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Killing of Human Malaria Parasites by Macrophage Secretory Products

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The susceptibility of the human malaria parasite, *Plasmodium falciparum*, to killing in vitro by macrophage secretory products was investigated. The effect of O_2 radicals and tumor necrosis factor on parasite viability was assessed both morphologically and by following the uptake of [^{3}H]hypoxanthine. H_2O_2 produced by the interaction of glucose and glucose oxidase was found to reduce viability; this effect was reversed by the addition of exogenous catalase. Further studies indicated that the catalase level within the erythrocyte was not altered upon parasite invasion. O_2 radicals produced during the xanthine-xanthine oxidase interaction also killed *P. falciparum*. The addition of various O_2 radical scavengers (including catalase) did not reverse this effect; therefore, it was not possible to determine which of the O_2 radicals were involved in the killing process. Samples from three different sources containing tumor necrosis factor, a nonspecific soluble mediator derived from *Mycobacterium bovis* BCG-activated macrophages treated with endotoxin, also killed the parasite. There was no evidence that tumor necrosis factor or the products of the xanthine-xanthine oxidase interaction caused damage to the erythrocyte membrane that could be implicated as an important aspect of the killing process. These findings all strongly suggest that such macrophage products play an important role in immunity to malaria.

The activation of macrophages by agents such as *Mycobacterium bovis* BCG and *Propionibacterium acnes* (*Corynebacterium parvum*) may result in the nonspecific protection of the murine host against several species of rodent malaria (6, 7). Macrophages activated in this way release a variety of secretory products, including O_2-derived radicals (3). Furthermore, when mice are given *Mycobacterium bovis* BCG or *P. acnes* followed by endotoxin, or are treated with endotoxin during the course of a malarial infection, macrophages will release factors that can kill tumor cells (tumor necrosis factors, TNFs) (5, 9, 24). Recent reports have indicated that human and rodent malaria parasites are susceptible to killing by both categories of secretory products (2, 8, 10, 20, 24).

The administration of alloxan, a drug that causes damage to vascular endothelial cells and tocopherol-deficient erythrocytes by the release of O_2-derived free radicals, results in the reduction of the parasitemia in *Plasmodium vinckei*-infected mice (8). This reduction can be reversed by desferrioxamine, an iron-chelating agent that prevents the production of hydroxyl radicals, suggesting that they are involved in a parasite killing process. More specifically, the O_2 radical H_2O_2 alone has been shown to kill *Plasmodium yoelii* parasites in vivo and in vitro (10).

To investigate the possible role of macrophages in nonspecific immunity to the human malaria parasite, *Plasmodium falciparum*, we have tested in vitro susceptibility of this parasite to these secretory products. O_2-derived radicals were generated by the xanthine-xanthine oxidase system, and hydrogen peroxide was produced by the interaction of glucose and glucose oxidase. TNF was obtained from rabbit serum, and in supernatants from mouse and human macrophages stimulated by endotoxin to produce cytolytic factor in vitro. The effect of these products on parasite viability was assessed both morphologically and by measuring the uptake of [^{3}H]hypoxanthine over a 24-h period after treatment.

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MATERIALS AND METHODS

Parasites. *P. falciparum* (Wellcome/Liverpool strain) was maintained by routine cultivation procedures (26), using RPMI 1640 medium supplemented with 15% ARh$^+$ human serum and N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES) buffer. Tests were performed on parasites grown in 96-well microtiter plates (Titertek) in volumes of 25 μl at a hematocrit of 6%. Growth was initiated 24 h previously at a parasitemia of 0.5%. The plates were incubated at 37°C in a Flow modular incubator chamber containing a gas mixture of 92% N_2, 5% CO_2, 3% O_2.

Production of O_2 radicals derived from xanthine-xanthine oxidase. Each test well received (i) 1.5×10^{-4} M xanthine (Sigma Chemical Co.), (ii) 25 mU of xanthine oxidase (type 1, Sigma), both in 12.5-μl quantities, made up in 0.05 M phosphate-buffered saline (PBS) at pH 7.4. The control wells contained (i) PBS only, (ii) 1.5×10^{-4} M xanthine only, (iii) 25 mU of xanthine oxidase only. Detection of superoxide anion production in wells containing the enzyme-substrate system was carried out by following the reduction of nitrotetrazolium blue (Sigma), using the method of Beauchamp and Fridovich (4).

