termed medium). In all experiments, with both resident alveolar and resident peritoneal cells, >92% of the leukocytes in these suspensions were mononuclear cells on Diff-Quik- (American Scientific Products, McGraw Park, IL) stained cytocentrifuge preparations. The concentration of mononuclear cells was adjusted to 2×10^6/ml with medium, and a 300-μl aliquot of this suspension was inoculated into individual wells of an eight-chamber tissue culture slide (Lab-Tek Products, Naperville, IL). The cells were incubated for 2 h at 37°C in air containing 5% CO_2 and washed twice with PBS. The monolayers were used immediately or after 18 h of further incubation in medium. As mouse alveolar cells were less plentiful than rat cells, they were inoculated into each culture chamber in a 50-μl dot placed at the center of the chamber (5); we were careful not to dislodge this dot of the cell suspension as the slide was placed into the incubator. >95% of the cells in the monolayers were mononuclear phagocytes, as assessed by their ability to ingest neutral red.

Organisms. Tachyzoites of the RH strain of *T. gondii* were harvested and processed by methods described previously (18), suspended in medium at concentrations of $2–12 \times 10^6$/ml, and immediately added to the macrophage monolayers. The concentration of the Toxoplasma suspension used in most experiments was 6×10^6/ml, since 300 μl of this suspension was usually sufficient to infect 20–55% of macrophages. Yeast-phase *Candida albicans*, previously killed by boiling for 30 min and stored at 4°C in PBS, were suspended in medium also at a concentration of $2–12 \times 10^6$/ml. Killed *Corynebacterium parvum*, 7 mg/ml, (Wellcome Research Laboratories, Beckenham, England) was used undiluted.

Special Reagents. Reagents were obtained from Sigma Chemical Co., St. Louis, MO, and dissolved in medium immediately before use, unless indicated otherwise. PMA was dissolved in DMSO at a concentration of 0.3 mg/ml, separated into 50-μl aliquots, and stored at −70°C (29, 30).

Scavengers of oxygen metabolites (19, 23, 31) were used at the concentrations shown. They included catalase from bovine liver recrystallized twice (2.5 and 1.3 mg/ml) as a scavenger of hydrogen peroxide; superoxide dismutase, type III, 3,000 U/mg (2.5 and 1.3 mg/ml) as a scavenger of superoxide anion; and histidine (10 mM) and diazabicyclooctane (DABCO, 1 mM) as scavengers of singlet oxygen radicals. The scavengers of hydroxyl radicals used were mannitol (50 mM), benzoic acid (10 mM), DMSO (150 mM), tetramethylurea (25 mM), thiourea (25 mM), methanol (315 mM), and ethanol (80 mM).

Infection of Macrophage Monolayers and Assessment of Intracellular Killing. A 300-μl aliquot of the Toxoplasma suspension was added to each monolayer, the organisms were incubated with the cells for 1 h, and then extracellular organisms were removed by washing the monolayers five times with PBS. Immediately thereafter (0 h), and at varying intervals later, duplicate monolayers were fixed in cold (4°C) aminoacridine fixative (5),

[1] *Abbreviations used in this paper:* CGD, chronic granulomatous disease; DABCO, diazabicyclooctane; IMDM, Iscove's modified Dulbecco's medium; medium, serum-free IMDM containing gentamicin 40 mg/liter; NBT, nitroblue tetrazolium dye.

stained with Diff-Quik, and examined for morphologically intact intracellular Toxoplasma (32). A rosette was defined as eight or more Toxoplasma in a single vacuole. The number of infected macrophages, the number of Toxoplasma per 100 macrophages, and the number of macrophages that contained rosettes were determined by counting at least 200 cells in each monolayer. All counts were performed on duplicate monolayers and the mean values calculated.

Toxoplasmacidal activity was also assessed by acridine orange fluorescence microscopy (19, 23, 33, 34). Macrophage monolayers were incubated with acridine orange (5 μg/ml in HBSS) for 20 min at 37°C, washed, and incubated in dye-free medium for 1 h. Viable or formalin-killed Toxoplasma were then added to the monolayers. After 30 min, the slides were washed, then examined either immediately by epifluorescence microscopy or after incubation in medium for varying intervals. Toxoplasma were scored as dead if they showed uniform yellow-green, orange, or red staining of their cytoplasm. However, if they showed only punctate orange lysosomal staining, faint dull green staining, or no discernable cytoplasmic staining, they were not scored as dead. The total number of intracellular Toxoplasma (both morphologically intact and partially digested) was determined from slides run in parallel that were fixed and stained with Diff-Quik stain. Control experiments were performed with Toxoplasma in suspension; viable Toxoplasma stained with acridine orange were killed by adding either hydrochloric acid (to lower the pH to 4.5) or formalin (to give a final formaldehyde concentration of 4%) to the suspension, and examined by epifluorescence microscopy after varying intervals of incubation at 37°C.

Intracellular Reduction of Nitroblue Tetrazolium (NBT) Dye. NBT dye, at a concentration of 0.25 mg/ml medium, was incubated for 1 h with macrophage monolayers in the presence of live tachyzoites of *T. gondii* or heat-killed Candida, in eight-chamber tissue culture slides (Lab-Tek Products) (18). The slides were then rinsed in saline, fixed in methanol, and counterstained with 0.2% safranin. The percentage of macrophages that contained formazan granules within phagocytic vacuoles was determined microscopically by counting 100–200 cells that contained organisms. Cells were scored as positive only if they contained organisms surrounded by deep blue staining. Macrophages that contained only diffuse fine deposits of formazan were present in similar numbers in challenged and unchallenged monolayers; they were therefore scored as negative.

