INFECTION AND IMMUNITY, Jan. 1984, p. 238–244
0019-9567/84/010238-07$02.00/0

Vol. 43, No. 1

Polyamine Oxidase-Mediated Intraerythrocytic Killing of *Plasmodium falciparum*: Evidence Against the Role of Reactive Oxygen Metabolites

CHRISTINE M. RZEPCZYK,[1]* ALLAN J. SAUL,[1] AND ANTONIO FERRANTE[2]

Queensland Institute of Medical Research, Herston, Brisbane, Q 4006, Australia[1] and Immunology Unit, Department of Paediatrics, University of Adelaide, Adelaide Children's Hospital, South Australia 5006[2]

Received 27 June 1983/Accepted 20 September 1983

The polyamines spermine and spermidine, in the presence of polyamine oxidase, were shown to be cytotoxic in vitro to various isolates of *Plasmodium falciparum*. Neither polyamines nor polyamine oxidase alone was cytotoxic. This cytotoxicity was manifested by the degeneration of the parasites into crisis forms and by the inhibition of methionine incorporation by the parasites. Only 2 to 2.5 h of exposure to the reaction mixture (polyamine oxidase, 100 μg/ml; spermine, 1 mM) resulted in parasite death. It was shown that ammonia, hydrogen peroxide, and associated reactive oxygen intermediates produced during the oxidation of polyamines were not the cause of the parasite death observed in this system. This suggested that aldehydes or further breakdown products of these, e.g., acrolein (or both), need to be considered as the effector substances of the polyamine oxidase-mediated killing of *P. falciparum*.

The ability of non-antibody-mediated immune mechanisms to protect against intraerythrocytic stages of certain hemoprotozoan (malarial and babesial) infections has been well documented (5, 14). A characteristic feature of this type of immunity is the development of degenerate parasites within the host erythrocytes, the so-called crisis forms (8, 33). There is now good evidence that the appearance of crisis forms may be the result of the release of nonspecific factor(s) from cells of bone marrow origin in appropriately sensitized hosts (3, 7, 25). Although various substances produced by these cells such as tumour necrosis factor, interferon, polyamine oxidase (PAO), and reactive oxygen species (3, 6, 7, 22, 25–27) have been suggested as potential mediators of this nonspecific immunity, the factor(s) responsible for intraerythrocytic parasite death still remain(s) uncharacterized experimentally, and the mechanisms are unknown. For this reason we have studied further the possible involvement of PAO as a mediator of intraerythrocytic death.

PAO catalyzes the oxidative deamination of the polyamines spermine and spermidine. The oxidase activity has been identified in blood, liver, and other tissues. Elevation of this enzyme activity occurs during some infections (e.g., during *Babesia* sp. infections in mice) and in the sera of some patients with hepatitis (23). In addition there is evidence that activated macrophages contain higher levels of this enzyme and release it into culture medium (20). Polyamines are released from dividing and dead cells and are present in physiological fluids. In the presence of PAO these are oxidized. Oxidized polyamines are known to be toxic to a variety of cell types, including trypanosomes and intracellular protozoa (10, 21, 32), and we have demonstrated toxicity to *Plasmodium falciparum* in in vitro culture (A. Ferrante, C. M. Rzepczyk, and A. C. Allison, Trans. R. Soc. Trop. Med. Hyg., in press). These experiments showed that *P. falciparum* in the presence of PAO and either of the polyamines spermine or spermidine failed to develop and that the parasites degenerated into crisis forms. Under these conditions polyamines are oxidized, resulting in the formation of (i) aldehydes, which react to produce a variety of further products, e.g., acrolein and condensation products, (ii) hydrogen peroxide, which may react further to produce free radicals; and (iii) ammonia.

Currently, we are investigating the mechanism(s) of this toxicity. In this paper, the role of the generated ammonia and reactive oxygen intermediates such as superoxide (O_2^-), the hydroxyl radical ($OH^\cdot$) and hydrogen peroxide (H_2O_2) in the toxicity of the PAO-polyamine reaction is examined. Our findings suggested that these species are not the cause of the parasite death observed in this study.

MATERIALS AND METHODS

Parasites. Four established culture lines of *P. falciparum* maintained at Queensland Institute of Medical Research were used in this study. These included two Papua New Guinea isolates (FCQ-2/PNG, FCQ-27/PNG), a Thai isolate (KI), and an isolate from the Netherlands (NF36) (28). The lines were maintained in long-term culture by the method of Trager and Jensen (34) and were kept synchronized by a modification (24) of the sorbitol method of Lambros and Vanderburg (19). All experiments were initiated with the parasites in the ring stage at a parasitemia of 1.5 to 5%. A hematocrit of 5 to 10% was used throughout.