Scavengers of oxygen radicals. Test wells containing 1.5×10^{-4} M xanthine additionally received 5-μl quantities of 50 mM mannitol, 2,000 U of catalase (bovine liver), 200 U of superoxide dismutase (bovine blood), 1 mM diazabicyclooctane, or 10 mM histidine, all prepared in 0.05 M PBS (18). (All reagents were from Sigma). Finally, 25 mU of xanthine oxidase was added to each well. Control groups contained the scavenging chemicals only.

Production of hydrogen peroxide by using glucose-glucose oxidase. H_2O_2 was generated in test wells by adding 12.5-μl quantities of 50 mM glucose and 5, 25, and 50 mU of glucose

oxidase, respectively, prepared in 0.05 M PBS at pH 7.4. In addition, each well contained 2.5 mM glucose as a constituent of the parasite growth medium. Test wells contained (i), 2,000 U of catalase, (ii) 2,000 U of heat-inactivated catalase, (iii) 200 U of superoxide dismutase, (iv) 200 U of heat-inactivated superoxide dismutase, in addition to glucose-glucose oxidase. Control wells contained (i) 50 mM glucose, (ii) 50 mU of glucose oxidase, (iii) 0.05 M PBS, (iv) scavenging enzymes only, all in the absence of the enzyme-substrate system.

Catalase assay for parasitized and normal erythrocytes. The level of catalase in the lysates of mature human erythrocytes (both parasitized and nonparasitized) was assessed by following the decomposition of H_2O_2 spectrophotometrically (1). Stock lysates were prepared from 1-ml volumes of the cell samples by adding 4 ml of distilled water. Parasitized cells were separated by a density gradient centrifugation method, using Percoll (14). A 1:500 dilution of each lysate was prepared in PBS, and the hemoglobin content was assessed by the method of van Kampen et al. (28) outlined below. A 2-ml amount of this hemolysate was added to 1 ml of 10 mM H_2O_2 in 50 mM phosphate buffer at pH 7.0 in a quartz cuvette. After thorough mixing, the decrease in extinction at E_{240} was followed at 20°C, using a Pye-Unicam chart recorder.

Preparation of TNF samples. Rabbit serum containing TNF was obtained from rabbits infected with *Mycobacterium bovis* BCG and given endotoxin, by the method outlined by Taverne et al. (25). Supernatants of mouse macrophages containing TNF were prepared by endotoxin stimulation of peritoneal cells from mice infected with *Mycobacterium bovis* BCG, as described by Mannel et al. (18). Supernatants containing as anti-tumor cytotoxin produced by human monocytes stimulated with endotoxin were prepared by the method of Matthews (19). All samples were assayed for cytotoxicity against the L929 mouse fibroblast cell line (25).

Testing of TNF samples against *P. falciparum*. A 25-μl amount of the appropriate dilution of TNF sample was added to triplicate test wells. Dilutions were prepared in complete medium. Control wells received (i) complete medium only, (ii) 5μg of lipopolysaccharide W from *Escherichia coli* O55:B5 per ml (phenol extract, Difco Laboratories, Detroit, Mich.) in RPMI, (iii) supernatants from activated macrophages that had not been stimulated with endotoxin, (iv) normal rabbit serum, all in 25-μl quantities.

Parasitemia and parasite morphology. Smears of cells from each well were prepared at 4 or 24 h after the beginning of each test. These were methanol fixed and Giemsa stained, and the parasitemia was expressed as a percentage of 2,000 erythrocytes examined under oil immersion. Any alterations in parasite morphology observed during these examinations were noted.

Uptake of [^{3}H]hypoxanthine. A 5-μl (0.2 μCi) amount of [^{3}H]hypoxanthine (Amersham International Ltd.) was added to each well at the beginning of the tests. After 24 h, the contents of each well were filtered through Whatman 3MM filter paper (35 mm), then washed with 15 ml of distilled water and 30 ml of 0.9% saline solution. The disks were dried, placed in polyethylene vials (Packard Instrument Co., Inc.) with 10 ml of toluene scintillant (Packard), and counted with a Packard Tri-Cam scintillation counter. Standards were taken as the uptake of [^{3}H]hypoxanthine by parasitized cells in complete medium only, and by uninfected erythrocytes. (This method is described by S. Irare and D. C. Warhurst, manuscript in preparation).

