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Killing of *Plasmodium yoelii* by Enzyme-Induced Products of the Oxidative Burst

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The murine malaria parasite *Plasmodium yoelii* was killed in vitro when incubated with glucose and glucose oxidase, a system generating hydrogen peroxide, or with xanthine and xanthine oxidase, a system which produces the superoxide anion and subsequently other products of the oxidative burst. Catalase blocked the killing in both cases; superoxide dismutase and scavengers of hydroxyl radicals or singlet oxygen were ineffective in the xanthine oxidase system. Thus, hydrogen peroxide appears to be the main reactive oxygen species killing *P. yoelii*.

Previous publications have highlighted the importance of non-antibody-mediated mechanisms of immunity to malaria (2, 6, 29). The ability of sera from mice infected with *Mycobacterium bovis* BCG to induce crisis forms, parasites degenerating within erythrocytes (6), together with the absence of extensive erythrophagocytosis during infection, have suggested that parasites might be killed within erythrocytes in the circulation by nonspecific factors. For example, sera containing tumor necrosis factor can kill malarial parasites cultured in vitro (30) and reduce parasitemia when given to mice during infection (29). Sera from functionally immune Africans are also able to induce crisis forms and metabolic death in *Plasmodium falciparum* cultured in vitro (17).

Another group of nonspecific products is generated during the respiratory burst by macrophages and polymorphs (19), which include superoxide anions ($O_2^- \cdot$), hydrogen peroxide, and hydroxyl radicals ($OH \cdot$). These reactive oxygen intermediates have been shown to kill protozoa such as *Toxoplasma gondii* (24), *Leishmania donovani*, and *Leishmania tropica* (14, 23). Macrophages are activated during a malaria infection, and thus it is possible that secreted reactive oxygen intermediates might destroy malaria parasites within erythrocytes. We have shown that hydrogen peroxide, injected intravenously, caused a drop in parasitemia in mice infected with *Plasmodium yoelii* or *Plasmodium chabaudi* and could kill *P. yoelii* and *Plasmodium berghei* maintained in vitro (10). Recent work by Clark and Hunt (8) has demonstrated that injection of alloxan, a drug that generates oxygen radicals, into mice infected with *Plasmodium vinckei* causes a rapid reduction in parasitemia, accompanied by parasite damage and hemolysis. In this system, the iron-chelating agent desferrioxamine protected the parasites, which was interpreted to mean that hydroxyl radicals might mediate the observed effects. We now report killing of *P. yoelii* cultured in vitro with systems generating reactive oxygen intermediates. The addition of inhibitors suggested that in this system hydrogen peroxide is the major effector molecule against this parasite. Thus, such molecules could represent another mechanism of non-antibody-mediated killing of malarial parasites during infection.

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MATERIALS AND METHODS

Mice. Tuck no. 1 outbred female mice were obtained from Tuck & Sons, Battlesbridge, Essex, United Kingdom, and were used at 6 to 8 weeks of age.

Parasites. *P. yoelii* 17X, a nonlethal parasite, was used in all experiments. Mice were infected intravenously, and parasitemias were counted on blood films stained wtih Giemsa.

Parasite killing assay. A sample of blood containing approximately 10^4 parasitized erythrocytes (parasitemia, approximately 3%) was incubated in phenol red-free balanced salts solution (BSS) containing 10% fetal bovine serum (Flow Laboratories) (final volume, 1 ml) for 2 h at 37°C in an atmosphere of 5% CO_2 in air. RPMI 1640 medium was used instead of BSS in experiments done to test whether iron salts were required for killing. After incubation, three samples of 0.2 ml each were injected intravenously into mice, and their parasitemia was counted daily. The dose of parasites injected was calculated from the day on which parasitemia reached 0.5%, based on the inverse linear relationship between log viable parasite dose and time to patency (30).