Release of Superoxide From Rat Alveolar Macrophages. The amount of superoxide released from monolayers of rat alveolar macrophages in response to PMA (200 nM), heat-killed Candida (at organism/macrophage ratios of 3:1 and 6:1), or live tachyzoites of *T. gondii* (at the same organism/macrophage ratios) was measured by the method of Pick and Mizel (35), which uses reduction of ferricytochrome *c*. Macrophages (3×10^5 per well) were incubated in quadruplicate in 96-well flat-bottomed tissue culture plates (Costar, Cambridge, MA), and measurements of absorbance at 550 nm made in a micro-ELISA reader (Dynatech Laboratories Inc., Alexandria, VA). The amount of superoxide produced was calculated from the formula (35): nanomoles of superoxide per well = (absorbance at 550 nm × 100)/6.3.

Intracellular Killing of T. gondii After Pretreatment of Rat Alveolar Macrophages With PMA. Rat alveolar macrophages were incubated with medium containing 200 ng/ml of PMA and 0.07% of DMSO (vol/vol) for 90 min at 37°C in 5% CO_2 to exhaust their respiratory burst (29). The monolayers were washed twice, reincubated in medium alone for 2 h (29), and then infected with tachyzoites of *T. gondii* and assessed for toxoplasmacidal activity. The impaired capacity of the macrophages to generate a respiratory burst after pretreatment with PMA was confirmed by assessing their reduction of NBT dye in the presence of heat-killed Candida. In control experiments, the macrophages were pretreated with 0.07% DMSO alone.

Effects of Scavengers of Oxygen Metabolites on the Toxoplasmacidal Activity of Rat Alveolar Macrophages. Rat alveolar macrophages were incubated with scavengers of oxygen metabolites dissolved in medium (at the concentrations stated under special reagents above), or with medium alone, for 3 h before infection with *T. gondii*, during the period of infection, and for 18 h thereafter (19). Slides were fixed and stained at 0, 6, and 18 h,

and examined to determine the number of infected cells and the number of morphologically intact Toxoplasma per 100 cells. Control experiments were performed with murine peritoneal macrophages that had been activated to kill *T. gondii* by intraperitoneal injection of the mice with 1.4 mg of killed *C. parvum* 21–30 d earlier (5, 36).

Effect of Permeant Weak Bases on the Toxoplasmacidal Activity of Rat Alveolar Macrophages. To determine whether acidification of the phagosome by lysosomes plays an important part in the toxoplasmacidal activity of rat alveolar macrophages, monolayers of these cells were incubated with weak bases that have been shown to raise the intralysosomal pH of phagocytes (37, 38). The monolayers were incubated with 300 μl of ammonium chloride (10 mM), methylamine (10 mM), or medium alone for 15 min (38), then 33 μl of a suspension of Toxoplasma containing 6×10^7 organisms/ml was added to each well. After 1 h of further incubation in the presence of the base, extracellular Toxoplasma were removed by washing, and the slides were fixed, stained, and examined microscopically, either immediately or after varying periods of incubation with the same weak base or medium alone. Parallel experiments were performed with mouse peritoneal macrophages as a control.

To confirm that the intralysosomal pH of the macrophages had actually been raised, we determined the ability of the macrophages to concentrate acridine orange into their lysosomes, a process that is dependent on low intralysosomal pH (34, 39). This was done in parallel with the studies with weak bases described above. Monolayers from the same suspensions of macrophages were incubated with 10 mM ammonium chloride, 10 mM methylamine, or medium alone for 15 min, 6 h, or 18 h. At the end of each of these time periods, a small volume of acridine orange was added to the incubation solution to give a final acridine orange concentration of 5 μg/ml. After 20 min of incubation, the monolayers were washed five times with weak base or control medium to remove extracellular acridine orange. They were reincubated for 1 h with the same weak base or control medium but without acridine orange. They were then washed and examined by epifluorescence microscopy to determine the percentage of cells in each monolayer that had lysosomes that were stained bright orange.

Viability of Toxoplasma Attached to the Surface of Rat Alveolar Macrophages. To examine whether Toxoplasma are killed at the surface of alveolar macrophages, we determined the viability of Toxoplasma that were attached to the cells. Toxoplasma incubated with acridine orange (10 μg/ml) for 30 min were added to monolayers of rat alveolar macrophages that had also been incubated with acridine orange (10 μg/ml). It was necessary to use 10 μg/ml of acridine orange and to prelabel the macrophages, as well as the Toxoplasma, because macrophages and Toxoplasma compete for the dye, and when these procedures were not followed there was a tendency for acridine orange in the Toxoplasma suspension to be redistributed to the macrophages, thus making it difficult to visualize lysosomal staining in Toxoplasma. The Toxoplasma and macrophages were incubated together for 20 min, extracellular organisms were gently washed off once, and the Toxoplasma attached to the macrophages were examined for viability by epifluorescence microscopy.

Since Toxoplasma (both live and dead) are internalized rapidly by macrophages, the experiment was repeated with sodium fluoride to block phagocytosis of the organisms. Sodium fluoride has previously been shown to prevent entry of latex beads (40) and yeast cells (41) into macrophages, although it may have a wide range of effects of cells (42). Monolayers of rat alveolar macrophages were incubated for 1 h with medium that contained acridine orange (10 μg/ml) and 5 mM sodium fluoride, preliminary experiments having shown this to be the lowest concentration of fluoride that would prevent ingestion of Toxoplasma by these cells. The incubation fluid was then aspirated and replaced by a suspension of *T. gondii* in HBSS that contained 10 μg/ml of acridine orange and 5 mM sodium fluoride. After 2 h, extracellular Toxoplasma were gently washed off once, and the Toxoplasma attached to the macrophages were examined for viability by epifluorescence microscopy (19, 23, 33, 34). In control experiments, the suspension of Toxoplasma was incubated with sodium fluoride and acridine orange, but without rat alveolar macro-