Reinvasion assay. A 0.5-ml packed volume of unparasitized erythrocytes was washed once in 0.15 M PBS (pH 7.4) and then incubated in 4 mg of fluorescein isothiocyanate (dissolved in PBS) per ml for 10 min at 20°C, following the method of Lamont et al. (16). The cells were then washed five times in PBS and suspended in complete medium, and 5 $\times$ 10^5 cells in 25-μl quantities were placed in the wells of a microtiter plate. After 8 h of incubation with equal quantities of (i) TNF samples, (ii) 1.5 $\times$ 10^{-4} M xanthine and 25 mU of xanthine oxidase, (iii) xanthine only, (iv) xanthine oxidase only, or (v) complete medium only, the supernatant was removed from each well and medium containing 5 $\times$ 10^5 erythrocytes (2 $\times$ 10^4 parasitized) was added. A further incubation of 18 h at 37°C was carried out. Smears were then prepared, methanol fixed, and stained with 2 μg of ethidium bromide per ml dissolved in water for 15 min at 20°C. After being washed in water, slides were air dried and examined under a Vickers UV microscope, to establish whether orange fluorescing parasite DNA was visible in both fluorescein isothiocyanate-labeled and unlabeled erythrocyte populations.

Assay for hemoglobin in the supernatant. Using the method of van Kampen et al. (28), supernatants from various test groups were added in volumes ranging from 0.2 ml up to 1 ml to a solution containing 1.63 nmol of phosphate buffer, 0.75 nmol of potassium cyanide per liter, 0.6 nmol of potassium ferricyanide per liter, and 5% sodium azide, to give a total volume of 3 ml in silica cuvettes. The formation of cyanmethemoglobin was followed by reading the absorbance at 546 nm at 25°C, using a Pye-Unicam spectrophotometer with a 1-cm light path. The standard used was distilled water.

RESULTS

Killing of *P. falciparum* by O_2 radicals produced by xanthine-xanthine oxidase. The interaction of xanthine and xanthine oxidase results in the production of a range of O_2 radicals, primarily superoxide anions (3). This interaction was therefore used to produce O_2 radicals in vitro and to assess their effect on the viability of *P. falciparum*. Parasite viability was severely reduced by products of the system, and there was close correlation between the two methods used to assess parasite viability (Fig. 1 and 2). A reduction in parasite viability was seen within 2 h of the parasitized cells being exposed to O_2 radicals produced in the system (Fig. 2). It should be noted that controls containing very high concentrations of xanthine oxidase alone showed a decrease in parasite viability. This was possibly due to the enzyme utilizing an alternative substrate, such as acetaldehyde (15).

Effect of O_2 radical scavengers on parasite killing by xanthine-xanthine oxidase. Various scavengers of the O_2 radicals produced by xanthine-xanthine oxidase were subsequently added to the system for an investigation of whether parasite killing was reversed upon the removal of particular radicals. The scavengers used were catalase (H_2O_2 scavenger), superoxide dismutase (superoxide anions), mannitol (hydroxyl radicals), diazabicyclooctane and histidine (singlet oxygen scavengers). None of the scavengers used were found to effectively reverse parasite killing (Table 1). The scavenging enzymes used were found to be fully active upon subsequent testing.

Killing of *P. falciparum* by H_2O_2 produced in vitro by glucose-glucose oxidase. The interaction of glucose and glucose oxidase results in the production of hydrogen peroxide only. The addition of these reagents into the test system resulted in a similar reduction in parasite viability (Fig. 3). When a range of O_2 radical scavengers were included in the

system, catalase was found to be the only scavenger that could partially reverse parasite killing (Table 2). This suggested the involvement of H_2O_2 in the killing process.

Catalase levels in infected and noninfected erythrocytes. As catalase was found to partially reverse parasite killing by hydrogen peroxide produced in the glucose-glucose oxidase system, assays of the intracellular levels of this enzyme were carried out on both infected and noninfected human erythrocytes, so that we could investigate whether protection against H_2O_2 differed in the two cell types. After three assays, the mean levels of catalase in these two groups was found to be 23.64 *K*/ml (± 0.67) and 24.40 *K*/ml (± 1.0), respectively, (where *K* = rate constant for a first-order reaction). Thus, it appeared that there was no significant difference between the levels of this enzyme in the two types of erythrocyte.