Reagents. RPMI 1640 (GIBCO Diagnostics, Madison, Wis.) modified for malaria culture (34) was the diluent for all reagents. PAO was the commercial, ex bovine plasma amine oxidase preparation from Miles Laboratories, South Africa, used previously (Ferrante et al., in press). The enzyme was stored at −20°C at a concentration of 1 mg/ml and was routinely used at a concentration of 100 μg/ml, except where otherwise stated. Polyamines (spermine tetrahydrochloride and spermidine trihydrochloride; Sigma Chemical Co., St. Louis, Mo.) were likewise stored at stock concentrations of 10 mM. They were used in the in vitro assay at a maximum concentration of 1 mM. All other reagents were made up as required. Catalase (bovine liver) and superoxide dismutase (SOD; bovine erythrocytes), (Calbiochem, Carlingford, Australia) were used at the following unit concentrations: catalase, 5,860 and 586 U/ml; SOD, 35,000 and 3,500 U/ml. Sodium diethyldithiocarbamate (DDC; Sigma) was added to

* Corresponding author.

cultures to give final concentrations of 50 to 400 μM, and ammonium chloride (NH_4Cl), (BDH Australia) was used at concentrations of 10 to 1,000 μM in the assays.

Oxidation kinetics. The rate of oxidation of spermine by PAO was measured by a modification of the technique of Allen et al. (2). Reaction rates were measured at 37°C in tissue culture medium containing human serum at an enzyme concentration of 1.5 μg/ml with a Rank (Cambridge, England) cell with 2-ml volume with 586 U of catalase added to decompose H_2O_2 formed during the reaction.

Measurement of parasite growth. Parasite growth was monitored from parasitemias and differential parasite counts obtained from Giemsa stained thin blood films and by measuring the incorporation of [^{35}S]methionine (specific activity, 1,500 Ci/mmol; 14.39 mCi/ml; Radiochemical Centre, Amersham, Bucks). In this study rings were defined as vacuolated parasites occupying <30% of the diameter of the erythrocyte; trophozoites as parasites with solid basophilic staining with <3 nuclei and occupying >30% of the diameter of the erythrocyte; schizonts as parasites with >2 nuclei and crisis forms as rounded, very condensed parasites with darkly staining nuclei occupying <30% of the diameter of the erythrocyte.

Experimental procedure. The basic procedure was as follows: 20 μl of medium, 10 μl each of PAO and polyamine (spermine or spermidine), and 60 μl of parasite suspension were added in succession to flat-bottom microtiter wells (Nunclon Delta, Denmark). Finally, 10 μl (0.4 μCi) of [^{35}S]methionine was added to each well; the total volume per well was 100 μl. In some experiments, part or all of the initial 20 μl of medium was replaced by medium containing potentiators or inhibitors. In control experiments an equal volume of medium replaced the enzyme or substrate. All cultures were set up in quadruplicate. After 24 h of incubation, Giemsa-stained thin films were prepared from one well, and the remaining three wells were harvested on GF/C glass filters (Whatman, England) with distilled water and an automated multiple sample harvester (Dynatech Laboratories, Inc., Alexandria, Va.). The filters were dried; after adding scintillant, incorporated radioactivity was determined with a Beckman LS 100 liquid scintillation counter. The results of the labeling experiments are either shown as mean counts per minute (cpm) of incorporated methionine ± standard error of the mean, or have been converted to show the test counts as a percentage of control values. In Fig. 1, the percentage growth inhibition was calculated from the following formula: percent inhibition = 100 − (cpm of test samples)/(cpm of control samples), where the control samples had no additions, i.e., contained medium and parasites only. Statistical analyses were done where appropriate with Student's *t* test.

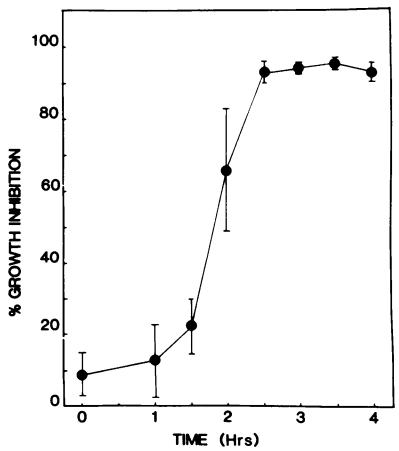

FIG. 1. Effect on parasite growth of different periods of exposure to PAO and spermine.

RESULTS

Oxidation kinetics. The PAO used in this study had a V_{max} of 35 nmol of O_2 per min per mg under the conditions used. The K_m for spermine was approximately 6 μM; however, the accuracy with which this value could be determined was limited by the sensitivity of the electrode used. Previously (2), a value of 2 μM was reported in phosphate buffer at 20°C. Both spermine and monooxidized spermine were apparently oxidized at a similar rate, as no evidence of biphasic kinetics was observed. At an enzyme concentration of 1.5 mg/ml, 125 μM spermine was fully oxidized in 6 min.

