Nonoxidative Microbicidal Activity in Normal Human Alveolar and Peritoneal Macrophages

JAMES R. CATTERALL,1,2* CAROLYN M. BLACK,1,2 JACK P. LEVENTHAL,3 NORMAN W. RIZK,3 JOHN S. WACHTEL,4 AND JACK S. REMINGTON1,2*

Department of Immunology and Infectious Diseases, Research Institute, Palo Alto Medical Foundation, Palo Alto, California 943011; Division of Infectious Diseases, Department of Medicine, Stanford University Medical Center, Stanford, California 943052; and Division of Pulmonary Diseases1 and Department of Obstetrics and Gynecology,4 Palo Alto Medical Clinic, Palo Alto, California 94301

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Although Toxoplasma gondii multiplies within normal murine alveolar and peritoneal macrophages, it is killed by normal rat alveolar and peritoneal macrophages. The killing by rat macrophages is by a nonoxidative mechanism. Studies on normal human alveolar macrophages have reported disparate results in regard to their ability to inhibit or kill T. gondii. We considered it of interest to explore further the effect of normal human alveolar and peritoneal macrophages on T. gondii. Unstimulated alveolar macrophages from each of seven individuals demonstrated a marked ability to kill or inhibit multiplication of T. gondii in vitro (e.g., the number of parasites per 100 alveolar macrophages was 31 at time zero and 2 at 18 h, whereas this value increased from 37 at time zero to 183 at 18 h in murine macrophages assayed in parallel). In quantitative assays of superoxide, alveolar macrophages released a substantial amount of superoxide when exposed to phorbol myristate acetate or to candidae. In contrast, alveolar macrophages incubated with T. gondii released no more superoxide than when in medium alone. Scavengers of superoxide anions, hydrogen peroxide, singlet oxygen, and hydroxyl radicals failed to inhibit killing of T. gondii by alveolar macrophages. Peritoneal macrophages from each of six normal women undergoing laparoscopy killed T. gondii in vitro; results of quantitative superoxide assays and scavenger experiments demonstrated that no oxidative burst was triggered in these macrophages by exposure to T. gondii. These data indicate that normal human alveolar and peritoneal macrophages can kill an intracellular parasite by nonoxidative mechanisms and suggest that these mechanisms are important in inhibition or killing of other opportunistic intracellular pathogens.

Macrophages play an important role in host resistance to infection (9, 10, 12, 17, 19, 34–36). Their clinical importance is suggested by the high incidence of life-threatening infections in patients with abnormal macrophage function (50) or impaired cell-mediated immunity (27). However, the mechanisms by which human macrophages kill microorganisms are poorly understood. Most studies of the antimicrobial functions of macrophages have emphasized their ability to produce toxic metabolites of oxygen (23, 26, 29, 49). Indeed, in normal human macrophages under aerobic conditions, this is the only mechanism of killing intracellular pathogens that has been shown to be effective in the intact cell. We now report oxygen-independent killing of an intracellular parasite by human alveolar and peritoneal macrophages.

Our findings were made during studies of host resistance to Toxoplasma gondii. We were interested in T. gondii because it causes life-threatening infection, including pneumonia (3, 7, 43, 46), in immunocompromised patients, whereas normal human subjects are relatively resistant to this organism (32). We found that resident human macrophages can kill T. gondii in vitro and that this occurs without the involvement of toxic metabolites of oxygen.

MATERIALS AND METHODS

Collection of macrophages. Human alveolar macrophages were collected by bronchoalveolar lavage (13) from seven subjects (six males and one female) aged 20 to 53 years. Five were healthy volunteers with no evidence of respiratory disease, one was undergoing bronchoscopy and bronchoalveolar lavage because of pulmonary sarcoidosis, and in the other patient bronchoscopy was performed because of unexplained hemoptysis. None of the subjects had an upper respiratory tract infection within 6 months of the bronchoscopy, and none smoked. The chest roentgenogram was normal in all subjects except the patient with sarcoidosis, whose radiograph showed bilateral lower zone pulmonary infiltrates. Two subjects had a toxoplasma dye test titer of greater than 1:16, but neither of these had serological evidence of recent toxoplasma infection. The remaining five subjects were either tested and found to be negative in the dye test or were not tested.