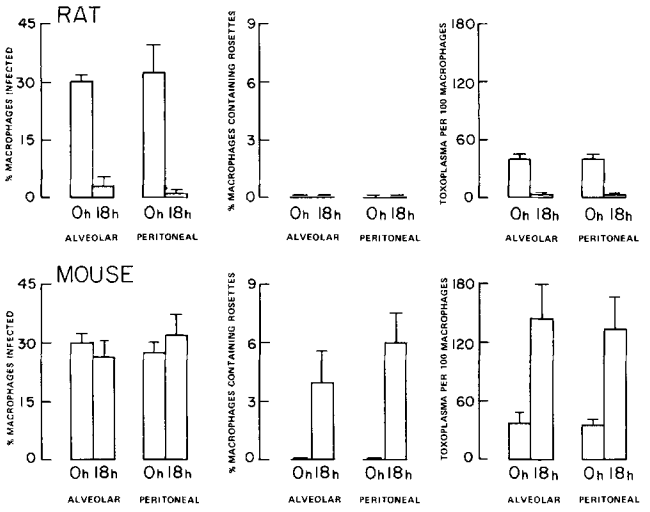

FIGURE 1. Fate of *T. gondii* in macrophages from rats and mice. Results are the mean ± SEM of four experiments performed in duplicate, except for those with mouse alveolar macrophages, which are the mean ± SEM of two experiments performed in duplicate. In all experiments with rat macrophages, the viability of the inoculum of *T. gondii* tachyzoites was confirmed by documenting their ability to multiply in resident murine peritoneal macrophages.

phages. Also, acridine orange–stained Toxoplasma were incubated with monolayers of rat alveolar macrophages in the absence of sodium fluoride.

Values are expressed as means ± SEM. Differences between values were determined by Student's *t* test.

Results

Fate of T. gondii in alveolar and Peritoneal Macrophages From Rats and Mice. Resident rat alveolar macrophages killed *T. gondii* in vitro. 18 h after entering these cells, most of the Toxoplasma had disappeared and almost all of the remaining organisms had lost their morphological integrity; none had multiplied. In contrast, organisms from the same Toxoplasma suspensions multiplied readily inside resident murine alveolar macrophages (Fig. 1). This marked difference between rats and mice in the resistance of their alveolar macrophages to *T. gondii* was also noted in experiments run in parallel with peritoneal macrophages from the same animals (Fig. 1). Because our main interest was in pulmonary defenses, all subsequent experiments were performed with alveolar macrophages.

In these and all subsequent experiments with rats, Lewis strain animals were used. In a separate series of experiments, alveolar and peritoneal macrophages from Sprague-Dawley rats were found to have an ability to kill *T. gondii* similar to that observed with the same cells from Lewis rats (data not shown).

Toxoplasmacidal Activity of Rat Alveolar Macrophages. The toxoplasmacidal activity of rat alveolar macrophages was both rapid (Fig. 2) and efficient (Table I).

To determine how quickly rat alveolar macrophages kill *T. gondii,* infected monolayers were examined at varying times after infection. Even when morphological criteria were used to assess destruction of the organism in fixed prepara-

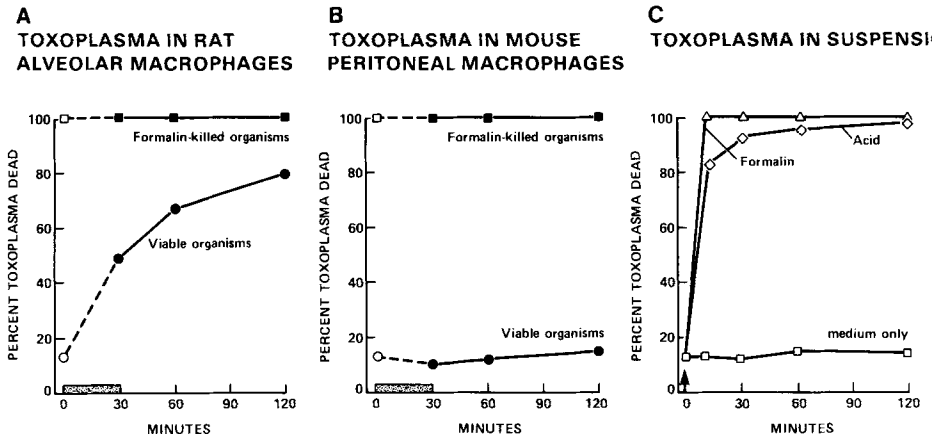

FIGURE 2. Fate of *T. gondii* in rat alveolar macrophages, mouse peritoneal macrophages, and medium containing formalin or acid, as indicated by acridine orange staining of the organism (see text for details). In *A* and *B*, macrophages whose lysosomes had been prelabeled with acridine orange were incubated for 30 min (▭) with viable (○, ●) or formalin-killed (□, ■) *T. gondii*, washed, and examined microscopically, either immediately or after varying periods of further incubation. The total number of intracellular organisms was determined in Diff-Quik-stained fixed preparations, and the number of dead organisms was determined by acridine orange fluorescence microscopy. In *C*, viable Toxoplasma stained with acridine orange were suspended in medium, and acid (◇) or formalin (△) added at the time marked by the arrow. The open symbols represent Toxoplasma suspended in medium, the closed symbols represent intracellular organisms. The results are the mean of three separate experiments.

TABLE I

Fate of T. gondii in Rat Alveolar Macrophages and in Activated Mouse Peritoneal Macrophages

Macrophages used:	Percent macrophages infected*	Toxoplasma per 100 macrophages*
Rat alveolar macrophages:		
0 h	71 ± 13	208 ± 56
18 h	2 ± 1	2 ± 1
Activated mouse peritoneal macrophages:‡		
0 h	53 ± 7	139 ± 23
18 h	8 ± 3	30 ± 15

* The results are the means ± SEM of three experiments.

‡ Peritoneal macrophages from mice infected with C56 strain of *T. gondii* 6–8 wk earlier (see text).

tions, 76% (range 70–86% in three experiments) of the parasites that appeared morphologically intact at the end of the hour-long infection period had become shrunken remnants, without the usual clear distinction between nucleus and cytoplasm, 2 h later. However, studies with acridine orange suggested that most of the organisms were killed during their first hour of exposure to the monolayers (Fig. 2).