Effect of PAO and polyamines on ring-stage cultures of *P. falciparum*. The results of experiments with three isolates are shown in Table 1. In all instances, the development of *P. falciparum* was arrested, and crisis forms resulted in cultures containing both PAO and polyamine (either spermine or spermidine). Parasites in control cultures containing either no additions, polyamine (spermine or spermidine) alone, or PAO alone had developed normally through to either the trophozoite or to the schizont stage when the cultures were examined after 24 h. These findings were confirmed by the results of [^{35}S]methionine incorporation experiments.

Time course of killing. In experiments designed to show the minimum exposure to PAO and polyamine required for parasite killing, *P. falciparum* cultures were exposed to spermine and PAO for set intervals of time (from 15 min to 4 h). After each time interval, the cultures were thoroughly washed (as were "no additions" control cultures set up in conjunction) and suspended in fresh medium. [^{35}S]methionine was added to all cultures 4 h after the commencement of the experiment, i.e., after all cultures had been exposed and residual PAO and polyamine were washed out. All cultures were harvested after a further 20 h. The averaged results of four such experiments are shown in Fig. 1. Maximum inhibition of parasite growth required between 2 and 2.5 h of exposure to the reaction mixture.

Effect of enzyme and substrate concentrations on cytotoxicity. The results of a checkerboard titration with twofold serial dilutions of PAO and spermine are shown in Table 2. Cytotoxicity was shown to be dependent on both enzyme and substrate concentration. Maximum inhibition of [^{35}S]methionine incorporation was obtained with concentrations of PAO as low as 25 μg/ml and spermine concentrations of 125 μM, but there was still some inhibition of parasite growth at the lowest concentrations of PAO and spermine tested, i.e., 6.25 μg/ml and 62.5 μM, respectively. An examination of blood films made of cultures at these low enzyme and substrate concentrations confirmed these findings (Table 2). Parasites in cultures containing PAO and spermine concentrations down to 125 μM showed mainly crisis forms. In the instances where stages other than crisis forms were seen, these seemed abnormally dense and appeared to be degenerating. When only 62.5 μM of spermine was used in conjunction with various concentrations of PAO in the cultures, the proportion of crisis forms was markedly lower; of the remaining parasites, the majority seemed to be degenerating, but there were some parasites (predominately rings) of apparently normal morphology.

TABLE 1. Effect of PAO and polyamines on *P. falciparum* in vitro

Expt	Parasite isolate	Culture conditions	Parasitemia	Differential parasite counts (%)				Methionine incorporation[a]	Significance (*P*)[b]
				Rings	Trophozoites	Schizonts	Crisis forms		
1	FCQ-27/PNG	0 h	5.5	85	11	4	0		
		24 h							
		No additions	5.3	13	51	35	1	100	
		Spermine alone	5.5	5	60	34	1	140	<0.1
		Spermidine alone	6.4	5	49	46	0	112	>0.1
		PAO alone	4.6	9	54	36	1	130	>0.1
		PAO + spermine	6.6	0	0	0	100	17	<0.001
		PAO + spermidine	6.4	0	0	0	100	21	<0.002
2	FCQ-2/PNG	0 h	3.2	96	4	0	0		
		24 h							
		No additions	4.0	1	84	13	2	100	>0.1
		Spermidine alone	2.5	3	84	11	2	92	>0.1
		PAO alone	2.2	5	81	13	1	96	>0.1
		PAO + spermidine	2.2	10	0	0	90	13	<0.001
3	K1	0 h	2.7	97	3	0	0		
		24 h							
		No additions	3.3	7	78	15	0	100	
		Spermine alone	3.0	10	73	16	1	107	>0.1
		PAO alone	3.2	12	75	12	1	94	>0.1
		PAO + spermine	3.1	2	0	0	98	11	<0.001

[a] Expressed as a percentage of "no addition" control values.
[b] *P* value calculated against "no additions" controls; critical value, $P < 0.05$.

Further data supporting these observations are presented in Fig. 2. This shows the results of an experiment with the FCQ-27/PNG strain of *P. falciparum* in which PAO was serially diluted from a starting concentration of 100 μg/ml, with the concentration of polyamine (spermine or spermidine) kept at a constant 1 mM.

We have also observed that spermine at the highest concentration used in these experiments (1 mM) often resulted in enhanced incorporation of [^{35}S]methionine (cpm higher than "no additions" control) when added in the absence of PAO to cultures. No such effect on methionine incorporation was observed when spermidine was added to the cultures (Fig. 2).