In the subjects with a normal chest roentgenogram, the lingula or middle lobe was lavaged. In the patient with sarcoidosis, the lavage was performed in the right upper lobe. Briefly, the upper airway was anaesthetized with 5 to 10 ml of 2% lidocaine, the bronchoscope was wedged into a segmental bronchus, and a further 5 to 10 ml of 2% lidocaine was injected into the bronchus to prevent coughing. Six 50-ml aliquots of saline were instilled and withdrawn through the bronchoscope, and the lavage fluid was collected in plastic tubes kept on ice. To remove mucus, we filtered the bronchoalveolar lavage fluid twice through coarse gauze before centrifugation.

Human peritoneal macrophages were collected from six women, aged 23 to 39 years, who were undergoing laparoscopy for purposes of tubal ligation or infertility (44a). No
evidence of inflammation, infection, or neoplasia was present in any of the women. One woman had a toxoplasma dye test titer of 1:32 and a negative immunoglobulin M titer by enzyme-linked immunosorbent assay. The other five patients were either negative in the dye test or were not tested. At the start of the laparoscopic procedure, 30 to 50 ml of sterile physiological saline was introduced and then aspirated from the peritoneal cavity. The peritoneal lavage fluid was kept on ice for 0.5 to 2 h during transport to the laboratory and then centrifuged as described below for culturing monolayers of macrophages.

Murine peritoneal macrophages were collected from Swiss Webster female mice that were 6 to 8 weeks old (Simonsen Laboratories, Gilroy, Calif.) as previously described (49).

Human monocytes were isolated from 50 to 100 ml of heparinized (10 U/ml) peripheral venous blood drawn from volunteers. Mononuclear cells were obtained as previously described (41).

Preparation of macrophage monolayers. The alveolar and peritoneal lavage fluids were centrifuged at 225 x g for 10 min at 4°C. The cells were washed with phosphate-buffered saline, pH 7.2 (GIBCO Laboratories, Grand Island, N.Y.) and recentrifuged. Alveolar macrophages were suspended in Iscove modified Dulbecco medium (GIBCO Laboratories) containing 40 mg of gentamicin per ml (hereafter termed medium). Peritoneal macrophages and peripheral blood mononuclear cells were suspended in the same medium containing 10% fetal calf serum. In all experiments, greater than 91% of the leukocytes in these suspensions were mononuclear cells in Diff-Quik (American Scientific Products, McGaw Park, Ill.)-stained cytocentrifuge preparations. The concentration of mononuclear cells was adjusted to 2 x 10^6/ml with medium, and 200 µl of this suspension was placed into individual wells of eight-chamber tissue culture slides (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.). It was previously determined that under these conditions, human alveolar and peritoneal macrophages suspended at the same concentration will produce similar densities of adhered monolayers. The cells were incubated for 2 h at 37°C in air containing 5% CO2 and were washed twice with phosphate-buffered saline. The monolayers were used immediately or after 18 h of further incubation in medium. It has been previously observed in our laboratory that activities in our assay system of monolayers that have been incubated overnight are no different from those used immediately. Over 95% of the cells in the monolayers of alveolar macrophages were mononuclear phagocytes as assessed by their ability to ingest neutral red.

Percentages of macrophages, monocytes, and granulocytes were determined on cytocentrifuge preparations of peritoneal fluid and on adhered mononuclear phagocytes. The concentration of mononuclear phagocytes was 2 x 10^6/ml, and the concentration of mononuclear phagocytes was 1 x 10^5/ml. The percentage of mononuclear phagocytes was determined as mononuclear phagocytes and granulocytes as determined by myeloperoxidase staining.

Microorganisms. Tachyzoites of the RH strain of T. gondii were harvested and processed by methods described previously (49), suspended in medium at concentrations of 2 x 10^6 to 8 x 10^6/ml, and immediately added to the macrophage monolayers. Heat-killed Candida albicans cells were opsonized with heterologous normal human serum and suspended in medium, also at a concentration of 2 x 10^6 to 8 x 10^6/ml.

Special reagents. Reagents were obtained from Sigma Chemical Co. unless indicated otherwise. The following scavengers of oxygen metabolites (25, 39, 42) were used: superoxide dismutase, type III, 3,000 U/mg (2.5 mg/ml) as a scavenger of superoxide anion; mannitol (50 mM) as a scavenger of hydroxyl radicals; and histidine (10 mM) as a scavenger of singlet oxygen radicals. Phorbol myristate acetate (PMA) was dissolved in dimethyl sulfoxide at a concentration of 0.3 mg/ml, separated into 50-µl aliquots, and stored at −70°C (49).