Chemicals and enzymes. Xanthine (Sigma Chemical Co., St. Louis, Mo.; sodium salt) and hypoxanthine were prepared in 50 mM KH_2PO_4 buffer (pH 7.8) containing 10^{-4} M EDTA and used at a final concentration of 1.5×10^{-4} M. Histidine hydrochloride and sodium benzoate were used at 10 mM, and mannitol was used at 50 mM (24). Sodium urate was used at a final concentration of 1.2 μM, and allopurinol (Sigma), an inhibitor of xanthine oxidase, was used at 0.05%. Hydrogen peroxide (20 volumes; 6% [wt/vol]) was obtained from BDH Chemicals, Poole, England. Catalase (Sigma; thymol free; 11,000 U/mg) was used at 600 μg/ml, and superoxide dismutase (Sigma; 2,900 U/mg) was used at 100 μg/ml; catalase was heat inactivated at 100°C for 20 min and resuspended by sonication. Horseradish peroxidase (type II; 165 U/mg), lactoperoxidase (79 U/mg), glucose oxidase (from *Aspergillus niger*; 1,400 U/ml), and xanthine oxidase (from milk; 4 U/ml) were all obtained from Sigma. Enzyme and inhibitor solutions were diluted in phosphate-buffered saline (PBS) (pH 7.2), 0.15 M.

Assay of hydrogen peroxide. To measure the amount of hydrogen peroxide generated by glucose and glucose oxidase, 100 μl of test supernatant was added to 2.9 ml of 0.01

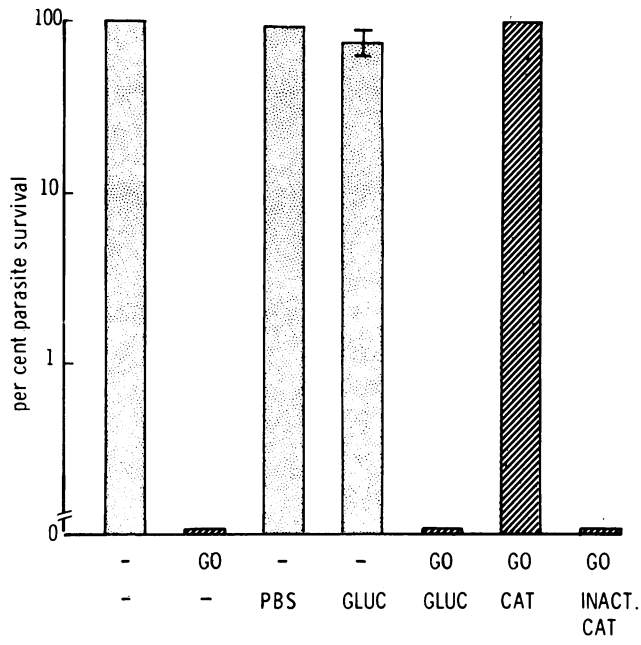

FIG. 1. Killing of *P. yoelii* by glucose oxidase-glucose. Parasitized blood containing approximately 10^4 parasitized erythrocytes was incubated for 2 h in BSS containing 10% fetal bovine serum. Test wells contained 10 mU of glucose oxidase (GO), PBS plus 10% fetal bovine serum replacing the BSS (PBS), increased glucose (0.2%) (GLUC), 10 mU of glucose oxidase with 0.2% glucose (GO GLUC), or glucose oxidase with 600 μg of catalase (GO CAT) or heat-inactivated catalase (GO INACT. CAT). After incubation, the percent parasite survival was estimated by injecting samples into mice. The results are expressed as mean ± standard deviation.

M phosphate buffer (pH 7.4) containing 6 U of horseradish peroxidase and 100 μg of *p*-OH phenyl acetic acid and read on a fluorescence spectrometer using excitation and emission wavelengths of 315 and 410 nm, respectively (12). The assay was standardized using known quantities of reagent-grade hydrogen peroxide, measured spectrophotometrically at an absorption of 230 nm using an extinction coefficient of 67 M^{-1} cm^{-1} (20).

RESULTS

Glucose and glucose oxidase system. To test whether hydrogen peroxide generated in vitro killed *P. yoelii*, glucose and glucose oxidase, an enzyme system generating hydrogen peroxide, but not the other reactive oxygen intermediates, was chosen. When parasites were incubated with glucose oxidase and glucose for 2 h, complete killing was observed down to 10 mU of glucose oxidase per ml. This killing was not due to depletion of glucose; doubling the glucose concentration to 0.2% did not alter parasite survival or killing by glucose oxidase. Moreover, the parasites can survive for 2 h in PBS containing 10% fetal bovine serum without added glucose (Fig. 1). Catalase, 600 μg/ml, but not heat-inactivated catalase, completely blocked killing (Fig. 1).