Effect of catalase and SOD on PAO-mediated killing of *P. falciparum*. The addition of catalase or SOD had no appreciable effect on the inhibition of growth caused by PAO and spermine (Table 3), even when the concentration of PAO and spermine used were reduced to the lowest levels which caused near-maximal inhibition of parasite growth (Table 2; spermine, 125 μM; PAO, 25 μg/ml). In one experiment (Table 3, experiment 3) there was a statistically significant reduction in the PAO-mediated inhibitory effect on methionine incorporation with some concentrations of these enzymes. However, these enhancing effects on incorporation were only marginal, and no dose dependence was demonstrable.

The addition of these enzymes to control cultures did not show any consistent effects, except that SOD at the highest concentrations used (35,000 U/ml) tended to be inhibitory (Table 3, experiments 1 and 2) in control cultures. We have also observed this inhibition in similar unpublished experiments.

Effect of DDC on PAO-mediated killing of *P. falciparum*. DDC had neither a suppressive nor an enhancing effect on parasite methionine incorporation when added to cultures containing both PAO and polyamine; it did, however, have a suppressive effect on methionine incorporation by parasites in control cultures (Table 4).

Effect of ammonium chloride on *P. falciparum* cultures. An examination of Table 5, shows that the addition of various concentrations (10 to 1,000 μM) of ammonium chloride to cultures of *P. falciparum* had either no or only minimal effect on parasite growth as determined by methionine incorporation.

TABLE 2. Checkerboard titration showing the effect of various PAO and spermine concentrations on PAO-mediated killing of *P. falciparum* NF36

Concn of spermine (μM)	Mean [^{35}S] methionine incorporation[a] at the following concn of PAO (μg/ml):					
	100	50	25	12.5	6.25	0
1,000	212 ± 27 (100)	269 ± 38	348 ± 57[b]	348 ± 55[b]	540 ± 25[b] (92)	1,575 ± 329[b]
500	190 ± 16	236 ± 36	271 ± 16	328 ± 16[b]	502 ± 64[b] (99)	1,509 ± 187[c]
250	163 ± 18	196 ± 15	225 ± 17	421 ± 51[b]	499 ± 121[b] (100)	1,361 ± 87[c]
125	208 ± 19	228 ± 8	263 ± 18	381 ± 35[b] (100)	499 ± 47[b] (94)	1,390 ± 229[c]
62.5	434 ± 121 (68)	343 ± 22[b] (75)	430 ± 67[b] (74)	619 ± 50[c] (43)	689 ± 115[b] (31)	1,322 ± 89[c]
0	1,359 ± 98[b]	1,322 ± 17[c]	1,381 ± 69[c]	1,630 ± 25[c]	1,545 ± 59[c]	1,385 ± 48[c] (1)

[a] Mean [^{35}S]methionine incorporation (cpm ± standard error of the mean). The percentage of crisis forms present at 24 h is shown within parenthesis.
[b] Significantly different ($P < 0.05$) from control mean (212 ± 27 cpm; PAO, 100 μg/ml; spermine, 1 μM).
[c] Highly significantly different ($P < 0.001$) from control mean.

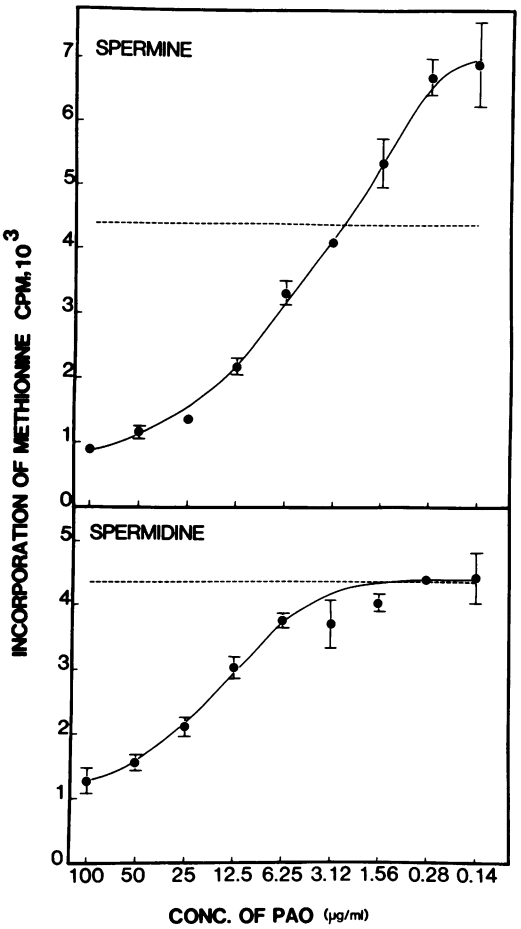

FIG. 2. Effect of different concentrations of PAO on the antimalarial action of the PAO-polyamine reaction. Symbols: (●——●) cultures containing PAO and 1 mM spermine or spermidine; (- - - -) control cultures (no additions).

