Killing of *Plasmodium falciparum* by Eosinophil Secretory Products

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The multiplication of two strains of *Plasmodium falciparum* in culture, as measured by [^{3}H]hypoxanthine incorporation, was inhibited in a dose-dependent manner by granule proteins secreted by purified eosinophils obtained from patients with the hypereosinophilic syndrome. Morphological examination revealed the presence of abnormal parasites inside erythrocytes, indicating that they were killed in situ, and the later stages of the developmental cycle were found to be most susceptible to these toxic effects. A monoclonal antibody against eosinophil cationic protein partially blocked the inhibitory effect, suggesting that it was caused by more than one of the eosinophil granule proteins. Thus some of the antimalarial effects of molecules such as the tumor necrosis factor, which activates eosinophils, may be mediated through the enhanced production of eosinophil secretion products.

The multiplication of malarial parasites in the infected host can be influenced by nonspecific defense mechanisms. Since such protection can be induced by agents known to activate macrophages (4, 5), attention has been paid to the inhibitory effect on the parasites of cytotoxic substances secreted by these cells. Thus, the asexual blood stages of both rodent and human malarial parasites have been shown to be susceptible to intraerythrocytic killing in vitro by reactive oxygen intermediates (7, 16, 32) and also by a factor or factors present in tumor necrosis serum (3, 8, 28, 32), induced by administration of endotoxin to animals whose macrophages had been activated previously (for example, by infection with *Mycobacterium bovis* BCG) (28). Another serum factor that inhibits the multiplication of *Plasmodium falciparum* in vitro, causing the appearance of intracellular crisis forms in parasite cultures, has been discovered in blood samples from people living in the Sudan who are clinically immune to malaria (11). This factor is different from both antibody and the tumor necrosis factor (TNF) (3). Its nature is still undefined, and its cellular origin has yet to be determined, but so far its presence seems to be associated with cultural or genetic features peculiar to a human community living in malarial areas in the southern Sudan (12).

Malarial parasites inside erythrocytes may also be killed when exposed in vitro to normal myeloid cells, including monocytes (29), neutrophils (13, 29), and eosinophils (29), and to neutrophils from children with acute malaria (1). It was reported recently that neutrophils from patients with malaria are activated in terms of increased oxygen consumption and that the neutrophils can be triggered to release free O_2 radicals by erythrocytes parasitized by *P. falciparum* in the presence of immune serum (19).

It has now been shown that recombinant TNF increases the phagocytic ability of neutrophils (21), enhances their antibody-dependent cytotoxicity (probably by increasing the oxidative burst) (21), and also enhances the toxicity of eosinophils for the larvae of *Schistosoma mansoni* (22). We have found that although recombinant TNF does not affect the viability of rodent malarial parasites in vitro (J. Taverne,

J. Tavernier, W. Fiers, and J. H. L. Playfair, Clin. Exp. Immunol., in press) nor, apparently, the multiplication of the human parasite *P. falciparum* in culture (17), it inhibited the multiplication of *Plasmodium yoelii* in mice and significantly prolonged the survival of mice infected with a lethal variant of this parasite, presumably by acting through some cellular intermediary. Since eosinophils can be activated by recombinant TNF (22) and their secretion products can now be studied (26), we have tested such preparations to see if they inhibit the multiplication of malarial parasites in vitro.

MATERIALS AND METHODS

Eosinophil secretion products. Eosinophils from the blood of three patients with the hypereosinophilic syndrome (23) were separated on metrizamide density gradients, and secretion products were then obtained by stimulating the cells with zymosan-C3b (26) to give supernatants containing eosinophil granule proteins. The concentration of secreted eosinophil cationic protein (ECP) was assayed by a double immunoassay (25). Supernatants were stored at −20°C.

Removal of ECP. A monoclonal antibody, EG1, which is specific for ECP (25) was used to absorb ECP from the secretion products. A 1/20 dilution of purified EG1 antibody (1 mg/ml) and a 1/4 dilution of rabbit anti-mouse immunoglobulin G antiserum were added to an equal volume of secretion products and the mixture was kept at 4°C overnight and then centrifuged at 1,500 × *g*. When the resulting preparations were tested for ECP by radioimmunoassay (26), they were always negative. As a control, a sample of the supernatant was treated with another monoclonal antibody of the same isotype (immunoglobulin G1) but with a different specificity (against rabbit cytochrome p450).