Killing of *P. falciparum* by TNF samples. TNF samples from three sources appeared to cause parasite killing at low dilutions. From Fig. 4, it is apparent that the sample from the supernatants of human monocytes gave the most effective reduction in parasite viability. Once again, the two methods used to assess viability appeared to be closely comparable, as indicated by the similarity between the effects of mouse macrophage-derived TNF illustrated in Fig. 4 and 5. Although they are not represented in these figures, we carried out a series of experiments to investigate the time at which killing could first be detected. The effect was observed between 6 and 8 h after the parasites were exposed to the TNF samples. This is similar to the period required for the killing of *P. yoelii* (24).

Detection of erythrocyte damage caused by O_2 radicals and TNF. (i) Hemoglobin assay. To detect whether parasite killing in the test systems was accompanied by large-scale damage to the erythrocyte membrane, we tested supernatants from wells containing parasitized and unparasitized cells exposed to TNF and O_2 radicals for the presence of hemoglobin. None was detected in any of the samples tested, indicating that substantial membrane "leakage" was not occurring in the test systems.

(ii) Reinvasion assay. In addition, the possibility that O_2 radicals or TNFs modify the erythrocyte surface membrane such that parasite invasion is affected was investigated by testing the ability of parasites to invade fluorescein isothiocyanate-labeled normal erythrocytes that had been previously exposed to these products. Such erythrocytes were incubated with non-fluorescein isothiocyanate-labeled erythrocytes, a proportion of which were parasitized. Finally, parasite material was stained with ethidium bromide for detection of whether invasion of both populations of erythro-

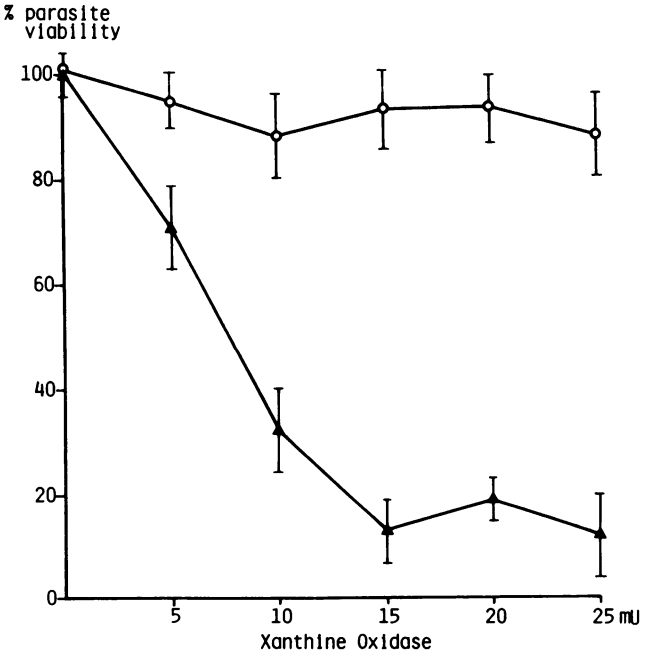

FIG. 1. Effect of increasing xanthine oxidase concentrations on *P. falciparum* viability in the presence of 1.5×10^{-4} M xanthine (▲) and in the presence of complete medium only (○). Viability was assessed by measuring the uptake of [^{3}H]hypoxanthine over 24 h; in all figures, levels of viability are expressed as the mean viability of six wells in each group. A 100% viability level was equivalent to approximately a 5 to 6% level of parasitemia. Each well contained a final volume of 50 μl at a hematocrit of 3%.

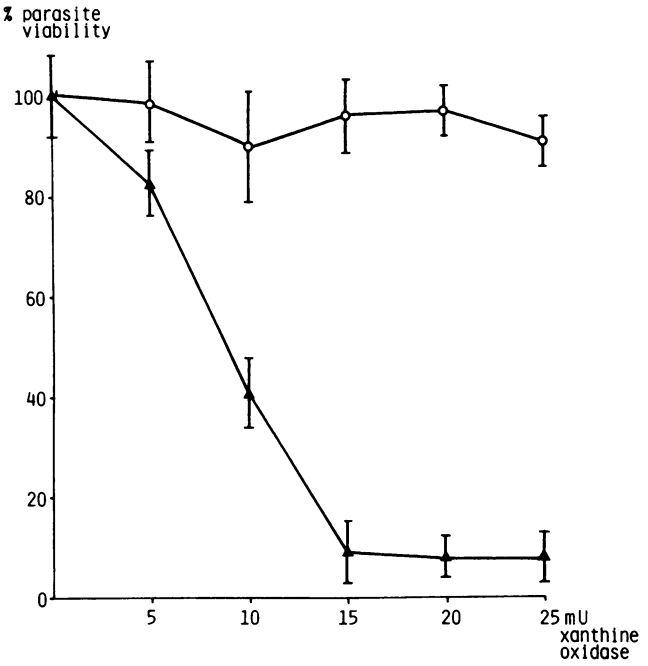

FIG. 2. Effect of increasing xanthine oxidase concentrations on *P. falciparum* viability in the presence of 1.5×10^{-4} M xanthine (▲) and in the presence of complete medium only (○). Viability was assessed morphologically after a 2-h incubation period.