DISCUSSION

The oxidation of the polyamines spermine and spermidine by PAO results in the formation of (i) aldehydes and further breakdown products such as acrolein, (ii) hydrogen peroxide, and (iii) ammonia (20). As the reaction is oxygen dependent, reduced forms of oxygen other than H_2O_2 may be produced, e.g., O_2^- and the $OH^{\cdot}$. We have previously reported that the presence of the oxidation products of polyamines in cultures of *P. falciparum* results in the parasites degenerating into crisis forms (Ferrante et al., in press). In the present study we have extended these preliminary observations. We have shown that PAO-mediated cytotoxicity was not restricted to a particular parasite isolate; two Papua New Guinea isolates (FCQ-27/PNG; FCQ-2/PNG), one Thai isolate (K1), and NF36, which was isolated in the Netherlands, were shown to be susceptible to these mechanisms (Tables 1 and 2). All of these isolates, with the exception of NF36, are chloroquine resistant. In addition, it was determined that the killing of *P. falciparum* required a relatively short exposure to the reaction mixture, as maximum inhibition of parasite growth was evident after the parasites were exposed to the enzyme and its substrate for between 2 and 2.5 h (Fig. 1). PAO-mediated cytotoxicity was shown to be dependent on the concentration of both PAO and substrate. Concentrations of PAO as low as 6.25 µg/ml and spermine concentrations as low as 125 µM still resulted in the cultured parasites being predominantly crisis forms (i.e., degenerate) when the cultures were examined after 24 h of incubation (Table 2).

The results of our experiments on the effects of ammonia on *P. falciparum* clearly suggested that the ammonia released during the oxidation of polyamines by PAO need not be considered further as an agent causing parasite death. The addition of ammonium ions to the cultures far in excess of the number generated in the PAO reaction had either no or only a marginal effect on methionine incorporation by the parasites (Table 5).

Hydrogen peroxide is a known product of the oxidation of the polyamines spermine and spermidine (20). However, as the addition of enough catalase to the culture to decompose the produced H_2O_2 failed to prevent PAO-mediated killing (Table 3), it is unlikely that H_2O_2 is the toxic agent in this system. These results are not surprising, since the high levels of endogenous catalase activity present in the erythrocytes (16) would ensure the breakdown of H_2O_2 would be sufficiently rapid to result in very low residual concentrations.

It has been proposed that the superoxide radical (O_2^-) may be an important mediator of intraerythrocytic parasite death in vivo (3). The lack of protection afforded by additional SOD (Table 3) indicates that the O_2^- is also unlikely to be the cause of toxicity. Again, the high levels of endogenous erythrocytic SOD (9) would provide some protection. It is unlikely that significant levels of O_2^- are generated in this system at all, since the addition of DDC, which is able to inhibit both parasite and erythrocyte SOD activity (31) to these cultures, did not potentiate PAO-mediated cytotoxicity in any way (Table 4). SOD (at the highest concentration used, 35,000 U/ml) and DDC were both found to be inhibitory when added to control cultures of *P. falciparum* (Tables 3 and 4). This inhibition by SOD and DDC was not reflected in the PAO-mediated killing experiments, suggesting that these inhibitory effects are distinct from those of the PAO-mediated reaction, and that they act either less rapidly or at a later stage than the toxic products of the PAO-mediated reaction.

Although we used DDC in these experiments primarily because of its reported inhibition of SOD, it needs to be emphasized that the results of experiments with DDC need to be interpreted with caution, as other effects of DDC on intraerythrocytic stages of malaria have been reported, namely, its adverse effects on parasite growth probably resulting through its inhibition of parasite metalloprotein enzymes and its effect on parasite glycolysis (29, 30).

Of all the oxygen intermediates, the hydroxyl radical is generally regarded as being most damaging to cells, and recently it was proposed as a potentially significant mediator of malarial parasite death in vivo (4, 6). In these published studies, when alloxan and *t*-butylhydroperoxide were used, both in vivo and in vitro, as generators of reactive oxygen intermediates, intraerythrocytic death accompanied by a transient hemolysis resulted. Since both of these effects could be largely prevented by the use of the iron chelators DDC and desferrioxamine, it was suggested that parasite death and erythrocyte damage was mediated by the generation of hydroxyl radicals. In the absence of compounds such as *t*-butylhydroperoxide, $OH^{\cdot}$ is formed by the interaction of O_2^- and H_2O_2, particularly in the presence of a catalytic metal such as iron (15). In our system, it is unlikely that $OH^{\cdot}$ would be formed, because the presence of high levels of erythrocytic SOD and catalase would have kept the concen-

