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Endotoxin-Induced Serum Factor Kills Malarial Parasites In Vitro

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We investigated the possibility that malarial parasites may be killed by nonspecific soluble mediators, such as those in tumor necrosis serum, that are obtained from mice given macrophage-activating agents like *Corynebacterium parvum* or *Mycobacterium bovis* BCG, followed by endotoxin. Such sera killed parasites in vitro after overnight incubation; killing was measured directly by using an in vivo infectivity assay. Parasite infectivity was not decreased by incubation in sera from mice given *C. parvum* or BCG alone (no endotoxin) or by incubation in sera from normal mice given endotoxin. *Plasmodium yoelii*, its lethal variant, and *Plasmodium berghei* were equally susceptible to inactivation. Sera obtained from mice given endotoxin during the course of infection with these parasites also contained parasite-killing factor. The activity of this factor appeared to be proportional to parasitemia in that it was higher in the sera from mice infected with the lethal parasites than in the sera from mice with infections which resolved either spontaneously or after vaccination.

Much evidence is accumulating to suggest that activated macrophages play an important role in protection against a variety of intracellular pathogens and tumors. Furthermore, macrophages may be stimulated in a number of different ways, which have specific and nonspecific effects. Activation of macrophages by agents such as *Corynebacterium parvum* and *Mycobacterium bovis* BCG has been shown to induce nonspecific protection of mice against various species of *Plasmodium* and *Babesia* (5, 6, 9), and both of these agents are known to provide protection against tumors. The observation of damaged parasites within the erythrocytes of mice protected by these agents (5, 6) led to the proposal that protection was mediated by a nonspecific soluble effector substance, and a parallel was drawn between the inhibition of parasite growth in mice given BCG (8) and the inhibition of tumor cell growth by tumor necrosis serum (TNS) obtained from animals given *C. parvum* or BCG and then injected with endotoxin (3, 13). The hypothesis that endotoxin causes the release of a tumoricidal factor from activated macrophages has now been confirmed in vitro with separated cell populations (18).

Parasites degenerating within erythrocytes (crisis forms) are known to occur in human malaria, and they have also been observed in rodents at about the time of recovery from infections which resolve spontaneously (7, 25). By analogy, it seems likely that parasites are converted to crisis forms by soluble products released from macrophages that have been activated specifically during the course of infection. Indeed, Clark (4) has proposed that the malarial parasite may act like endotoxin to trigger the release of toxic substances from macrophages that it has activated itself, either directly or indirectly through the mediation of T cells. Further evidence that soluble effector substances also contribute to parasite death in circumstances of specific protection is provided by the observation that crisis forms develop in mice vaccinated against a lethal variant of *Plasmodium yoelii* at about the time that they recover from a challenge infection, and there is reason to believe that the liver may be an important site for the destruction of parasites (24).

The probable involvement of macrophages and their products in parasite destruction is supported by the findings that macrophages in the liver are activated during infection (11) and that cell populations associated with parasite killing in vitro are of myeloid origin (Taverne, Dockrell, and Playfair, Parasite Immunol., in press). Carbon clearance studies have shown that the activity of the reticuloendothelial system is enhanced at some time during malarial infection (2, 10, 16), and activated macrophages, as assessed by increased size, spreading, and phagocytic ability, have been shown to be present in the spleens of mice infected with *Plasmodium berghei* (29). Furthermore, adherent

spleen cells from mice infected with *P. berghei* or *P. yoelii* have been shown to release soluble factors which affect mitogen-induced proliferation of thymocytes (33).

Mice whose macrophages are activated are known to possess enhanced sensitivity to the effects of endotoxin. For example, the concentration required to enhance the tumoricidal activity of macrophages from mice given BCG was 10,000-fold less than normal (31). Similarly, after injection of BCG or *C. parvum*, the lethal effect of endotoxin increased with macrophage activation, as measured in terms of resistance to *Listeria monocytogenes* (1), and macrophages from BCG-infected mice were killed by lower concentrations of lipopolysaccharide (LPS) in vitro (22). In mice infected with *Plasmodium* or *Babesia* spp., the 50% lethal dose of endotoxin was nearly 100-fold less than normal, and susceptibility to the lethal effect increased with parasitemia (4).