TABLE 1. Lack of effect of O_2 radical scavengers on killing of *P. falciparum* by xanthine-xanthine oxidase, assessed by [^{3}H]hypoxanthine uptake over a 24-h period[a]

Scavenger	% Parasite viability
None	17 ± 4.26
2,000 U of catalase	16.5 ± 4.18
2,000 U of heat-inactivated catalase	17 ± 4.57
50 mM mannitol	16 ± 5
10 mM histidine	15.5 ± 6
200 U of superoxide dismutase	17 ± 5
200 U of heat-inactivated superoxide dismutase	16.2 ± 4.3
1 mM diazabicyclooctane	15 ± 5.3

[a] Control wells containing the scavengers alone, without the enzyme-substrate system (data not shown), all maintained parasite viability at levels of 98 to 100%.

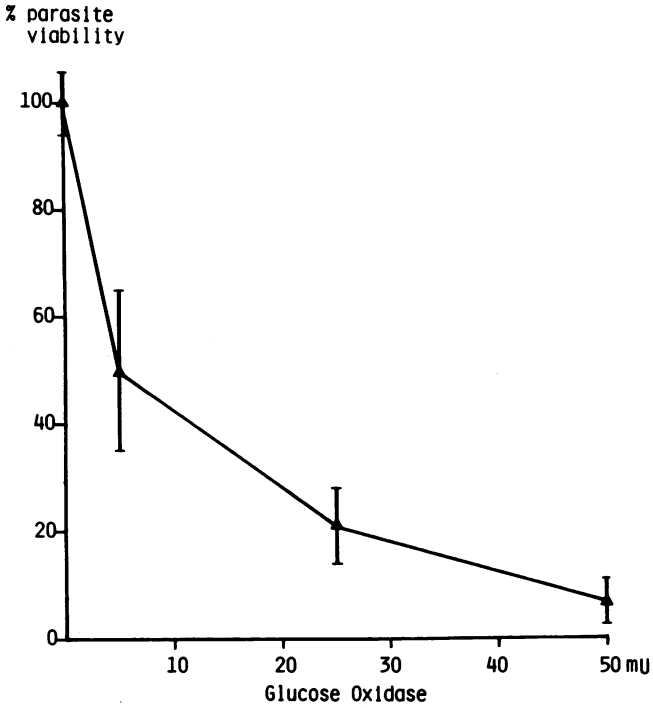

FIG. 3. Effect of increasing glucose oxidase concentrations in the presence of excess glucose on *P. falciparum* viability, assessed by measuring the uptake of [^{3}H]hypoxanthine over 24 h. (As glucose is a vital requirement for the metabolism of *P. falciparum*, it was not possible to include control wells with no available substrate.)

cytes had taken place. Orange fluorescing parasite DNA was apparent in both populations, indicating that O_2 radicals and TNFs did not modify the erythrocyte membrane in a way that precluded parasite invasion.

DISCUSSION

O_2 radicals released by the interaction of xanthine and xanthine oxidase, and H_2O_2 released by the interaction of glucose and glucose oxidase, were shown to kill the human malaria parasite, *P. falciparum*, in vitro. These findings are consistent with other reports of the susceptibility of protozoan parasites of O_2 radicals (21–23). In all tests, scavengers of various radicals were included in an attempt to elucidate which radicals were responsible for parasite killing. The significant reversal of killing achieved by the addition of catalase in the glucose-glucose oxidase system suggested the involvement of H_2O_2, as found for rodent malaria parasite killing (10, 21). The altered red-brown appearance of erythrocytes exposed to the products of this system suggested that hemoglobin oxidation had taken place. However, the absorption spectra of pigments extracted from such cells are currently being measured, and initial results indicate that there is no methemoglobin formation.