TABLE 3. Effect of catalase and SOD on PAO-mediated killing of *P. falciparum* NF36

Culture conditions	Expt 1		Expt 2		Expt 3	
	Methionine incorporation[a]	Significance (*P*)[b]	Methionine incorporation	Significance (*P*)	Methionine incorporation	Significance (*P*)
PAO + spermine[c]	21		8		48	
+ SOD (35,000 U/ml)	20	>0.1	8	>0.1	51	>0.1
(3,500 U/ml)	20	>0.1	8	>0.1	60	<0.05
+ Catalase (586 U/ml)[d]	ND[e]	ND	10	>0.1	53	>0.1
(58.6 U/ml)	ND	ND	7	>0.1	60	<0.02
No additions (control)	100	<0.001	100	<0.001	100	<0.01
+ SOD (35,000 U/ml)	(1,857 ± 28)		(10,532 ± 119)		(1,584 ± 153)	
(3,500 U/ml))	88	<0.001	40	<0.05	98	<0.001
+ Catalase (586 U/ml)	98	<0.001	89	<0.001	121	<0.001
(58.6 U/ml)	ND	ND	97	<0.001	122	<0.02
	ND	ND	73	<0.001	97	<0.01
PAO	86	<0.001	ND	ND	83	<0.01
+ SOD (35,000 U/ml)	67	<0.01	72	<0.01	91	<0.02
(3,500 U/ml)	84	<0.05	79	<0.01	ND	ND
+ Catalase (586 U/ml)	ND	ND	90	<0.001	122	<0.002
(58.6 U/ml)	ND	ND	71	<0.01	ND	ND
Spermine	112	<0.01	ND	ND	95	<0.002
+ SOD (35,000 U/ml)	62	<0.001	79	<0.001	119	<0.002
(3,500 U/ml)	120	<0.001	86	<0.001	ND	ND
+ Catalase (586 U/ml)	ND	ND	138	<0.001	117	<0.002
(58.6 U/ml)	ND	ND	114	<0.001	ND	ND

[a] Methionine incorporation expressed as a percentage of control values. The mean cpm ± standard error of the mean is shown within parentheses.

[b] Values obtained in cultures containing both PAO and spermine were used as the control values in the *t* test. Critical value, $P < 0.05$.

[c] Experiments 1 and 2: PAO concentration, 100 μg/ml; spermine concentration, 1 mM. Experiment 3: PAO concentration, 25 μg/ml; spermine concentration, 125 μM.

[d] In experiment 1, SOD and catalase were added to cultures together with PAO, polyamines, and parasitized blood at zero time. In experiments 2 and 3 they were preincubated (37°C, 15 min) with the parasitized blood before the addition of PAO and polyamines.

[e] ND, Not determined.

TABLE 4. Effect of DDC on PAO-mediated killing of *P. falciparum* NF36

Culture conditions	Expt 1		Expt 2	
	Methionine incorporation[a]	Significance[b] (*P*)	Methionine incorporation	Significance (*P*)
PAO (25 μg/ml) + spermine (125 μM)	48		36	
+ DDC (400 μM)	43	>0.1	28	>0.1
(200 μM)	44	>0.1	41	>0.1
(100 μM)	53	>0.1	37	>0.1
(50 μM)	46	>0.1	25	<0.1
No additions (control)	100 (1,584 ± 153)	<0.01	100 (1,040 ± 103)	<0.01
+ DDC (400 μM)	72	<0.002	60	<0.05
(200 μM)	77	<0.01	53	<0.05
(100 μM)	92	<0.001	65	<0.02
(50 μM)	103	<0.001	76	<0.05
PAO (25 μg/ml)	83	<0.01	98	<0.01
+ DDC (400 μM)	51	>0.1	53	<0.05
(200 μM)	ND[c]	ND	56	<0.02
(100 μM)	ND	ND	69	<0.05
(50 μM)	ND	ND	67	<0.01
Spermine (125 μM)	95	<0.002	97	<0.01
+ DDC (400 μM)	58	<0.01	40	>0.1
(200 μM)	ND	ND	62	<0.1
(100 μM)	ND	ND	62	<0.1
(50 μM)	ND	ND	72	<0.05

[a] Expressed as a percentage of control values. The mean cpm ± standard error of the mean is shown within parentheses.

[b] *P* Value calculated against PAO (25 μg/ml) plus spermine (125 μM) control; critical value, $P < 0.05$.

[c] ND, Not determined.