Rat alveolar macrophages also showed a remarkable ability to kill large numbers of Toxoplasma. In three experiments (Table I), rat alveolar macrophages were incubated with two to three times the usual inoculum of *T. gondii*

to give high initial infection rates. 18 h later, only 2% of the original number of intracellular Toxoplasma appeared morphologically intact, and none had multiplied (Table I). We compared the toxoplasmacidal capacity of rat alveolar macrophages with that of mouse peritoneal macrophages that had been activated to kill *T. gondii* by intraperitoneal injection of 10^5 tachyzoites of the low-virulent C-56 strain of *T. gondii* 6–8 wk earlier. Macrophages were harvested at these times because in previous studies in our laboratory they were highly activated to kill or inhibit *T. gondii* (5). These activated peritoneal macrophages killed most of the intracellular Toxoplasma (Table I), but in two of three experiments, replication of the organism did occur in a few cells (Table I), suggesting that these populations of activated macrophages were more heterogeneous in their toxoplasmacidal activity than the populations of resident rat alveolar macrophages.

Release of Superoxide by Rat Alveolar Macrophages. When *T. gondii* were incubated with rat alveolar macrophages in the presence of NBT, <5% of the macrophages that contained intracellular Toxoplasma showed reduction of NBT, even though organisms from the same suspensions of Toxoplasma were killed by the macrophages. When monolayers of the same macrophages were incubated with heat-killed Candida, however, NBT reduction was seen in the majority (73–97% in five experiments) of rat alveolar macrophages that contained Candida.

The foregoing results indicated that rat alveolar macrophages killed intracellular *T. gondii* without their respiratory burst being triggered. To confirm these findings, activation of the respiratory burst was assessed by quantitating the liberation of superoxide from rat alveolar macrophages (Fig. 3). The macrophages readily released superoxide in response to PMA or heat-killed Candida (Fig. 3), but when incubated with Toxoplasma they released no more superoxide than resting cells (peak superoxide level per well with resting cells, 0.37 ± 0.21 nmol; with cells exposed to *T. gondii*, 0.51 ± 0.35 nmol, $p > 0.2$). In concurrent experiments, however, alveolar macrophages from the same animals ingested and killed organisms from the same suspensions of Toxoplasma (data not shown).

Fate of Intracellular T. gondii in Rat Alveolar Macrophages Pretreated With PMA. PMA, a phorbol diester, triggers and exhausts the respiratory burst of phagocytes (29). Pretreatment of rat alveolar macrophages with PMA (200 ng/ml) for 90 min did not significantly affect the rat alveolar macrophages' ability to kill intracellular Toxoplasma, even though the treated macrophages showed a greatly impaired capacity to reduce NBT in the presence of heat-killed Candida (Fig. 4). Because of a recent report (30) that PMA will trigger the respiratory burst of alveolar macrophages most effectively if the monolayers have been pre-exposed to serum for 24–48 h, this experiment with PMA was repeated using rat alveolar macrophages that had been cultured in IMDM containing 10% FCS for 48 h. The results obtained were similar to those without serum pretreatment and are included in Fig. 4.

Effect of Scavengers of Oxygen Metabolites. Previous experiments with activated murine peritoneal macrophages have shown that scavengers of oxygen metabolites can impair the ability of these cells to kill intracellular *T. gondii*, provided that the scavengers are incubated with the cells for at least 3 h before infection as well as during infection and the subsequent 18 h (19). However, when we

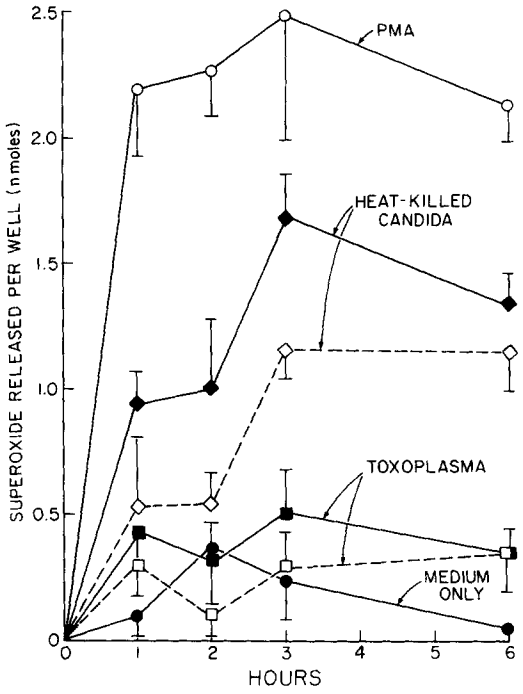

FIGURE 3. Production of superoxide by rat alveolar macrophages. Macrophage monolayers were made by seeding 3×10^5 mononuclear cells in 96-well flat-bottomed tissue culture plates. The monolayers were incubated with PMA (200 nM, ○), heat-killed Candida (at organism/macrophage ratios of 3:1, ◆, and 6:1, ◇, live tachyzoites of *T. gondii* (at organism/macrophage ratios of 3:1, ■, and 6:1, □) or medium alone (●). The amount of superoxide in each well was calculated from measurements of absorbance at 550 nm at the times indicated. The results are the means ± SEM of quadruplicate monolayers from one experiment. The figure is representative of two similar experiments.