When the production of hydrogen peroxide by glucose oxidase in the absence of erythrocytes was measured, over 100 nmol of hydrogen peroxide per ml was produced in 2 h, whereas the concentration previously shown to be toxic, 10^{-5} M (i.e., 10 nmol/ml) (10), was exceeded within 30 min (Fig. 2). The addition of the same number of erythrocytes as used in the parasite killing assay reduced the hydrogen peroxide concentration to 45 nmol/ml at 2 h, indicating that the erythrocytes, presumably via enzymes such as glutathione peroxidase and catalase, were scavenging some of the hydrogen peroxide. Catalase in the same amount as used in the parasite killing test reduced the hydrogen peroxide concentration to below 2.5 nmol/ml (Fig. 2).

Xanthine and xanthine oxidase system. To test whether other products of the oxidative burst were also toxic, *P. yoelii* was incubated with xanthine oxidase, which converts xanthine to uric acid (or hypoxanthine to xanthine) with the production of superoxide anion (3) and subsequently other reactive oxygen intermediates, including hydrogen peroxide, hydroxyl radicals, and singlet oxygen (4). When 10^4 parasites were incubated for 2 h with xanthine and 5 to 20 mU of xanthine oxidase per ml, all the parasites were killed (Fig. 3). Higher concentrations of enzyme killed some parasites even in the absence of added substrate. A concentration of 10 mU of xanthine oxidase per ml was chosen for the remaining experiments, as it induced greater than 99% parasite killing without itself causing a significant drop in viability. Similar killing was also observed when hypoxanthine was used as substrate (Fig. 4). Parasite survival was not diminished by incubation in 1.2 μM sodium urate, indicating that the breakdown product of xanthine was not itself toxic.

The time course of the killing occurring in this system was determined. It was found that significant killing had occurred by 15 min and that all the parasites were killed by 30 min (Fig. 5). However, as viability was assessed by injecting treated parasites into mice, they may only have been damaged during the incubation period, leading to parasite death or lysis of the erythrocyte later.

When inhibitors were added to the system, no blocking was seen with 100 μg of superoxide dismutase per ml, which converts $O_2^- \cdot$ to H_2O_2. Catalase, at 600 μg/ml, completely

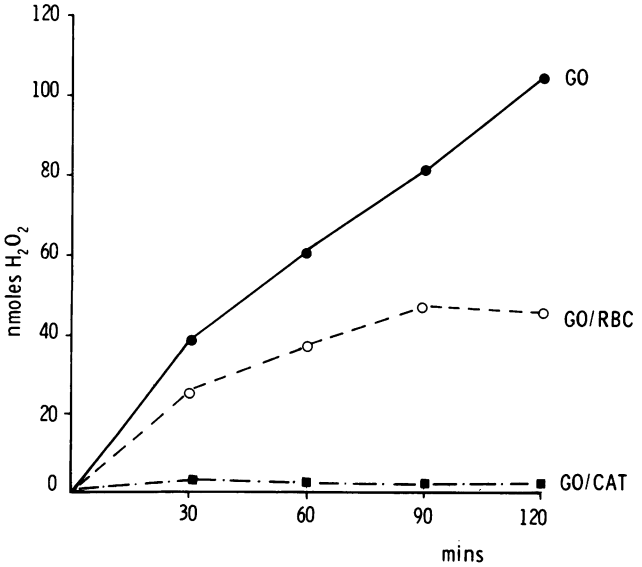

FIG. 2. Hydrogen peroxide production by glucose oxidase. Glucose oxidase (GO), 10 mU, was added to BSS containing 10% fetal bovine serum to give a final volume of 1 ml (closed circles). Additional wells contained a sample of parasitized blood containing approximately 10^4 parasitized erythrocytes (RBC) (open circles) or 600 μg of catalase (CAT) (closed squares). At 30, 60, 90, and 120 min, the supernatant was assayed for hydrogen peroxide (see text). The results are expressed as nanomoles of hydrogen peroxide per milliliter.

blocked killing (Fig. 6); heat-inactivated catalase did not. Neither enzyme decreased parasite survival in the absence of xanthine and xanthine oxidase; in fact, viability was occasionally improved by catalase. Allopurinol, an inhibitor of xanthine oxidase, also blocked killing (Fig. 6).