Parasite killing effected by the products of the xanthine-xanthine oxidase interaction could not be reversed by any of the O_2 radical scavengers that were tested. This finding contrasts with that of H. M. Dockrell and J. H. L. Playfair, (Infect. Immun., in press) that killing of the rodent parasite *P. yoelii* by these products was reversed by catalase. There are several possible explanations for this difference. It is possible that the major products of this system, superoxide anions, were not successfully scavenged by superoxide dismutase, and that they subsequently caused parasite damage. Alternatively, the breakdown of these anions, either spontaneously or by the action of superoxide dismutase, would result in H_2O_2 production. The elimination of this radical would depend on catalase levels inside and outside the erythrocyte and the internal level of the glutathione peroxidase-glutathione reductase system which is responsible for H_2O_2 detoxification within the cytoplasm. Finally, the interaction of H_2O_2 and superoxide anions by the iron-catalyzed Haber-Weiss reaction produces hydroxyl radicals. It has been suggested that these are involved in the killing of *P. vinckei* by the O_2 radical-generating drug alloxan, as killing can be reversed by pretreatment with desferrioxamine, an iron-chelating agent (8). However, in comparing rodent and human malaria parasite killing by O_2 radical-generating systems, it is important to consider the differing physical properties of the erythrocytes they invade and the proximity of these cells to the radicals, which is relative to the scale of the system used.

In any case, it is clear that the killing effect of the O_2 radicals produced by the xanthine-xanthine oxidase system must be influenced by the proximity of the radicals and the

TABLE 2. Effect of hydrogen peroxide and superoxide anion scavengers on killing of *P. falciparum* by glucose-glucose oxidase, assessed by [^{3}H]hypoxanthine uptake over a 24-h period[a]

Scavenger	% Parasite viability
None	10 ± 9.2
2,000 U of catalase	65 ± 17.6
2,000 U of heat-inactivated catalase	15.2 ± 17.4
200 U of superoxide dismutase	0.2 ± 8.9
200 U of heat-inactivated superoxide dismutase	0.5 ± 5.2

[a] Control wells containing the scavengers alone, without the enzyme-substrate system, all maintained parasite viability at levels of 100%.

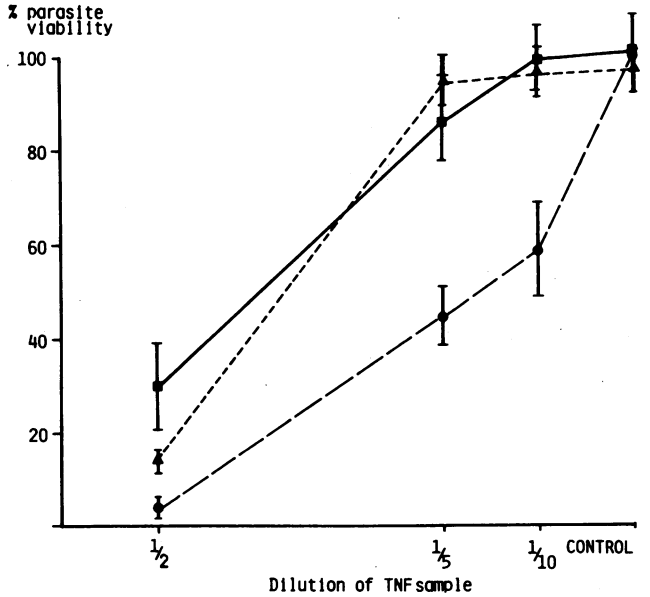

FIG. 4. Effect of differing dilutions of TNF samples on the viability of *P. falciparum*, assessed morphologically after 24 h. Symbols: ●, human monocyte supernatant; ▲, mouse macrophage supernatant; ■, rabbit serum. A 100% viability level was equivalent to approximately a 4 to 5% level of parasitemia. Each well contained a final volume of 50 μl at a hematocrit of 3%.

scavengers to the erythrocyte, the subsequent ability of both to traverse the erythrocyte membrane, and the levels of protective enzymes within the erythrocyte itself. Our initial studies have indicated that one such protective enzyme, catalase, remains at a constantly high level in both parasitized and nonparasitized cells. This contrasts with reports that the catalase levels in cells parasitized with the rodent malaria, *Plasmodium berghei*, may be raised by as much as fivefold (11), thus increasing protection against H_2O_2 damage. In the same context, it is also possible that levels of superoxide dismutase and glutathione peroxidase-glutathione reductase (responsible for the breakdown of superoxide anion and H_2O_2, respectively, within the cell cytoplasm) are altered during the process of parasite invasion.