TABLE 5. Effect of ammonium chloride on *P. falciparum* KI in vitro

Expt	NH_4Cl concn (μM)	Methionine incorporation[a]
1	0	100 (2,131 ± 16)
	10	102
	100	104
	500	93
	1,000	90
2	0	100 (660 ± 14)
	10	99
	100	106
	500	95
	1,000	97

[a] Expressed at a percentage of control values (no added ammonium chloride). The mean cpm ± standard error of mean is shown within parentheses.

trations of O_2^- and H_2O_2 low. In contrast to the above studies (4, 6), we did not observe erythrocyte lysis in our system, and we have not been able to block the killing reaction with the metal chelator DDC.

Comparison of the time course of parasite death (Fig. 1) with the rate of oxidation of spermine and the effect of spermine concentration on parasite death (Table 2) provides further evidence against the role of reactive oxygen species as the toxic agent. Since the K_m of spermine for PAO is much lower than the concentrations employed, the rate of oxidation will depend only on the PAO concentration and will remain virtually constant while spermine remains. Because of the short lifetimes of reactive oxygen species (12) and the high levels of catalase and SOD activity present in the erythrocytes, their concentrations would quickly come to a steady-state equilibrium while spermine remained and then rapidly fall when all the spermine was oxidized. At a concentration of 62.5 μM spermine and a PAO concentration of 100 μg/ml, it can be calculated either from the V_{max} or from the time PAO (1.5 mg/ml) took to oxidize 125 μM spermine that this occurs after approximately 45 min. Since it took 2 to 2.5 h at this PAO concentration and a higher spermine concentration to achieve comparable parasite death (Fig. 1 and Table 2), compounds with short half-lives cannot be the major toxic agent.

The dioxidized product of spermine has been proposed as the toxic product of the PAO reaction (13). However this compound decomposes further with a reported half-life of 47 min under similar conditions (17). Thus in the 2 to 2.5 h required to kill parasites at the PAO concentrations used, significant concentrations of further breakdown products (principally acrolein) would be present. Since acrolein is known to be toxic to eucaryotic (1) and procaryotic (18) cells, it is likely that this or related compounds may be the mediators of the PAO-mediated killing of *P. falciparum*. Further studies are now in progress to determine the effector molecules involved and to determine the manner in which this system may be manipulated to provide antimalarial protection in vivo.

ACKNOWLEDGMENTS

We thank P. G. Parsons (Queensland Institute of Medical Research) and A. C. Allison (Syntex Research) for their helpful advice. The competent technical assistance of Janelle Moss and Leanne Ingram is gratefully acknowledged.