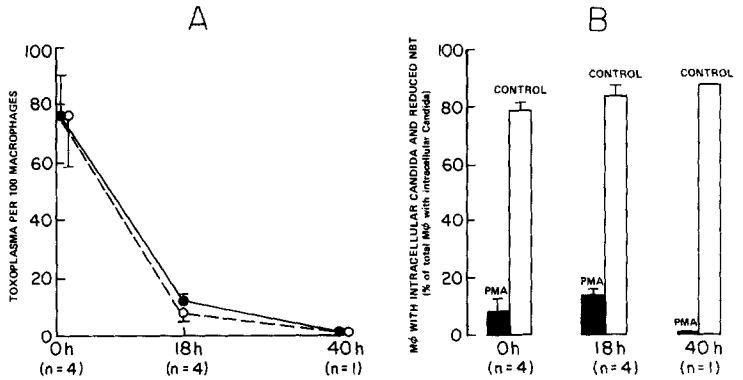

FIGURE 4. Fate of *T. gondii* in rat alveolar macrophages pretreated with PMA. Monolayers of rat alveolar macrophages were pretreated with PMA (200 ng/ml medium containing 0.07% DMSO; ●, solid bar) or with control medium (○, open bar) containing 0.07% DMSO alone. The ability of the cells to kill *T. gondii* (*A*), or their reduction of NBT dye during incubation with heat-killed Candida (*B*), were then assessed in parallel (see text for details). Results are the means ± SEM of four experiments run in duplicate, except for those at 40 h, which are the mean of duplicate results from one experiment. *Mϕ*, macrophages.

TABLE II
Effect of Scavengers of Oxygen Metabolites on the Toxoplasmacidal Activity of Rat Alveolar Macrophages

Medium supplemented with:	Number of experiments	Toxoplasma per 100 macrophages	
		0 h	18 h
Medium only*	4	40 ± 6‡	5 ± 2
Superoxide dismutase (2.5 mg/ml)	3	42 ± 5	3 ± 1
Catalase (2.5 mg/ml)	4	43 ± 7	2 ± 1
Histidine (10 mM)	4	46 ± 9	3 ± 2
DABCO (1 mM)	3	48 ± 5	3 ± 1
Mannitol (50 mM)	4	40 ± 3	3 ± 1
Benzoate (25 mM)	2	50 ± 4	5 ± 2
Tetramethylurea (25 mM)	2	48 ± 3	6 ± 5
Thiourea (25 mM)	2	41 ± 5	2 ± 1
DMSO (150 mM)	2	54 ± 9	4 ± 2
Ethanol (80 mM)	2	56 ± 3	9 ± 2
Methanol (315 mM)	2	58 ± 11	8 ± 3

* Macrophage monolayers were incubated in duplicate with medium alone, or with medium containing scavengers, for 3 h before infection, during infection, and for 18 h thereafter.
‡ The results are the means ± SEM of two to four experiments.

incubated our rat alveolar macrophages with scavengers of oxygen metabolites under the same conditions, their toxoplasmacidal activity was not affected by any of the 11 scavengers tested (Table II).

As a positive control for these studies, parallel experiments were performed with mouse peritoneal macrophages that had been activated to kill Toxoplasma by prior intraperitoneal injection of *C. parvum* (36). Most of the scavengers so tested (Fig. 5) did inhibit or slow the toxoplasmacidal activity of the activated murine peritoneal macrophages under these conditions, but the effect was clear only at 6 h (Fig. 5). Replication of Toxoplasma was seen in only one instance (an experiment with histidine, data not shown), and even in this instance there was a rise of only 3 Toxoplasma per 100 macrophages between 6 and 18 h because the replication of Toxoplasma in some macrophages was balanced by the killing of Toxoplasma in other cells. The scavengers also consistently altered the uptake of Toxoplasma by these activated murine peritoneal macrophages (Fig. 5), although this did not occur with the rat alveolar macrophages. Furthermore, the scavengers inhibited killing only if the interval between administration of *C. parvum* and harvest of the macrophages was >21 d. The scavengers had no obvious effect on the toxoplasmacidal activity of activated mouse peritoneal macrophages harvested <21 d after the *C. parvum* injection (data not shown), confirming previous observations (19) that the effects of scavengers in this (19) and similar (36) assays are critically dependent on the degree of activation of the macrophages.

Effect of Permeant Weak Bases. Treatment of rat alveolar macrophages with weak bases that are known to raise intralysosomal pH had no effect on their ability to kill *T. gondii* (Table III). This was not due to a direct toxic effect of the bases (or of raised intralysosomal pH) on either the Toxoplasma or the

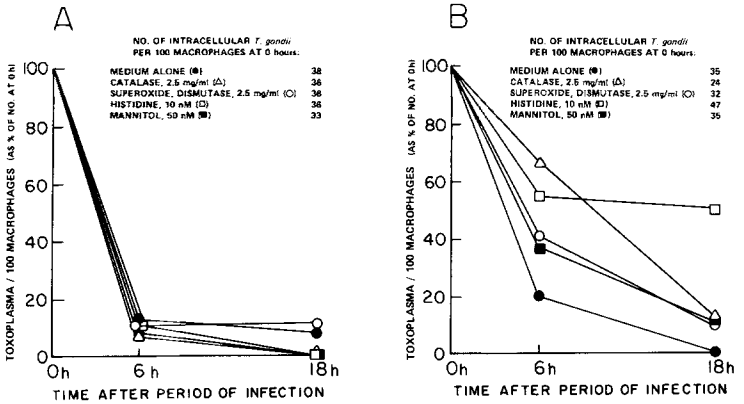

FIGURE 5. Effects of scavengers of oxygen metabolites on the Toxoplasmacidal activity of (*A*) resident rat alveolar macrophages and (*B*) murine peritoneal macrophages activated to kill *T. gondii*. The activated murine peritoneal macrophages were harvested 24 d after injecting the mice with killed *C. parvum*. Macrophage monolayers were incubated with medium alone or with medium containing scavengers for 3 h before infection, during infection, and 18 h thereafter. The experiments were performed in duplicate and in parallel. The figure is representative of two similar experiments.