Infection of macrophage monolayers and assessment of intracellular killing. A 300-µl sample 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 phosphate-buffered saline. Immediately thereafter (time zero) and at varying intervals thereafter, duplicate monolayers were fixed in cold (4°C) aminoacridine fixative (49), stained with Diff-Quik stain, and examined for morphologically intact intracellular T. gondii. The number of infected macrophages and the number of parasites per 100 macrophages were determined by counting at least 200 cells in each of duplicate monolayers. By observing any changes in the density of the monolayers over time and by comparing cell counts on monolayer supernatants, we determined that there is no preferential detachment of infected macrophages from the surface of the slides.

Intracellular reduction of NBT dye. Nitro Blue Tetrazolium (NBT) dyel at a concentration of 0.25 mg/ml of medium was incubated for 1 h with macrophage monolayers in the presence of live tachyzoites of T. gondii or opsonized heat-killed candida in eight-chamber tissue culture slides (Miles Scientific) (49). 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 to 200 cells which contained organisms.

Release of superoxide from human macrophages. The amount of superoxide released from macrophage monolayers in response to PMA (200 nM), opsonized heat-killed candida (at an organism/macrophage ratio of 4:1), or live tachyzoites of T. gondii (at the same organism/macrophage ratio) was measured by reduction of ferricytochrome c (31). In this procedure, blank wells consisting of the experimental conditions plus 400 U of superoxide dismutase were subtracted from each of the experimental readings.

Effect of scavengers of oxygen metabolites on toxoplasma-cidal activity of macrophages. Macrophage and monocyte monolayers were incubated with scavengers of oxygen metabolites dissolved in medium (at the concentrations stated above) or with medium alone for 3 h before infection with T. gondii, during the infection period, and for 18 h thereafter (25). Slides were fixed and stained at 0 and 18 h and examined to determine the number of infected cells and the number of morphologically intact parasites per 100 cells. Human peripheral blood monocytes are known to kill T. gondii primarily by oxygen-dependent mechanisms (49) and were used in this experiment as a positive control on the effectiveness of the scavenging agents.

RESULTS

Toxoplasma-cidal activity of human alveolar and peritoneal macrophages. Alveolar and peritoneal macrophages from all
The subjects killed *T. gondii* in vitro (Fig. 1). Immediately after the period of infection (0 h), 30 ± 6% (standard error of the mean [SEM]) of the alveolar macrophages and 56 ± 5% of the peritoneal macrophages contained at least one intracellular toxoplasma. Eighteen hours later, the proportion of infected alveolar and peritoneal macrophages had dropped to 7 ± 3% and 13 ± 7%, respectively, indicating that most of the *T. gondii* had been killed by both types of macrophages. Some of the surviving parasites did replicate in each group, however, indicating heterogeneity of antitoxoplasma activity among the populations of human alveolar and peritoneal macrophages.

The poor survival of *T. gondii* in human macrophages was not due to a low viability of the organisms in the infecting inoculum, since organisms from the same suspensions of parasites survived and multiplied readily inside resident murine peritoneal macrophages (Fig. 1). In the six experiments in which human alveolar macrophages and mouse peritoneal macrophages were studied in parallel, using organisms from the same suspensions of *T. gondii*, the number of parasites per 100 macrophages increased by 260 ± 61% (SEM) in the mouse peritoneal macrophages, but decreased by 43 ± 14% in the human alveolar macrophages. The alveolar and peritoneal macrophages from patients with positive toxoplasma dye test titers behaved no differently from macrophages from patients with negative dye test titers, nor was there any difference in this assay between the normal volunteers and either the patient with sarcoidosis or the patient with unexplained hemoptysis.

**Release of superoxide by human alveolar and peritoneal macrophages.** When *T. gondii* cells were incubated with human alveolar macrophages in the presence of NBT, fewer than 5% of the macrophages that contained intracellular *T. gondii* showed reduction of NBT, even though organisms from the same suspensions of *T. gondii* were killed by the macrophages. When monolayers of the same macrophages were incubated with opsonized heat-killed candidae, however, NBT reduction was seen in the majority (58 to 96%) in five experiments) of human alveolar macrophages that contained candidae.

Activation of the respiratory burst was also assessed by quantitating the liberation of superoxide (Fig. 2A and B). The human alveolar and peritoneal macrophages readily released superoxide in response to PMA or opsonized heat-killed candidae, but when incubated with *T. gondii* they released no more superoxide than did resting cells (Fig. 2A and B). In concurrent experiments, however, alveolar and peritoneal macrophages from the same preparations used in the superoxide assay ingested and killed organisms from the same suspensions of *T. gondii* (data not shown).