LITERATURE CITED

1. **Alarcon, R. A.** 1964. Isolation of acrolein from incubated mixtures of spermine with calf serum, and its effects on mammalian cells. Arch. Biochem. Biophys. **106**:240–242.
2. **Allen, J. C., J. Smith, J. M. Hussain, J. M. Thomas, and J. M. Gaugas.** 1979. Inhibition of lymphocyte proliferation by polyamines requires ruminant-plasma polyamine oxidase. Eur. J. Biochem. **102**:153–158.
3. **Allison, A. C., and E. M. Eugui.** 1982. A radical interpretation of immunity to malaria parasites. Lancet **ii**:1431–1433.
4. **Clark, I. A., W. B. Cowden, and G. A. Butcher.** 1983. Free oxygen radical generators as anti-malarial drugs. Lancet **i**:234.
5. **Clark, I. A., F. E. G. Cox, and A. C. Allison.** 1977. Protection of mice against *Babesia spp.* and *Plasmodium spp.* with killed *Corynebacterium parvum*. Parasitology **74**:9–17.
6. **Clark, I. A., and N. H. Hunt.** 1983. Evidence for reactive oxygen intermediates causing hemolysis and parasite death in malaria. Infect. Immun. **39**:1–6.
7. **Clark, I. A., J. L. Virelizier, E. A. Carswell, and P. R. Wood.** 1981. Possible importance of macrophage mediators in acute malaria. Infect. Immun. **32**:1058–1066.
8. **Clark, I. A., E. J. Willis, J. E. Richmond, and A. C. Allison.** 1977. Intra-erythrocytic death of the parasites in mice recovering from infections with *Babesia microti*. Parasitology **75**:189–196.
9. **Concetti, A., P. Massei, G. Rotilio, M. Brunori, and E. A. Rachmilewitz.** 1976. Superoxide dismutase in red blood cells: method of assay and enzyme content in normal subjects and in patients with β-thalassemia (major and intermedia). J. Lab. Clin. Med. **87**:1057–1064.
10. **Ferrante, A., A. C. Allison, and H. Hirumi.** 1982. Polyamine oxidase-mediated killing of African trypanosomes. Parasite Immunol. **4**:349–354.
11. **Forman, H. J., J. L. York, and A. B. Fisher.** 1980. Mechanism for the potentiation of oxygen toxicity by disulfiram. J. Pharmacol. Exp. Ther. **212**:452–455.
12. **Fridovich, I.** 1975. Oxygen: boon and bane. Am. Sci. **63**:54–59.
13. **Fukami, H., I. Tomida, T. Morino, H. Yamada, T. Oki, H. Kawasaki, and K. Ogata.** 1967. Phagocidal action of oxidized spermine and its analogues. Biochem. Biophys. Res. Commun. **28**:19–24.
14. **Grun, J. E., and W. P. Weidanz.** 1981. Immunity to *Plasmodium chabaudi adami* in the B-cell-deficient mouse. Nature (London) **290**:143–145.
15. **Haber, F., and J. Weiss.** 1934. The catalytic decomposition of hydrogen peroxide by iron salts. Proc. R. Soc. Lond. **147**:332–351.
16. **Hartz, J. W., S. Funakoshi, and H. F. Deutsch.** 1973. The levels of superoxide dismutase and catalase in human tissues as determined immunochemically. Clin. Chim. Acta **46**:125–132.
17. **Kimes, B. W., and D. R. Morris.** 1971. Preparation and stability of oxidized polyamines. Biochim. Biophys. Acta **228**:223–234.
18. **Kimes, B. W., and D. R. Morris.** 1971. Inhibition of nucleic acid and protein synthesis in *Escherichia coli* by oxidized polyamines and acrolein. Biochim. Biophys. Acta **228**:235–244.
19. **Lambros, C., and J. P. Vanderburg.** 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. J. Parasitol. **65**:418–420.
20. **Morgan, D. M. L.** 1980. Polyamine oxidase, p. 285–302. *In* J. M. Gaugas (ed.), Polyamines in biomedical research. Wiley/Chichester, New York.
21. **Morgan, D. M. L., and J. R. Christensen.** 1983. Polyamine oxidation and the killing of intracellular parasites. Adv. Polyamine Res. **4**:169–174.
22. **Morgan, D. M. L., J. R. Christensen, and A. C. Allison.** 1981. Polyamine oxidase and the killing of intracellular parasites. Biochem. Soc. Trans. **9**:563–564.
23. **Morgan, D. M. L., J. Fergula, and A. C. Allison.** 1980. Polyamine oxidase and macrophage function, p. 303–308. *In* J. M. Gaugas (ed.), Polyamines in biomedical research. Wiley/Chichester, New York.
24. **Myler, P., A. Saul, T. Mangan, and C. Kidson.** 1982. An automated assay of merozoite invasion of erythrocytes using highly synchronized *Plasmodium falciparum* cultures. Aust. J. Exp. Biol. Med. Sci. **60**:83–89.
25. **Rzepczyk, C. M.** 1982. Probable macrophage origin of the

lipopolysaccharide-induced cytostatic effect on intraerythrocytic malarial parasites (*Plasmodium vinckei*). Immunology **46**:261–270.

26. **Rzepczyk, C. M., and I. A. Clark.** 1981. Demonstration of a lipopolysaccharide-induced cytostatic effect on malarial parasites. Infect. Immun. **33**:343–347.
27. **Rzepczyk, C. M., and I. A. Clark.** 1982. Failure of bacterial lipopolysaccharide to elicit a cytostatic effect on *Plasmodium vinckei petteri* in C3H/HeJ mice. Infect. Immun. **35**:58–63.
28. **Sanderson, A., D. Walliker, and J. F. Molez.** 1981. Enzyme typing of *Plasmodium falciparum* from African and some other Old World countries. Trans. R. Soc. Trop. Med. Hyg. **75**:271–273.
29. **Scheibel, L. W., and A. Adler.** 1980. Antimalarial activity of selected aromatic chelators. Mol. Pharmacol. **18**:320–325.
30. **Scheibel, L. W., A. Adler, and W. Trager.** 1979. Tetraethylthiuram disulfide (Antabuse) inhibits the human malaria parasite *Plasmodium falciparum*. Proc. Natl. Acad. Sci. U.S.A. **76**:5303–5307.
31. **Suthipark, U., J. Krungkrai, A. Jearnpipatkul, Y. Yuthavong, and B. Panijpan.** 1982. Superoxide dismutase (SOD) in mouse red blood cells infected with *Plasmodium berghei*. J. Parasitol. **68**:337–339.
32. **Tabor, H., and C. W. Tabor.** 1964. Spermidine, spermine, and related amines. Pharmacol. Rev. **16**:245–300.
33. **Taliaferro, W. H., and L. G. Talliaferro.** 1944. The effect of immunity on the asexual reproduction of *Plasmodium brasilianum*. J. Infect. Dis. **75**:1–32.
34. **Trager, W., and J. B. Jensen.** 1976. Human malaria parasites in continuous culture. Science **193**:673–675.