A number of scavengers of other oxygen radicals were also tested. Mannitol and benzoate, which are hydroxyl radical scavangers (24), and histidine, which captures singlet oxygen (24), also did not block killing (Fig. 7). Histidine alone caused a slight drop in viability. As the BSS used in this test does not contain added iron and thus might restrict the production of hydroxyl and other radicals by the Haber-Weiss reaction (13), these experiments were also performed in RPMI 1640, which contains ferrous sulfate; identical results were obtained.

Role of lactoperoxidase and halide in hydrogen peroxide killing. In a number of microbicidal systems, peroxidase and halide enhance killing caused by hydrogen peroxide (19). Parasites were therefore incubated with hydrogen peroxide at concentrations between 10^{-3} and 10^{-6} M (10), with the addition of lactoperoxidase and potassium iodide. No enhancement of killing was observed.

DISCUSSION

To generate reactive oxygen intermediates and thus mimic the oxidative burst in the absence of cells, the enzymes glucose oxidase and xanthine oxidase were chosen. The bacterial enzyme glucose oxidase is useful because it generates hydrogen peroxide without other products of the oxidative burst (13). *P. yoelii* was killed when incubated with 10 mU or more of glucose oxidase per ml, which produced greater than 10 nmol of hydrogen peroxide per ml within 30 min, concentrations previously shown to be toxic with reagent-grade hydrogen peroxide (10). When erythrocytes were added to the hydrogen peroxide assay system at the same concentration as used in the parasite killing tests, they scavenged up to 56.6% of the hydrogen peroxide produced at 2 h but were unable to reduce the hydrogen peroxide concentration to nontoxic levels. Erythrocytes have also been shown to reduce chemiluminescence in a hypoxanthine-xanthine oxidase system (15). When catalase was present, the concentration of the hydrogen peroxide present was below 2.5 nmol/ml, confirming that the catalase was active; all the parasites survived under these conditions (Fig. 1).

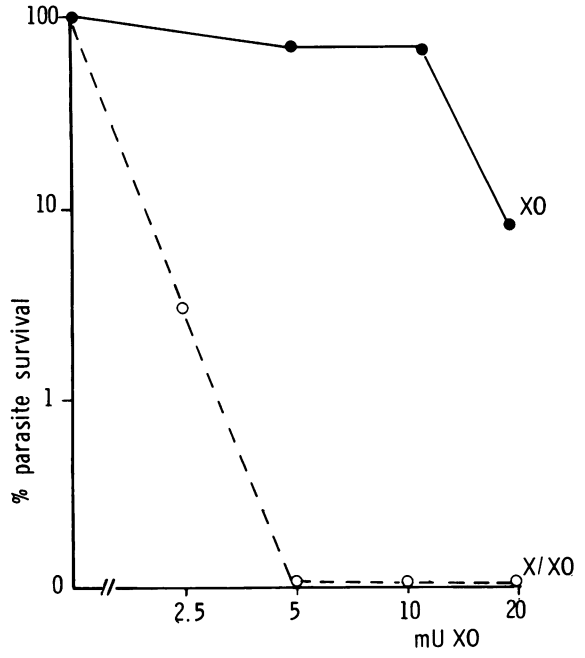

FIG. 3. Killing of *P. yoelii* by xanthine oxidase-xanthine (X/XO). A sample of parasitized blood containing approximately 10^4 parasitized erythrocytes was incubated for 2 h at 37°C in BSS containing 10% fetal bovine serum and 1.5×10^{-4} M xanthine. Test samples contained from 5 to 20 mU of xanthine oxidase (open circles). A control with xanthine oxidase without xanthine (XO) is also shown (closed circles). After incubation, parasite survival was estimated by injecting samples into mice.