To determine directly whether sera from mice whose macrophages were activated non-specifically with *C. parvum* or BCG could kill malarial parasites and to investigate the role of endotoxin in inducing the release of parasite-killing factors, we incubated erythrocytes infected with *P. yoelii* in various sera in vitro and measured the effects in terms of loss of infectivity. To assess changes in susceptibility to endotoxin that occurred during infection and thus possible activation of macrophages, we also tested serum samples that were collected at intervals from mice infected with *Plasmodium* spp. and then given endotoxin for the ability to kill parasites in vitro.

MATERIALS AND METHODS

Mice. Female outbred mice (Tuck no. 1; A. Tuck and Sons, Battlesbridge, Essex, England) were used throughout.

Parasites. *P. yoelii* strain 17X, its lethal variant (23), and *P. berghei* strain Anka were maintained by blood passage. In mice infected with *P. yoelii*, the parasitemia reached a peak 10 to 13 days after infection, when 10 to 30% of the erythrocytes were infected. Parasites disappeared completely from the blood of most mice by day 25. On average, mice infected with the lethal variant and with *P. berghei* survived 15 and 18 days, respectively.

Infectivity titrations. Infectivity titrations were performed by a modification of the method of Warhurst and Folwell (30), as previously described (Taverne et al., Parasite Immunol, in press), by injecting samples of parasites intravenously into groups of three mice and determining the mean time after injection when 0.5% of their erythrocytes became infected. The number of viable parasites originally injected was calculated by referring to a standard curve. This curve was based upon the fact that there is an inverse relationship between time to patency and log dose of parasites injected, and the curve was drawn by plotting the time taken for known numbers of parasites (varying from 10^1 to 10^6 parasites) to infect 0.5% of the erythrocytes. Infectivity was expressed in terms of $\log_{10}$ parasites per milliliter of original suspension.

Parasite cultures. Parasitized erythrocytes were suspended in 1-ml volumes of RPMI 1640 medium (Flow Laboratories, Inc., Rockville, Md.) supplemented with 0.2% $NaHCO_3$, 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 5 mM glutamine, 100 μg of streptomycin per ml, 100 U of penicillin G per ml, 20% heat-inactivated normal horse serum, and 2×10^8 washed normal mouse erythrocytes, and these suspensions were incubated overnight in multiwell plates (Linbro Plastics) at 37°C in an atmosphere of 5% CO_2 in air. The survival of the parasites in terms of infectivity was determined before and after incubation. The survival of parasites in test serum was always expressed as a percentage of the survival in the control serum at the same dilution incubated in parallel. Differences in survival of more than 1 $\log_{10}$ were reproducible and were considered significant.

Endotoxin-induced serum TNS. For each batch, sera were obtained from groups of at least three mice and were pooled.

(i) *C. parvum* TNS. Mice were injected intraperitoneally with 1.4 mg of killed *C. parvum* (Wellcome Laboratories). After 7 days, these mice were injected intravenously with 25 μg of LPS W from *Escherichia coli* O55:B5 (Difco Laboratories, Detroit, Mich.) and were bled 2 h later. Serum was stored at −20°C.

(ii) BCG TNS. Mice were injected intravenously with 3×10^6 colony-forming units of BCG bacilli (Glaxo Laboratories); 14 days later these mice were given LPS as described above and bled after 2 h.

(iii) Malaria TNS. Three groups of mice were injected intravenously with 10^4 cells of nonlethal or lethal *P. yoelii* or *P. berghei*. A fourth group, which had been vaccinated 3 weeks earlier with saponin-lysed, Formalin-fixed lethal *P. yoelii* together with *Bordetella pertussis* (23), was challenged with 10^4 lethal *P. yoelii* cells. At intervals during infection, some mice were bled for control serum, and others were injected intravenously with 25 μg of LPS and bled 2 h later.