Culture of *P. falciparum*. The NF54 Nijmegen (9) and Tanzanian (18) (Tz) strains of *P. falciparum* were used. They were maintained in human A$^+$ erythrocytes at a 5% hematocrit in a standard culture system (31) in RPMI 1640 medium containing 10% human A$^+$ serum, supplemented with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and 0.2% NaHCO$_3$ in an atmosphere of 5% CO$_2$–5% O$_2$–90% N$_2$. Cultures were synchronized by treatment with 5% sorbitol (14), and NF54 schizonts were purified from other stages by sedimentation through 1%
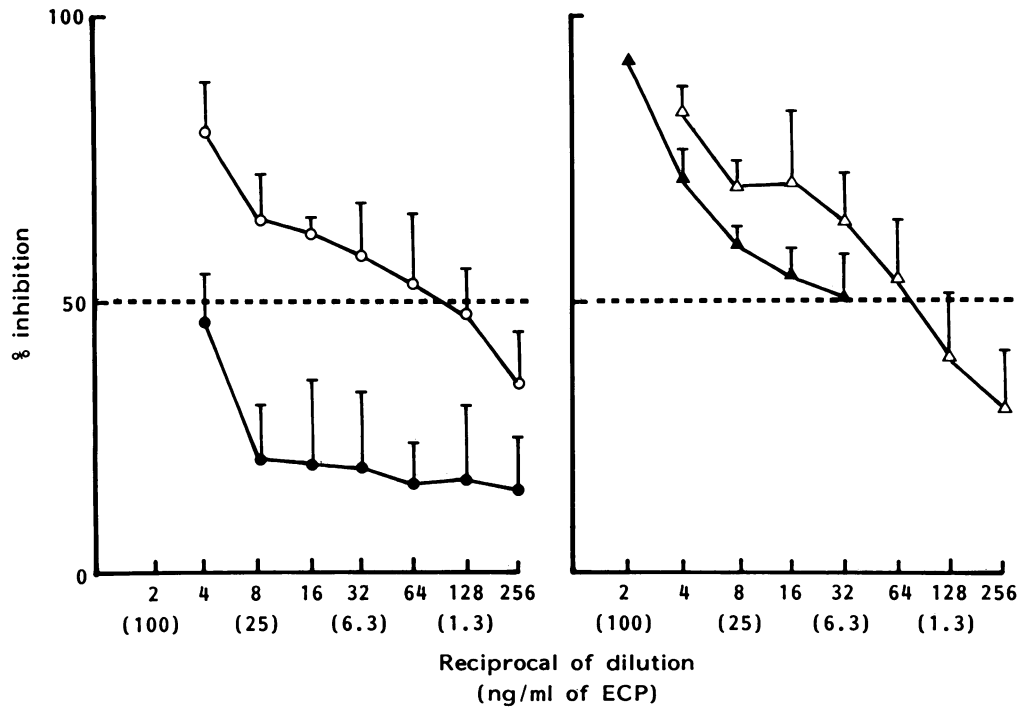
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FIG. 1. Inhibition of *P. falciparum* NF54 multiplication by eosinophil secretion products from patient 1. The undiluted preparation contained 200 ng of ECP per ml. Curves represent means ± standard deviations from two experiments in which cultures were unsynchronized (▲) or synchronized as ring forms (●), trophozoites (○), or schizonts (△).

gelatin (10). For cytotoxicity assays, 50-μl volumes of parasite suspensions were distributed in 96-well flat-bottom Falcon microtiter plates (Becton Dickinson Labware) at a concentration of 0.5 to 1% parasitized erythrocytes. Doubling dilutions of test samples were made in RPMI 1640 medium containing 10% A⁺ serum, and 50-μl volumes were then added to triplicate wells of the cultures. Tritiated hypoxanthine (0.4 μCi per well) was added in 10-μl volumes, and incorporation was measured in a Packard Tricarb 574 counter after 48 h of culture. Each experiment was repeated at least once. Results are given as mean percent inhibition of [³H]hypoxanthine uptake (± standard deviation) compared with findings for controls in which parasites were cultured in medium alone. Results were confirmed by examination of blood films, where it is possible to distinguish among different morphologies of the parasite, to measure their rate of development, and to identify morphologically abnormal crisis forms (11).

RESULTS

In our first experiments in which different concentrations of eosinophil secretion products from patient 1 were tested against *P. falciparum* NF54, significant inhibition of parasite multiplication was observed with dilutions up to 1/32 of the original preparation. When NF54 parasite suspensions were treated with sorbitol to give cultures synchronized to within 4 to 6 h, in which more than 90% of the parasites were at the same stage of development, cultures initiated from trophozoites or schizonts were still inhibited at dilutions of about 1/100, whereas cultures initiated from the ring stages only showed an effect at a 1/4 dilution (Fig. 1). In further experiments with eosinophil secretion products from patient 2, similar differences were observed for the susceptibility of the various stages. In addition, the Tanzanian strain of *P.*

falciparum was also shown to be susceptible to inhibition by these proteins. In both cases, cultures initiated with parasites at the later stages of development displayed greater inhibition than those started from ring forms. For purposes of comparison, results obtained with preparations containing 200, 120, and 30 ng of ECP per ml from three patients are shown in terms of the dilution of secretion products causing 50% inhibition of multiplication (Table 1); the level of cytotoxicity observed was clearly related to the amount of ECP present. A preparation which contained 30 ng ECP per ml (from patient 3) was not inhibitory. As this preparation originally contained only 50% eosinophils, it appears that