TABLE III

Effect of Permeant Weak Bases on the Toxoplasmacidal Activity of Rat Alveolar Macrophages

Medium supplemented with:	Toxoplasma per 100 macrophages	
	0 h	18 h
Medium only*	53 ± 6‡	2 ± 1
Ammonium chloride (10 mM)	56 ± 4	3 ± 2
Methylamine (10 mM)	43 ± 9	0 ± 0

* Macrophage monolayers were incubated in duplicate with medium alone or with medium containing a weak base, for 15 min before infection, during infection, and for 18 h thereafter.

‡ The results are the means ± SEM of two experiments.

macrophages, since Toxoplasma multiplied intracellularly when the experiment was repeated with mouse peritoneal macrophages (Fig. 6). Moreover, as shown in Fig. 6, the weak bases markedly reduced the macrophages' capacity to concentrate acridine orange, confirming that the intralysosomal pH of these cells was elevated throughout the experiment (38).

Viability of Toxoplasma attached to the Surface of Rat Alveolar Macrophages. Toxoplasma attached to the surface of macrophages were not killed, as judged by acridine orange staining. After gentle washing of monolayers that had been incubated with Toxoplasma for 20 min, only a few (<5%) of the macrophages had Toxoplasma attached to their surface, but the proportion of these organisms that appeared dead was similar to the proportion of dead organisms in suspensions of Toxoplasma.

Even when ingestion of Toxoplasma was prevented by incubation of the macrophages with sodium fluoride, thus allowing us to observe the organisms

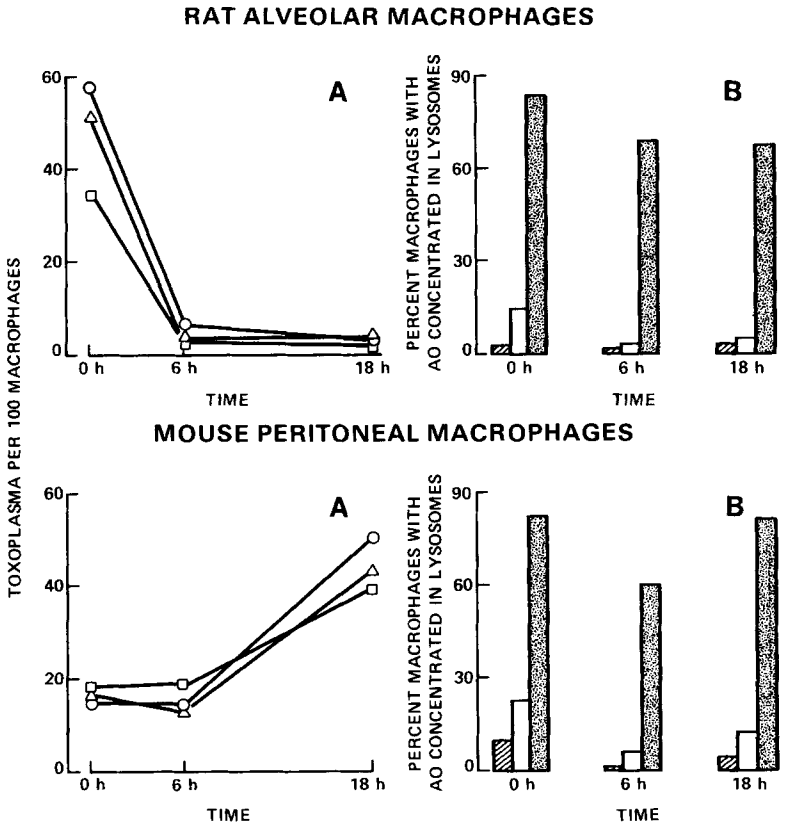

FIGURE 6. Effect of permeant weak bases on the Toxoplasmacidal activity of rat alveolar macrophages, the multiplication of *T. gondii* in mouse peritoneal macrophages, and the ability of the macrophages to concentrate acridine orange (AO) in lysosomes. Duplicate monolayers of rat alveolar macrophages were incubated with 10 mM ammonium chloride (△, striped bar), 10 mM methylamine (□, open bar) or medium alone (○, stippled bar). The fate of *T. gondii* in the macrophages (*A*), and the ability of the macrophages to concentrate acridine orange in lysosomes (*B*), were then determined. See Materials and Methods for details. The figure is representative of two similar experiments.

after they had been in contact with the cell surface for 2 h, we found no evidence that they were killed at the cell surface. In three experiments, ~20% of the macrophages had Toxoplasma attached to their surface after gentle washing of the monolayers, but these organisms were not killed, as judged by acridine orange staining. Only 18 ± 4% of the Toxoplasma attached to macrophages were scored dead after 2 h of incubation, a figure similar to the number scored dead (16 ± 4%) in Toxoplasma suspensions incubated with sodium fluoride. However, when acridine orange–labeled Toxoplasma from the same original suspension were incubated with rat alveolar macrophages that had not been treated with sodium fluoride, ingestion did take place and most (78 ± 7%) of the intracellular organisms appeared dead at 2 h.

Discussion

T. gondii rarely causes serious illness in healthy adult human subjects, but in the immunosuppressed individual it is an important cause of life-threatening

disseminated disease (13, 43, 44); pulmonary involvement is common in disseminated toxoplasmosis (11–14, 44) and may be a presenting feature of the infection (12, 14). Experimentally, *T. gondii* has proved to be remarkably useful as an intracellular probe of macrophage function (15–23, 27, 29, 45, 46); previous studies with this organism have contributed to our understanding of macrophage activation (21, 23, and reviewed in 46), macrophage antimicrobial processes (19, 23), and microbial pathogenicity (18, 45).

Our results indicate that resident rat alveolar macrophages kill *T. gondii* in vitro, and that this killing occurs without significant involvement of toxic metabolites of oxygen (18–20, 23). These findings have a number of implications: they suggest that rat alveolar macrophages possess a powerful, nonoxidative antimicrobial mechanism that is not present in resident murine alveolar and peritoneal macrophages (20, 21) or in normal human monocyte-derived macrophages (18, 20); they suggest that the toxoplasmacidal activity of rat alveolar macrophages may be a useful model for the study of nonoxidative killing by alveolar macrophages (21); and they help to explain the remarkable resistance of rats to *T. gondii* (26, 27).