RESULTS

Multiplication of parasites in vitro. Cells of the nonlethal strain of *P. yoelii* multiplied when they were incubated overnight in vitro (Fig. 1). With lower concentrations (up to about 10^4 parasites per ml), the amount of multiplication approached the amount which would be expected theoretically if every parasite was infective, each schizont giving rise to 16 merozoites and one cycle of growth occurring every 24 h, although the initial population was not synchronized. At higher concentrations, the amount of multiplication was less consistent, although we sometimes observed multiplication from numbers approaching 10^5 parasites per ml. In most experiments, we used 10^3 to 10^4 parasites per ml and at these low concentrations (<0.01% of the

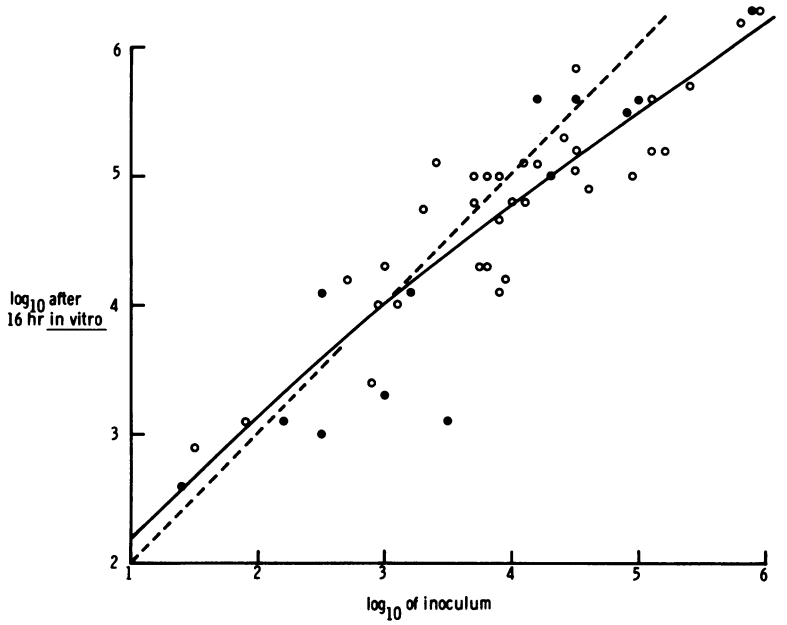

FIG. 1. *Multiplication of the nonlethal P. yoelii after 16 h in vitro. Symbols: ○, nonlethal P. yoelii; ●, lethal variant. Parasite cell numbers were determined before and after 16 h of incubation at 37°C, and the results are expressed as $\log_{10}$ infective parasite cells per milliliter. The solid line shows the actual infectivity obtained on average, and the dashed line shows the theoretical yield at 16 h drawn on the basis that each infective parasite cell yielded 16 daughters in 24 h.*

erythrocytes infected) it was impractical to evaluate the parasites morphologically. Parasitized erythrocytes taken from mice 20 to 24 days after infection (when they were being cleared from the blood) showed the same infectivity and multiplied to the same extent as parasites taken early in the linear phase of infection. Unless otherwise indicated, the nonlethal parasite was used in most experiments and at concentrations at which full multiplication was usually obtained. The lethal variant multiplied similarly in vitro (Fig. 1).

Killing by TNS. Some typical results are shown in Table 1. Parasites incubated overnight in the presence of serum obtained from normal mice 2 h after injection with LPS or in the presence of serum from normal mice given *C. parvum* 7, 14, or 21 days earlier (but no LPS) multiplied to the same degree as in normal serum. In contrast, TNS diluted 1:1 abolished all infectivity. Generally, at some higher dilutions TNS inhibited multiplication without reducing infectivity below the infectivity at the start of the experiment; few batches of TNS diluted more than 1:9 killed parasites. We found no significant differences in the potencies of TNS obtained 7, 14, and 21 days after injection of *C. parvum*. Although the TNS obtained on different occasions varied in potency, the degree of killing obtained with a given sample was reproducible (Table 1). TNS activity was not abolished by heating at 56°C for 30 min. Parasites taken from mice at different times during the course of infection, even at about the time when