TABLE 1. Dilutions of various preparations of eosinophil secretion products causing 50% inhibition of *P. falciparum* multiplication

Patient	Purity of eosinophil prepn (%)	ECP (ng/ml)	Parasite strain	Parasite stage at start of culture	Reciprocal of dilution at 50% inhibition (ECP concn [ng/ml])[a]
1	90	200	NF54	Unsynchronized	45 (4.4)
				Ring	4 (50.0)
				Trophozoite	115 (1.7)
				Schizont	83 (2.4)
			Tz	Unsynchronized	102 (2.0)
2	80	120	NF54	Ring	18 (6.7)
				Trophozoite	32 (3.8)
				Schizont	38 (3.2)
			Tz	Trophozoite	48 (2.5)
3	50	30	NF54	Schizont	4 (7.5)
				Trophozoite	4 (7.5)

[a] Means of at least two experiments.

neither the method of preparation itself nor products released from other leukocytes contaminating the eosinophil preparations contributed to the death of the parasites.

Ring trophozoites were consistently less susceptible to the inhibitory effects of eosinophil secretion products than were either developing trophozoites or schizonts (Table 1), perhaps because of the greater permeability of trophozoite- and schizont-infected erythrocyte membranes. To ensure that the differential effect was not due to the instability of the secreted proteins and consequent loss of activity, products of eosinophils of patient 1 were incubated for 24 h at 37°C before being used in the assay. This caused some loss of activity (about 20%), but the parasiticidal effects on late-stage asexual parasites were still significantly greater than against ring-infected erythrocytes. The Tanzanian strain of *P. falciparum* is, without sorbitol treatment, more synchronous in development than is strain NF54, so that cultures were always initiated at the late trophozoite stage. This explains the differences observed in the eosinophil products from patient 1. The dilutions of secretion products from patient 2 causing 50% inhibition of trophozoites of the two strains were not apparently different (1/32 compared with 1/48), a result obtained on six occasions.

It was not possible to measure directly the effect of purified ECP on parasite multiplication, since purified ECP is in the extracted rather than the secreted form, can only be maintained in sodium acetate buffer at pH 4.2 (conditions in which erythrocytes are lysed and malarial parasites do not survive), and precipitates if the pH is increased. Secreted ECP, by contrast, is stable at pH 7.0. Therefore, to see if the inhibitory effect of the granule proteins was due to their ECP content, the ECP was absorbed from secretion products from patient 2 and the inhibitory effect against *P. falciparum* was then assayed. Since the original preparation was diluted 1/3 by addition of the antibody, the control was similarly diluted either with medium or with an irrelevant antibody of the same isotype. The results of a representative assay are illustrated (Fig. 2). Treatment with specific antibody against ECP consistently diminished the inhibitory effect of the granule proteins, reducing the 50% inhibition point from 1:16 (2.5 ng/ml) to 1:6 (6.7 ng/ml), but did not block the inhibition of parasite multiplication altogether, suggesting that more than one of the secreted products from eosinophils can kill malarial parasites.

DISCUSSION

We have demonstrated that the multiplication of two strains of *P. falciparum* in culture is inhibited in a dose-dependent fashion by granule proteins secreted by activated human blood eosinophils. Morphological observation of the cultures revealed the presence of abnormal-looking parasites within erythrocytes, suggesting that they were killed in situ by the toxic effect of the secretion products, and experiments with synchronized cultures initiated by parasites largely at the same stage of development showed that parasites were more susceptible to inhibition at the trophozoite-schizont stages of their growth cycle. Some of this parasite death was attributable to ECP in the granule proteins, since the amount of inhibition was diminished by specific antibody to ECP. Eosinophils secrete many other molecules when they degranulate, including the major basic protein present in the crystalloid and some characteristic enzymes such as arylsulfatase B, phospholipase D, lysophospholipase, and a neurotoxin (24), and it seems that some of these may also have contributed to the death of the
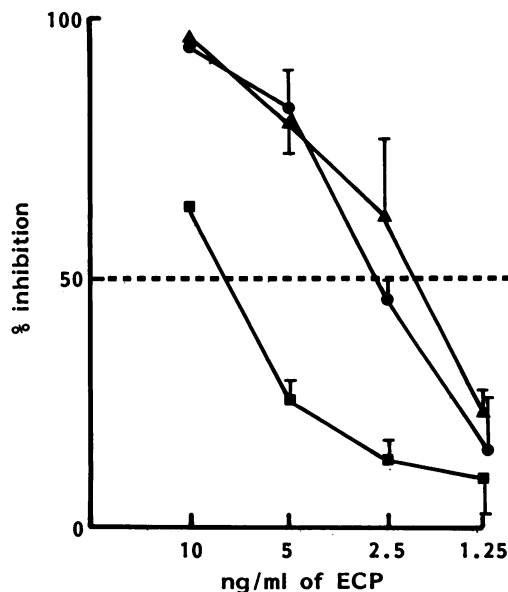