When Toxoplasma enter human monocyte-derived macrophages or resident murine peritoneal macrophages, they do so without triggering the respiratory burst of these cells (18, 23) and are thus able to survive and replicate. In our experiments with resident rat alveolar macrophages, *T. gondii* showed the same ability to avoid triggering the respiratory burst; nevertheless, the organisms were killed. This strongly suggests that rat alveolar macrophages possess a nonoxidative antimicrobial mechanism that is either not present in normal human monocyte-derived macrophages (18, 20) or murine peritoneal macrophages (5, 18, 19, 23), or is far less active in these cells (20). Such a mechanism could clearly be important for killing other organisms that can avoid or overcome the respiratory burst of macrophages (21); it might also enable the cell to kill some organisms during severely hypoxic conditions (47), such as exist in consolidated areas of lung during pulmonary infections. The exact significance of this nonoxidative toxoplasmacidal mechanism in the overall antimicrobial activity of the macrophage remains to be established, but the fact that it can kill large numbers of *T. gondii* suggests that it might play a significant role in the antimicrobial defenses of the rat alveolar macrophage.

The toxoplasmacidal activity of the rat alveolar macrophage appears to be a convenient model for the study of nonoxidative killing by alveolar macrophages. Our recent observation that human alveolar macrophages also kill *T. gondii* by nonoxidative processes is of particular interest (Catterall, J. R., and J. S. Remington, manuscript in preparation). Indeed, the model may be of direct clinical relevance to the increasing numbers of severely immunosuppressed patients, for there is evidence that bone marrow transplant recipients and patients with acquired immune deficiency syndrome, many of whom die of opportunistic pulmonary infection (7, 8), possess alveolar macrophages that are unable to kill *T. gondii* (10, 48). Unlike healthy individuals, these patients have impaired cell-mediated immunity and probably cannot activate macrophages in vivo (6, 49). However, further studies are needed to determine whether the nonoxidative antimicrobial activity of human alveolar macrophages is due to the same bio-

chemical mechanism as that in rat alveolar macrophages, and whether the alveolar macrophages of these and other immunosuppressed patient populations also show deficiencies in their oxygen-dependent antimicrobial activity.

Previous studies in mouse and hamster models have suggested that macrophages are important for the host's resistance to *T. gondii* (5, 15–17, 45, and reviewed in 46). Our finding that rat alveolar macrophages readily kill *T. gondii* is consistent with that view, since rats are highly resistant to the parasite (26, 27). Alveolar macrophages in particular are almost certainly important for this defense, since pulmonary toxoplasmosis is well recognized in a variety of animals (50), as well as in immunosuppressed patients (7, 11–14, 44). Our results differ from those of Chinchilla et al. (51), who found that *T. gondii* were killed by rat peritoneal macrophages but not by rat alveolar macrophages. However, their monolayers probably contained a mixture of cell types, for they included tissue from ground lung, and the proportion of cells that were macrophages was not defined. The presence of nonphagocytic cells in a macrophage monolayer could alter the results, since most nonphagocytes permit intracellular replication of intracellular Toxoplasma (52). In our experiments, >95% of the cells in the monolayers were macrophages.

Murray and colleagues (19), using scavengers of oxygen metabolites, showed the importance of oxidative mechanisms in the toxoplasmacidal activity of activated murine peritoneal macrophages. Sibley et al. recently made similar observations (23), and their results with activated murine peritoneal macrophages closely resemble our own (Fig. 6). In contrast, the same (and additional) scavengers failed to inhibit the toxoplasmacidal activity of our rat alveolar macrophages. These findings alone do not exclude oxidative mechanisms, however, as illustrated by our control experiments with activated mouse peritoneal macrophages; the scavengers inhibited the anti-Toxoplasma activity of these cells only when their degree of activation was decreasing (36). A similar phenomenon has previously been observed with activated murine peritoneal macrophages (19, 36) and it has been suggested (19) that the most active cells might be producing more oxygen metabolites than the scavengers could overcome. If this explanation is correct, it would be impossible to exclude oxidative killing mechanisms by using scavengers alone. This was not the explanation with our rat alveolar macrophages, however, for the entry of *T. gondii* into these cells did not trigger a respiratory burst, as assessed by both intracellular reduction of NBT dye and extracellular release of superoxide. The failure to trigger a respiratory burst does not exclude a minor or facilitative role in killing for the small quantities of oxygen metabolites produced continuously by the cells (Fig. 2), but even this seems unlikely since pretreatment with PMA (29), a procedure that exhausted the respiratory burst of nearly all the cells (Fig. 3), had no effect on their ability to kill Toxoplasma. Thus, although each of the experiments designed to assess the importance of oxidative killing has limitations, their combined results leave no reasonable doubt that the toxoplasmacidal activity of rat alveolar macrophages depends mainly on nonoxidative mechanisms.

Our results complement those from previous studies of nonoxidative killing of other organisms by macrophages. Oxygen-independent microbicidal mechanisms have been shown in subcellular macrophage fragments (21, 24) and in oxidatively

deficient cells (18, 20, 23, 25), including mononuclear phagocytes from patients with chronic granulomatous disease (CGD) (18, 20), but previous proof of nonoxidative antimicrobial mechanisms in intact macrophages has been restricted to one report of weak Chlamydiastatic activity in murine peritoneal macrophages activated in vitro (22). Although killing by phagocytes from patients with CGD provides very strong evidence for nonoxidative antimicrobial mechanisms, it is not absolute proof since these cells commonly can release small levels of oxygen metabolites (18, 21). It would also be impractical and unethical to collect large numbers of alveolar macrophages from patients with CGD, yet alveolar macrophages are of special interest because pulmonary infection is common, both in these and other immunosuppressed patients and in otherwise healthy subjects. It is of interest that activated monocyte-derived macrophages from patients with CGD exhibited toxoplasmastatic activity only (20), whereas our rat alveolar macrophages were toxoplasmacidal; whether this reflects quantitative or qualitative differences in the nonoxidative antimicrobial mechanisms of the two types of macrophage remains to be established.