TABLE 1. *Representative experiment demonstrating killing of the nonlethal P. yoelii by TNS in vitro*[a]

Serum donors	LPS	Serum dilution	No. of infective parasites ($\log_{10}$) at:			% Survival
			Zero time	16 h		
				Normal mouse serum	Test serum	
Normal	+	1:4	4.2	5.1	5.2	>100
C. parvum	−	1:4	3.7	4.6	4.6	100
C. parvum	+	1:1	4.1	4.9	0	0
		1:4	4.1	4.8	3.1	2.0[b]
		1:9	4.1	4.8	3.9	13.3

[a] Mice injected with 1.4 mg of *C. parvum* intraperitoneally 7 days earlier were given 25 μg of LPS intravenously and bled 2 h later. The parasites at the concentrations indicated at zero time were incubated for 16 h at 37°C in serum at the dilutions shown; then their infectivities were determined, and survival values were expressed as percentages of the survival obtained in normal mouse serum.

[b] For comparison, the mean ± standard deviation percent survival values, derived from at least three tests each for four different batches of TNS diluted 1:4, were 9.0 ± 3.6, 0.97 ± 0.9, 2.9 ± 1.8, and 2.8 ± 4.0%.

they were disappearing from the blood, were all equally susceptible to killing by TNS, as were the lethal variant of *P. yoelii* and *P. berghei*. The time courses of parasite inactivation by two different concentrations of TNS are shown in Fig. 2.

The proportion of parasites killed by a given sample of TNS at a particular dilution did not vary with the concentration of parasites used (Fig. 3). TNS obtained from mice given BCG behaved similarly to TNS obtained from mice given *C. parvum*. The serum obtained from mice given LPS 6 days after infection with the lethal *P. yoelii* variant also killed parasites in vitro, and serum obtained 9 days after infection was even more effective. Therefore, we investigated the time course of the ability of infected mice to produce a parasite-killing factor in their sera after the administration of LPS.

Endotoxin-induced serum factor from mice with malaria. Serum samples taken after injection of LPS into mice at different times after infection with one of the three parasites or after homologous challenge of mice vaccinated against the lethal *P. yoelii* variant were tested for the ability to kill nonlethal *P. yoelii* cells in vitro (Fig. 4). To exclude the possible effect of antibody, we always compared survival with survival in a control serum taken from the same group of mice on the same day; in fact, none of the sera from infected mice not treated with LPS killed parasites in vitro. When diluted 1:4, endotoxin-induced sera from mice infected with the nonlethal *P. yoelii* had little effect, although some activity was observed around day 13; sera taken after recovery were normal. Activity was revealed if the sera were tested at 1:1 dilutions, and sera taken between days 7 and 14 showed the greatest effect. In contrast, sera from mice infected with the lethal *P. yoelii* variant and given LPS killed parasites at 1:4 dilutions, and this activity increased with time, in parallel with parasitemia. The killing activity of endotoxin-induced sera from vaccinated mice infected with the lethal *P. yoelii* variant was never as great as the killing activity of sera from unvaccinated mice, and, as with the nonlethal infections, the killing activity returned to normal when the mice recovered. Sera from mice infected with *P. berghei* suggested the existence of two phases of susceptibility to endotoxin induction for the parasite-killing factor.

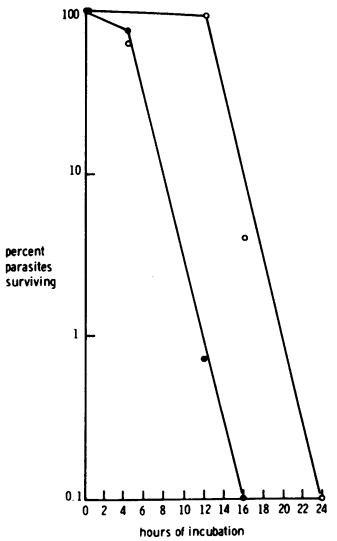

FIG. 2. *Typical experiment showing the time course of the killing effect of TNS on the nonlethal P. yoelii in vitro. Symbols: ●, TNS diluted 1:1; ○, TNS diluted 1:4. Parasites at a concentration of 10^4 cells per ml were incubated at 37°C in endotoxin-induced serum obtained from mice given C. parvum 7 days earlier. Parasite infectivity was determined at different times, and survival is expressed as a percentage of survival in normal mouse serum at zero time.*