FIG. 2. Effect of monoclonal antibody EG1 to ECP on the inhibition of *P. falciparum* Tz multiplication by eosinophil secretion products. Eosinophil secretion products from patient 2 were diluted 1/3 initially by the addition of monoclonal antibody and rabbit anti-mouse antiserum. Serial dilutions were then prepared from this mixture and incubated with a culture of *P. falciparum* Tz which consisted of 99% late trophozoites. Multiplication was compared with that of control cultures diluted 1/3 with medium or with an irrelevant monoclonal antibody. Indicated are secretion products (obtained from patient 2) untreated (●), treated with EG1 antibody to ECP (■), or prepared with the irrelevant antibody (▲).

parasites. The amounts of ECP present in our preparations were, however, within the range of concentrations found in the sera of patients with the hypereosinophilic syndrome. As the levels in the immediate microenvironment of the cells secreting it are probably considerably higher than in serum, it is likely that parasites would be killed even in healthy individuals in the vicinity of activated eosinophils. ECP (M_r, 18,000 to 21,000) induces pore formation in the plasma membrane of target cells, and at concentrations around 10^{-7} M, it causes lysis of sheep erythrocytes (33). In our experiments, preparations that contained less than 10^{-8} M ECP (for example, 1/2 dilutions of a sample from patient 1) inhibited all parasite multiplication but did not cause obvious hemolysis in the cultures. It seems possible that parasitized erythrocytes are more susceptible to such damage than are uninfected ones.

Eosinophil secretion was induced here by incubation with zymosan-C3b. In the infected host, it is possible that the cells may be stimulated to degranulate by malarial parasites coated with antibody, with or without complement, or by antigen-antibody complexes, just as degranulation can be induced by helminths coated with immunoglobulin G, complement, or both (2). Like other granulocytes, eosinophils when stimulated release reactive oxygen metabolites, which are also toxic to malarial parasites (7, 16, 32), but these active molecules are short lived and were not present in the preparations tested, although they may be important in vivo.

The activated eosinophils used in our experiments were obtained from patients suffering from the hypereosinophilic syndrome (23). Monocytes from such patients can release products which enhance the cytotoxicity of normal eosino-

phils for the larvae of *Schistosoma mansoni* (30). Some activity may be due to the presence of TNF (29a), and recombinant TNF has been shown to induce similar enhancement (22). Macrophages from mice with malaria are activated and can release TNF when triggered by endotoxin (6, 27). Activation may be mediated by T-cell factors, including interferon gamma, and for mouse peritoneal cells, macrophage activating factors can substitute for endotoxin (unpublished findings); possibly parasites or their products may do so too. Similarly, interferon gamma in the presence of interleukin 2 has been shown to induce the production of TNF by human monocytes (15). Eosinophil differentiation is regulated by a lymphokine synthesized by T cells (20), so that the generation of activated eosinophil products is under complex T-cell control and is influenced by immune status and perhaps by appropriate vaccination.

It is possible that some of the inhibitory activity termed crisis-forming factor against malarial parasites found in sera from people in the Sudan who are clinically immune to malaria (3, 11, 12) may be due to eosinophil secretory products, in view of the widespread occurrence of eosinophilia caused by helminthic infections in the same populations. This cannot account for all the activity, however, since people in other tropical countries such as Indonesia are likely to be similarly infested but their sera do not induce crisis forms (12).

A possible role for eosinophils in nonspecific immunity in malaria has previously not been considered, perhaps because levels of blood eosinophils have not obviously correlated with immunity. In view of the marked toxic effects of physiological concentrations of eosinophil secretory products on *P. falciparum* and now that the importance of various cell-to-cell interactions mediated by extremely small quantities of a number of cytokines is better appreciated, the behavior of eosinophils in malaria seems worthy of further investigation.

ACKNOWLEDGMENTS

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