**Effects of scavengers of oxygen metabolites.** The toxoplasma macidal activity of human alveolar and peritoneal macrophages was not affected by any of the scavengers of oxygen metabolites tested (Fig. 3). In two experiments the number of parasites per 100 alveolar and peritoneal macrophages decreased during 18 h of incubation with medium alone or with superoxide dismutase, mannitol, or histidine. In contrast, when peripheral blood monocytes were tested in parallel with alveolar and peritoneal macrophages, the presence of oxygen-scavenging agents completely inhibited the ability of the monocytes to kill *T. gondii* (Fig. 3).

**DISCUSSION**

Previous studies in this laboratory have shown that *T. gondii* can avoid the oxidative antimicrobial mechanisms of macrophages (49). When *T. gondii* enter human monocyte-derived macrophages or resident murine peritoneal macrophages, they do so without triggering the respiratory burst of these cells, and the organisms survive and subsequently replicate (49). In the present experiments with human alveolar and peritoneal macrophages, *T. gondii* demonstrated the same ability to avoid triggering the respiratory burst; nevertheless, the organisms were killed. These findings have a number of implications. They may help to explain the normal resistance of human subjects to *T. gondii*; they illustrate the limitations of monocyte-derived macrophages as a model for human tissue macrophages; and they provide a clear demonstration, under aerobic conditions, that a normal intact human phagocyte can kill an intracellular microorganism by an oxygen-independent mechanism.

Most studies of host resistance to *T. gondii* have been performed with mice (14, 35, 38, 39) or hamsters (12). These
species are highly susceptible to *T. gondii*, and their resident macrophages are unable to kill the organism. In contrast, resident alveolar and peritoneal macrophages from rats are capable of killing large numbers of *T. gondii* (4, 20), and we recently postulated that this might account for the remarkable resistance of rats to *Toxoplasma* infection (4, 15, 33). The results of the present study are consistent with that view, since human subjects are also relatively resistant to *T. gondii* and their macrophages also kill this parasite. The resistance of healthy subjects to *T. gondii*, therefore, may depend in part on the innate toxoplasmacidal ability of their resident macrophages. Alveolar macrophages in particular are likely to be important, since pneumonia is a common finding in immunosuppressed patients who have died of toxoplasmosis (3, 7, 43, 46).

However, not all the intracellular *T. gondii* were killed by the resident tissue macrophages that we studied. In most experiments there was an occasional macrophage which permitted intracellular replication of parasites. This was most likely due to heterogeneity within the macrophage populations. Recent studies indicate that the antitoxoplasma activity of human alveolar macrophages can be enhanced by lymphokines or by recombinant gamma interferon (24). The resistance of immunocompetent adults to *T. gondii*, therefore, may depend not only on the antimicrobial activity of resident normal macrophages but also in part on the ability of their T lymphocytes to produce gamma interferon for the activation of occasional permissive macrophages which may be present in normal populations.

There is considerable evidence that tissue macrophages are derived from circulating monocytes (44). Human monocytes can kill *T. gondii* (21, 48), and our results suggest that they retain some, though not all (21, 24), of this toxoplasmacidal activity when they differentiate into alveolar or peritoneal macrophages. In contrast, monocytes which have been cultured in vitro for 7 to 10 days lose their ability to kill *T. gondii* (1, 26, 49). These observations suggest that the maturation of monocytes into macrophages in vivo differs from their maturation in vitro and that monocyte-derived macrophages have limitations as a model for tissue macrophages. The importance of the environment in the differentiation of mononuclear phagocytes has been emphasized by studies of macrophages from laboratory animals. Against some microorganisms, for example, including *Nocardia* (2), *Pasteurella* (6), *Aspergillus* (37), and *Candida* (41) species, there are even differences in antimicrobial

### FIG. 2

(A) Release of superoxide by human alveolar macrophages when incubated with PMA (*n* = 4), opsonized heat-killed candidae (*n* = 2), *T. gondii* (*n* = 4), or medium alone (*n* = 4). (B) The same experiments done with human peritoneal macrophages (*n* = 2 for all groups). Results are mean ± SEM (bars) of *n* experiments.