DISCUSSION

Our experiments showed that sera from mice that received *C. parvum* or BCG followed by endotoxin killed malarial parasites in vitro. The nonlethal *P. yoelii* was no more susceptible to killing than the lethal parasites (*P. berghei* and the lethal variant of *P. yoelii*), and parasites taken from mice when the infection was resolving spontaneously 20 to 24 days after infection were no more susceptible than those tested 2 to 3 days after infection, when they were in the logarithmic phase of growth. Thus, previous coating by specific antibody or possible deleterious changes induced by nonspecific factors present at the time of resolution did not increase the susceptibility of parasites to the effects of TNS, nor was the ability of such parasites to multiply in vitro diminished. Quinn and Wyler (25) reported that the multiplication of crisis forms of *P. berghei* in recipient rats was indistinguishable from the multiplication of morphologically normal parasites and concluded that crisis is a reversible process. We have confirmed that cell for cell, *P. yoelii* tested at the time of spontaneous resolution had the same infectivity in vivo as young parasites at the start of infection and that this was also true for the lethal *P. yoelii* variant taken from vaccinated mice as they were eliminating a challenge infection (unpublished data).

Sera from mice given *C. parvum* or BCG but not endotoxin not only failed to kill parasites in vitro, but also supported multiplication to the

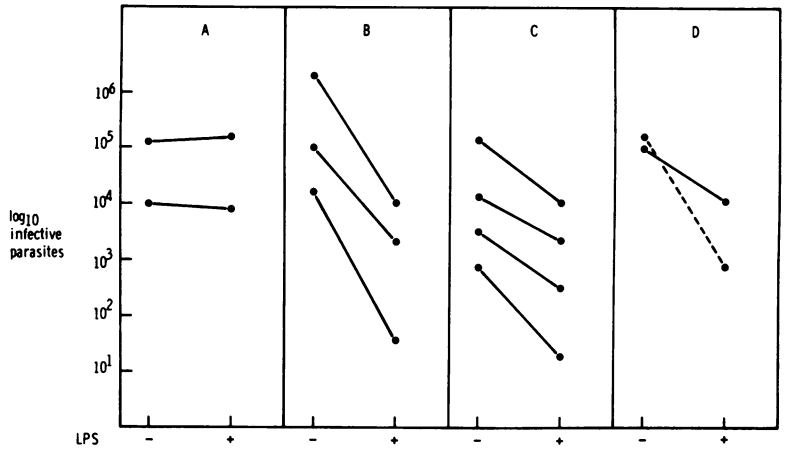

FIG. 3. *Killing of parasites by sera from mice activated by different agents and then given LPS: $\log_{10}$ infective parasites present after incubation for 16 h in normal mouse serum or in the various samples of TNS. Mice were injected intravenously with 25 μg of LPS 7 days after C. parvum injection, 14 days after BCG infection, and 6 days (solid line) or 9 days (dashed line) after infection with the lethal variant of P. yoelii and bled 2 h later. (A) Normal serum diluted 1:3. (B) C. parvum serum diluted 1:3. (C) BCG serum diluted 1:7. (D) P. yoelii serum diluted 1:4.*