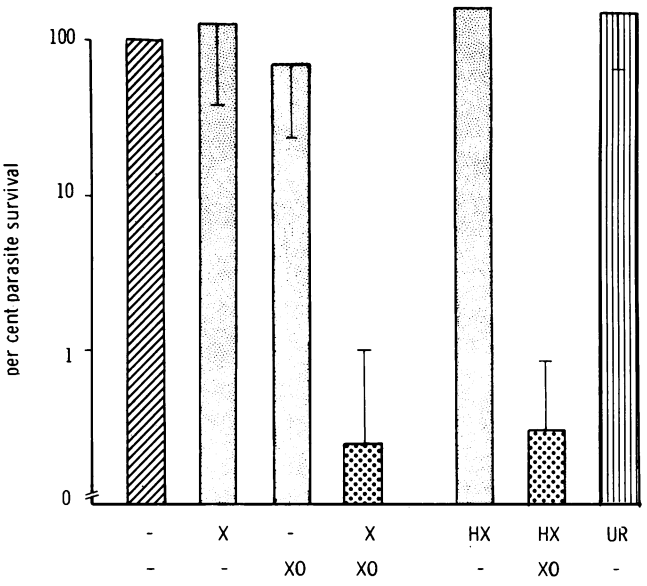

FIG. 4. Killing of *P. yoelii* by xanthine oxidase-xanthine. A sample of parasitized blood containing approximately 10^4 parasitized erythrocytes was incubated for 2 h at 37°C in BSS containing 10% fetal bovine serum. Test wells contained 1.5×10^{-4} M xanthine (X), 10 mU of xanthine oxidase (XO), xanthine and xanthine oxidase (X XO), 1.5×10^{-4} M hypoxanthine (HX), hypoxanthine and xanthine oxidase (HX XO), or 1.2 μM sodium urate (UR). After incubation, samples were injected into mice to estimate parasite survival. The results are expressed as mean ± standard deviation of a minimum of three experiments.

The action of xanthine oxidase on its substrate xanthine generates the superoxide anion $O_2^- \cdot$ (3) and uric acid; hydrogen peroxide, hydroxyl radicals, and singlet oxygen are subsequently formed (4, 13, 19). Xanthine oxidase is, however, relatively nonspecific as regards its substrate; not only hypoxanthine but other compounds such as acetaldehyde can also be utilized (18). Although the levels of such compounds in the serum added would be extremely low and xanthine oxidase itself is present in fetal bovine serum, this may account for the killing of *P. yoelii* seen here using high concentrations of enzyme without added xanthine.

The killing effect of xanthine oxidase-xanthine occurred very rapidly and was usually complete by 30 min. A longer incubation period, 2 h, was chosen for comparative experiments to minimize differences in incubation time between groups when large numbers of animals were injected. In fact, the parasites may not actually be killed within 30 min, but merely damaged, leading to subsequent lysis, as has been observed in the xanthine oxidase-acetaldehyde system in which erythrocytes lysed 2 h later (18).

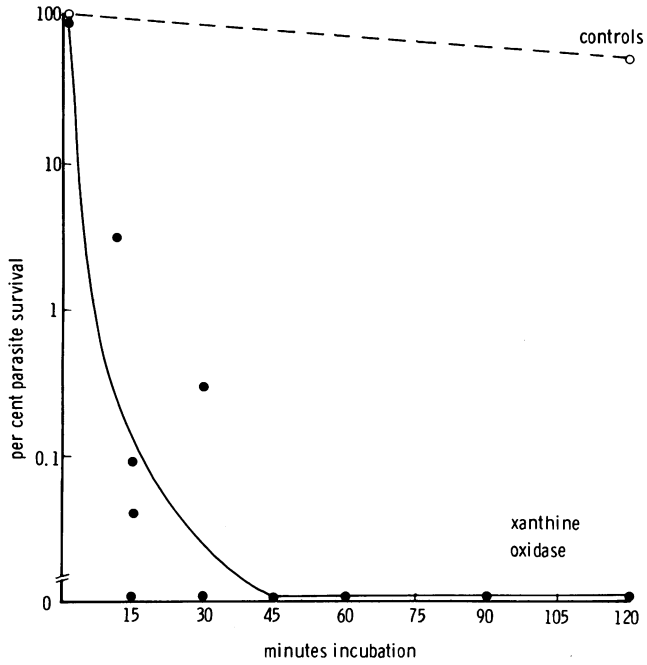

FIG. 5. Time course of killing of *P. yoelii* by xanthine oxidase-xanthine. A sample of parasitized blood containing approximately 10^4 parasitized erythrocytes was incubated at 37°C in BSS containing 10% fetal bovine serum and 1.5×10^{-4} M xanthine (open circles). Test wells contained 10 mU of xanthine oxidase (solid circles). After incubation, samples were injected into mice to estimate the percent parasite survival.

Inhibition of killing by catalase implicates hydrogen peroxide as the chief effector molecule. Thus, superoxide dismutase would be expected to be ineffective, as it would produce more hydrogen peroxide; moreover, the superoxide anion is relatively nontoxic in other systems (19). The failure to show inhibition with scavengers of hydroxyl radicals or singlet oxygen also implies that these products are not toxic in this system. It has been suggested that xanthine itself can scavenge these radicals (4), but doubling the xanthine concentration did not decrease the killing observed. However, it is possible that molecules such as the superoxide anion and hydrogen peroxide may cross the erythrocyte membrane and generate further radicals within the cell, where they would be inaccessible to scavengers such as mannitol.