TNFs containing serum and macrophage supernatants from three sources had a similar killing effect a low dilutions. The factor responsible for killing in each case has not yet been identified; only a single factor might be involved, since the time to death of both rodent and human malarias after exposure to the various TNF samples was similar. TNF-containing samples that have undergone further purification by ion exchange and gel filtration are currently being tested, and initial results appear to indicate good correlation between the killing activities shown against malaria parasites and tumor cells. It has been suggested that the detection of low levels of endotoxemia in *P. falciparum*-infected patients (27) indicates the possibility of localized TNF production, with host or parasite-derived material providing endotoxin-like activity (9).

To investigate whether parasite killing by O_2 radicals and TNFs were brought about indirectly by initial damage to the host cell, we tested supernatants from test wells in which killing was observed for the presence of hemoglobin. None was detected, indicating that no large-scale damage had occurred. Differential staining techniques indicated that the exposure of normal erythrocytes to these cytotoxic agents did not damage the cells to an extent that prevented parasite invasion. It is possible that the agent in each case could pass through the membrane and effect the parasite directly. There

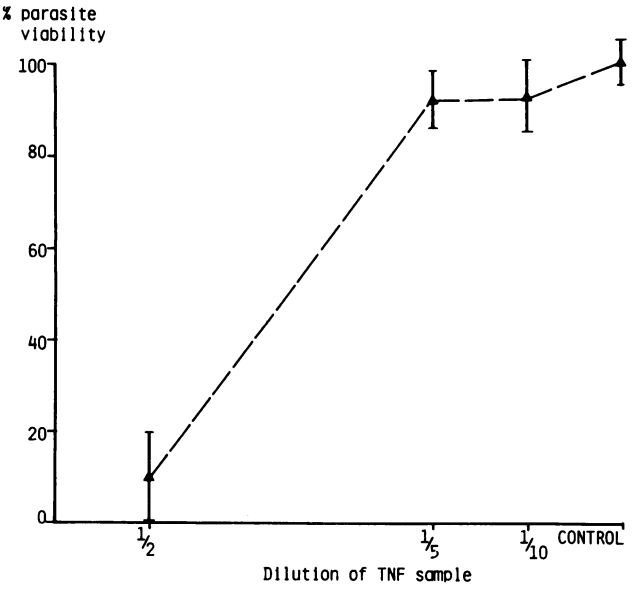

FIG. 5. Effect of differing dilutions of TNF-containing mouse macrophage supernatant on the viability of *P. falciparum*, assessed by measuring the uptake of [^{3}H]hypoxanthine over 24 h.

is evidence, for example, of superoxide anions traversing the membranes of erythrocyte ghosts (17). However, it must still be considered that parasite damage could be secondary to host cell damage caused by phenomena such a lipid peroxidation and altered membrane permeability (12). *P. falciparum* development in vitro is relatively synchronous, and it was observed that the schizont stage of the parasite was more susceptible than ring trophozoites to killing by both sorts of macrophage products. In many cases, the erythrocyte membrane of schizont-infected cells was seen to have ruptured prematurely, at a time when damaged ring forms of the parasite were still contained within intact erythrocytes. Electron microscopy is currently being used to provide more information about the killing process.

In summary, the killing of *P. falciparum* parasites by these two categories of macrophage products supports other findings that the macrophage plays an important role in nonspecific and specific immunity to malaria. This could be particularly relevant when parasitized cells are brought into close proximity to fixed populations of macrophages, such as in the sinusoids of the liver and spleen. Allison and Eugui (2) propose that antibodies facilitate the binding of macrophages to the surface of parasitized cells, underlining the probable involvement of several components of the immune response. Jensen et al. (13) provide evidence that mechanisms similar to those investigated here operate in vivo. Sera from patients with a strong clinical immunity inhibited parasite growth in vitro and resulted in the production of "crisis" forms. This could not be correlated directly with antimalarial antibody titers and appeared to be due to potent nonantibody factors. Clark and co-workers have suggested that various macrophage-derived mediators also contribute to the pathogenesis of acute malaria by causing localized damage to host tissue as well as to the parasite (9). It remains to be established whether these mediators can be exploited to the advantage of the host—for example, by drugs or by appropriate methods of vaccination which might focus and trigger their production.

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