Because of recent reports that activated, oxidatively deficient macrophage cell lines (25) and activated murine peritoneal macrophages under anaerobic conditions (47) are capable of killing extracellular targets, we attempted to determine whether the killing of Toxoplasma by rat alveolar macrophages occurred intracellularly or extracellularly. Although most of the Toxoplasma were killed within 1 h of initial exposure to the macrophage monolayers, the killing probably did not occur until after ingestion, for we found no evidence that they were killed at the cell surface. Even when Toxoplasma were allowed to attach to the macrophages for 2 h, their entry into the cells being prevented by sodium fluoride, the organisms remained viable. These results suggest that phagosome formation is probably necessary for the killing of Toxoplasma. However, it must be stressed that they do not provide absolute proof of this, since sodium fluoride can have wide-ranging metabolic effects (42). Therefore, it is impossible to exclude a direct inhibitory effect of sodium fluoride on a putative killing mechanism at the cell surface, although Lehrer et al. (41), using *Candida albicans*, found that sodium fluoride had no effect on the ability of rabbit alveolar macrophages to kill yeasts.

Our studies showing transfer of acridine orange from lysosomes of rat alveolar macrophages to intracellular Toxoplasma (Fig. 2) raise the possibility that lysosomal contents could have come into contact with the Toxoplasma (34, 53), but they do not prove that phagosome-lysosome fusion is necessary for killing of the organism, since previously killed Toxoplasma are incapable of blocking phagosome-lysosome fusion (46) and their is evidence from studies with yeast cells that dead organisms can even take up acridine orange without phagosome-lysosome fusion (34). Indeed, our inability to block the toxoplasmacidal activity of rat alveolar macrophages with weak bases that raise intralysosomal pH (37, 38) is in keeping with a recent observation that the toxoplasmastatic activity of macrophages from CGD patients occurs without phagosome-lysosome fusion (23). On the other hand, the results of these experiments with weak bases do not exclude an earlier acidification of the phagosome, since weak bases do not raise the pH of phagosomes before they fuse with lysosomes (37). This may be pertinent, for

Toxoplasma cannot survive at low pH (Fig. 3, and reference 54), and recent studies suggest that phagosome acidification occurs before fusion with lysosomes during the killing of antibody-coated *T. gondii* by mouse peritoneal macrophages (54).

The results of our studies do not necessarily imply that oxygen-independent antimicrobial mechanisms are more important in alveolar macrophages than in macrophages from other anatomical compartments, for recent studies in our laboratory suggest that rat peritoneal macrophages also kill *T. gondii* by nonoxidative mechanisms (McCabe, R. E., and J. S. Remington, manuscript in preparation). Nor do they imply that alveolar macrophages kill all microorganisms by nonoxidative mechanisms. Toxic metabolites of oxygen almost certainly contribute to the killing of *Mycobacterium tuberculosis* by guinea pig alveolar macrophages (55) and *Legionella pneumophila* by primate alveolar macrophages (56), for example, although there is evidence that nonoxidative mechanisms in rabbit alveolar macrophages may account for the ability of these cells to kill *Aspergillus* spores (57) and possibly *Candida albicans* (41).

Rat alveolar macrophages possess both oxidative and nonoxidative antimicrobial mechanisms, and each is effective in the intact cell. Whether an organism survives in the cell will depend on its ability to avoid or overcome each of these processes. The innate ability of *T. gondii* to avoid triggering of the respiratory burst renders it particularly useful for studying nonoxidative antimicrobial mechanisms, and the similarities between rat and human alveolar macrophages suggest that this model may be relevant to human pulmonary defenses. Further studies are needed to determine the exact nonoxidative event, or series of events, responsible for killing *T. gondii*, but our findings suggest that it is an intracellular process and that exposure of the organisms to low intralysosomal pH is unlikely to be the crucial step. Whatever their nature, the nonoxidative killing mechanisms of rat alveolar macrophages are remarkably effective against *T. gondii* and may play a significant role in the cell's antimicrobial functions.

Summary

We have found that normal alveolar macrophages can kill an intracellular parasite by a mechanism that does not involve toxic metabolites of oxygen. We studied the interaction between *Toxoplasma gondii* and rat alveolar macrophages in vitro. We were interested in Toxoplasma because it causes pneumonia in immunosuppressed patients but not in healthy individuals, and we chose the rat because it resembles immunocompetent human subjects in being resistant to *T. gondii*. Resident rat alveolar macrophages could kill large numbers of *T. gondii*. This occurred without a respiratory burst as judged by intracellular reduction of nitroblue tetrazolium and quantitative release of superoxide. Furthermore, scavengers of toxic oxygen metabolites had no effect on the toxoplasmacidal activity of the alveolar macrophages, nor did prior exhaustion of their respiratory burst with PMA. Whereas acid pH (e.g., 4.5–6.0) rapidly kills extracellular *T. gondii*, raising of the intralysosomal acid pH of rat alveolar macrophages by incubating them with weak bases did not inhibit their ability to kill *T. gondii*. Killing of Toxoplasma occurred within 1 h of initial exposure to the alveolar macrophages. However, there was no evidence that killing preceded ingestion;

Toxoplasma attached to the surface of the cell appeared viable, and when phagocytosis was blocked with sodium fluoride the organisms survived. These results indicate that rat alveolar macrophages possess a powerful nonoxidative microbicidal mechanism, which is distinct from acidification of the phagolysosome but which probably involves phagosome formation. This mechanism may be clinically relevant, for we have recently observed that human alveolar macrophages also kill *T. gondii* by an oxygen-independent process.

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