Hydrogen peroxide, generated by xanthine oxidase, has been shown to be toxic for a range of organisms, including *Leishmania* sp. (14) and *Escherichia coli* (3). Other organisms, such as *T. gondii* (24) and *Staphylococci epidermidis* (3), seem to be killed by a combination of both superoxide anions and hydrogen peroxide, suggesting that hydroxyl radicals might be involved. In some of these systems, the cytotoxicity seen with hydrogen peroxide is enhanced by peroxidase and potassium iodide (19). No additional killing of *P. yoelii* was observed in our system when lactoperoxidase and potassium iodide were included, but the presence of serum, essential for parasite viability, may act as a scavenger of reactive halide anions. Measurement of hydrogen peroxide generated by glucose oxidase in the presence or absence of serum indicated that although the serum reduced hydrogen peroxide levels by up to 16%, toxic levels were still produced (H. M. Dockrell, unpublished results).

There have been a number of other recent studies on oxidative radicals and malaria parasites. The drug alloxan, which generates reactive forms of oxygen by redox cycling between alloxan and dialuric acid, causes a rapid decrease in parasitemia in mice infected with *P. vinckei* (8). The compound *t*-butyl hydroperoxide, previously shown to cause physicochemical changes in erythrocytes, including decreased deformability and osmotic fragility (9), is toxic for *P. falciparum* in vitro (7). Incubation of *P. falciparum* with glucose oxidase or xanthine oxidase results in the death of the parasites (32). It is also possible that the killing of *P. chabaudi* by the enzyme polyamine oxidase (22) is mediated by oxidative radicals, as the reaction of this enzyme with spermine generates hydrogen peroxide (1).

What is less clear in all these studies is why the parasites die. One possibility is that hydrogen peroxide causes lipid peroxidation of the erythrocyte membrane (18), leading to changes in ion transport and decreased membrane deformability (9). Erythrocyte defenses against hydrogen peroxide, such as glutathione peroxidase and catalase, may also be overwhelmed in infected erythrocytes already under oxidative stress (11). Toxic effects might result from oxidation of hemoglobin (11), or there may be a more direct cytotoxic action on the parasites themselves.

The observation that a reaction involving the enzyme xanthine oxidase can kill malaria parasites is particularly interesting, as this enzyme is a normal component of mammalian tissues (16), and levels have been reported to be raised during a malaria infection (27, 31). Allopurinol, an inhibitor of xanthine oxidase, was found to increase mortal-

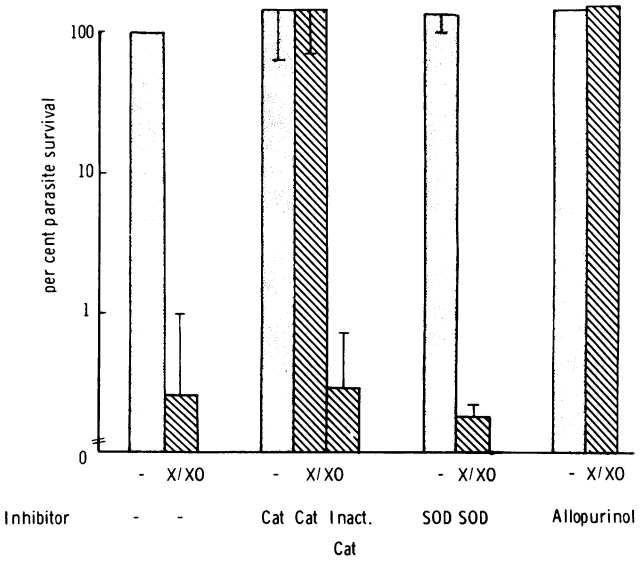

FIG. 6. The effect of inhibitors on the killing of *P. yoelii* by xanthine oxidase-xanthine. A sample of parasitized blood containing approximately 10^4 parasitized erythrocytes was incubated for 2 h at 37°C in BSS containing 10% fetal bovine serum. Test wells contained 1.5×10^{-4} M xanthine and 10 mU of xanthine oxidase (X/XO), 600 μg of catalase (Cat), or heat-inactivated catalase (Inact. Cat) with xanthine and xanthine oxidase, 100 μg of superoxide dismutase (SOD), superoxide dismutase and xanthine-xanthine oxidase (X/XO SOD), or 0.05% allopurinol or allopurinol and xanthine-xanthine oxidase (Allopurinol X/XO). After incubation, samples were injected into mice to estimate the percent parasite survival. Results are expressed as mean ± standard deviation of a minimum of three experiments.