### FIG. 3

Effects of scavengers of oxygen metabolites on the antitoxoplasma activity of human peripheral blood monocytes, alveolar macrophages, and peritoneal macrophages. Monolayers were incubated with medium alone (□), with 2.5 mg of superoxide dismutase per ml (●), with 50 mM mannitol (●), and with 10 mM histidine (○) for 3 h before infection, during infection, and for 18 h thereafter. Peripheral blood monocytes were tested in parallel with alveolar and peritoneal macrophages, but alveolar and peritoneal macrophages were tested in separate experiments. Data shown are representative of two similar experiments performed in duplicate.
activity between alveolar macrophages and peritoneal macrophages from the same animal. However, with our human tissue macrophages, we found no significant difference between peritoneal and alveolar macrophages in their ability to kill *T. gondii* and no evidence that the mechanism of their toxoplasmoidal activity was different.

Our results appear to confirm the observations of McLeod and colleagues that resident human alveolar macrophages kill *T. gondii* in vitro (21). However, it is not clear from the data presented by those authors whether there was killing or survival of the parasite in their mouse macrophage control since the data were presented solely as the percentage of macrophages infected rather than the number of parasites per 100 macrophages. In a recent study by Murray and co-workers (24), resident human alveolar macrophages supported the intracellular growth of *T. gondii*. However, during the same time period, these authors also observed an increase in the number of parasites per 100 human monocytes, in contrast to previous studies in which intracellular *T. gondii* were rapidly killed by human monocytes (21, 26, 48). The reasons for these apparent differences are unclear.

The absence of a respiratory burst during killing of *T. gondii* by human macrophages is very strong evidence that the killing was nonoxidative. However, even resting macrophages release small amounts of toxic oxygen metabolites (Fig. 2A and B), and it is conceivable that even these small amounts could have killed the parasites. To explore this possibility, we determined whether killing was affected by scavengers of oxygen metabolites which under certain conditions (25) have previously been shown to impair the toxoplasmoidal activity of activated murine peritoneal macrophages (25, 39). However, the same scavengers under the same conditions had no effect on the toxoplasmoidal activity of our human macrophages. Taken together, these results strongly suggest that the killing of *T. gondii* by human tissue macrophages occurs independently of toxic metabolites of oxygen.

Most studies of microbial killing by human phagocytes have emphasized the importance of oxidative mechanisms. Previous evidence for nonoxidative intracellular killing has been limited to chemical analysis of cell fragments (8, 11, 22), experiments performed under anaerobic conditions (5, 18, 30, 45), and studies of cells from patients with chronic granulomatous disease (23, 26, 28, 40, 49). Although killing by phagocytes from patients with chronic granulomatous disease provides strong evidence for nonoxidative antimicrobial mechanisms in intact cells, it is not absolute proof since these cells commonly can release small levels of toxic oxygen metabolites (29). Wilson and Haas (47) recently reported nonoxidative killing of *T. gondii* by monocyte-derived macrophages which had been activated in vitro by lymphokines. However, other authors using the same system have obtained different results with activated monocyte-derived macrophages (26). In the present study, we used freshly harvested resident macrophages from subjects with no known enzyme deficiency, and the studies were performed with intact cells under aerobic conditions.

Our studies do not imply that human macrophages kill all microorganisms by an oxygen-independent mechanism. Although the macrophages failed to generate a respiratory burst when incubated with *T. gondii*, they did release toxic metabolites of oxygen when incubated with opsonized heat-killed candidae or PMA. Thus, our results confirm those of previous authors that human tissue macrophages (24) are capable of producing a respiratory burst, provided they are stimulated appropriately. Studies in animal models suggest that different microorganisms have different abilities to stimulate the respiratory burst of macrophages. However, we are aware of no such studies involving living microorganisms and human tissue macrophages.

What is the exact biochemical nature of the oxygen-independent antimicrobial activity that we observed? This question will be difficult to answer by studying human macrophages alone, since the number of human tissue macrophages available for study is limited by both ethical and practical considerations. However, we have recently observed that resident alveolar and peritoneal macrophages from rats also kill *T. gondii* by an oxygen-independent mechanism (4, 20). The interaction of rat alveolar macrophages and *T. gondii* in vitro may be highly relevant to the antimicrobial functions of human macrophages and should be a useful model for the study of oxygen-independent antimicrobial mechanisms.

In conclusion, we found that human alveolar macrophages and human peritoneal macrophages possess both oxygen-dependent and oxygen-independent antimicrobial activities and that both are effective in the unstimulated cell. The exact biochemical nature of the oxygen-independent antimicrobial activity requires further elucidation, and we suggest that *T. gondii* will be a useful organism for such studies because of its inherent ability to avoid triggering the respiratory burst of macrophages. Further studies are also needed to determine the relative importance of oxidative and nonoxidative killing mechanisms in the overall antimicrobial activity of human macrophages.

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LITERATURE CITED