ity in mice infected with *P. berghei* (31); however, in another study in which allopurinol was shown to increase parasitemia in rats infected with *P. berghei*, this finding was interpreted as being due to the increased concentrations of xanthine produced rather than to decreased production of toxic oxygen products (5). Our preliminary results obtained with *P. yoelii* indicate that allopurinol can increase parasitemia and induce death at certain times during the infection (H. M. Dockrell, unpublished results). Thus, the classical oxidative burst based on NADPH oxidase may not provide the only source of reactive oxygen intermediates during infection.

The question of whether sufficient reactive oxygen intermediates are actually generated in vivo during a malaria infection to play a role in controlling parasitemia remains to be settled. A study of peritoneal macrophages incubated with *P. berghei* parasites in vitro using chemiluminescence showed that an oxidative burst could be triggered in some cases (21). It is possible that macrophages in sites such as the liver and spleen, polymorphs in the blood, natural killer cells (2, 25), and even endothelial cells, which have been shown to contain xanthine oxidase (16), could be involved in releasing reactive oxygen intermediates. These products would be most effective in localized microenvironments, such as the sinusoids of the liver and spleen, where parasitized erythrocytes are in close proximity to macrophages; it has been estimated that in the space between closely apposed cells, localized hydrogen peroxide concentrations could reach 10^{-2} M (28); hydrogen peroxide release between monocytes and tumor target cells has been visualized with electron microscopy (26). The release of such products in confined spaces might also cause tissue damage (8).

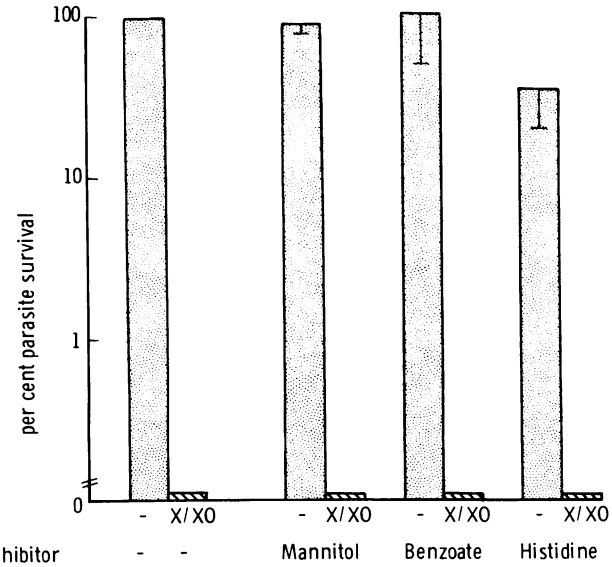

FIG. 7. The effect of oxygen radical scavengers on the killing of *P. yoelii* by xanthine-xanthine oxidase. A sample of parasitized blood containing approximately 10^4 parasitized erythrocytes was incubated for 2 h at 37°C in BSS containing 10% fetal bovine serum. Test wells contained 1.5×10^{-4} M xanthine and 10 mU of xanthine oxidase (X/XO), and 50 mM mannitol, 10 mM sodium benzoate, or 10 mM histidine alone or with xanthine-xanthine oxidase. After incubation, samples were injected into mice to estimate the percent parasite survival. Results are expressed as mean ± standard deviation of a minimum of three experiments.

These oxygen intermediates are extremely reactive, short-lived products which would be quickly scavenged or broken down in plasma. Thus, cytotoxic factors detected in serum (6, 17) are probably more stable products such as tumor necrosis factor (29, 30) or other as yet unidentified factors. We are now investigating the relationship of tumor necrosis factor release to the release of reactive oxygen intermediates during malaria infection. Although it has not yet been possible to assess the relative contributions of these two types of nonspecific products to parasite killing in vivo, we feel that hydrogen peroxide, in addition to factors like tumor necrosis factor, could well play an important role in host defense.

ACKNOWLEDGMENT

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