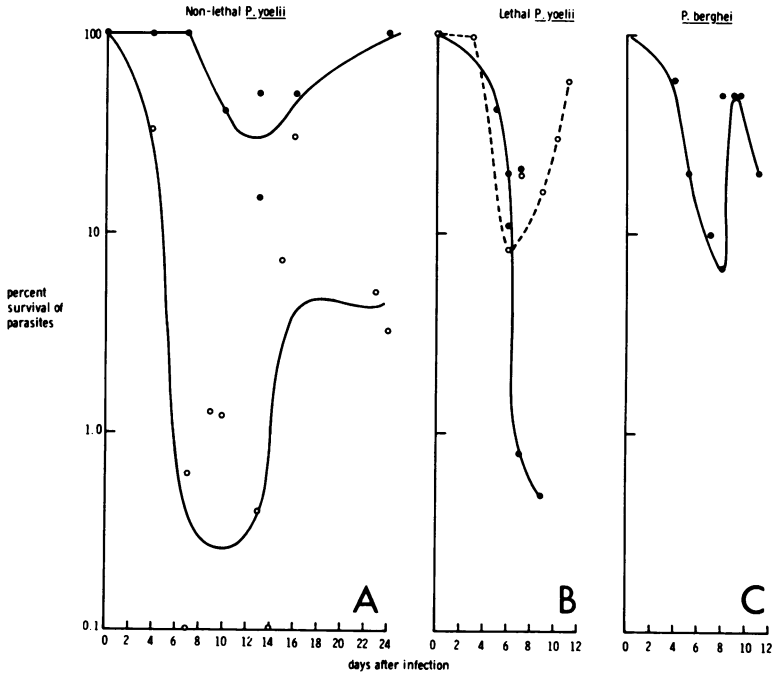

FIG. 4. *Killing of the nonlethal P. yoelii by endotoxin-induced sera obtained from mice after infection with the nonlethal P. yoelii (A), the lethal P. yoelii variant (B), or P. berghei (C). Parasites were incubated for 16 h in vitro in endotoxin-induced serum; then their infectivities were determined, and survival values were compared with the survival values in control sera taken from the same groups of infected mice on the same day. Symbols: ●——●, serum diluted 1:4; ○——○, serum diluted 1:1; ○— —○, serum from vaccinated mice after homologous challenge, diluted 1:4. Freehand lines have been drawn to connect the points.*

same degree as normal mouse serum. Such mice have been shown to be protected against a variety of parasites (5, 6, 8), and protection is thought to be due to soluble mediators. This difference may be explained by localization in vivo of the site of killing (with perhaps higher local concentrations of killing factors) or, possibly, by differences in the regimens used to activate the mice.

We do not know at which stage of development parasites are inactivated by TNS. However, the fact that at higher concentrations infectivity was abolished completely (Table 1) suggests that TNS did not merely inhibit multiplication. Preliminary experiments in which sorbitol-treated parasites were used (14) indicated that at least the early stages were susceptible to TNS.

The activity of TNS against tumors was first demonstrated in vivo (3), and mice have also been protected against *Klebsiella pneumoniae* and *L. monocytogenes* by passive transfer of TNS (21). When injected repeatedly into mice infected with *P. yoelii,* rabbit TNS caused a delay in the appearance of parasites in the blood and prevented death from the lethal variant (unpublished data).

The time required for TNS to kill parasites in vitro is similar to the time reported for tumor cells. Thus, significant loss of parasite infectivity was first apparent after 8 h of incubation with a high concentration of TNS and after 16 h with a lower concentration, whereas cytotoxicity for L cells was first detected after 8, 16, or 24 h (3, 17, 19). Although the conditions under which the parasite-killing factor was produced were the same as those necessary for the production of TNS, it is not certain that the effector molecule was the same. Endotoxin is known to cause the release of many enzymes (12, 28) and other soluble factors from activated macrophages (20); sera also contain migration inhibition factor (26) and interferon (26, 34), and Schultz et al. have claimed that malarial parasites are susceptible to interferon (27).

The results obtained with sera from infected mice given endotoxin at intervals during the course of infection apparently support the observation that susceptibility to endotoxin varies with parasitemia (4), at least in terms of the capacity to release the parasite-killing factor. However, vaccinated mice might have been expected to show greater activity, in that *B. pertussis* has immunological properties in common with *C. parvum* and BCG, but, in the few tests done, injection of *B. pertussis* alone (without killed free parasites) followed by endotoxin produced sera that had little or no activity (unpublished data). If the capacity to release the parasite-killing factor reflects macrophage activation, then such activation is greater in lethal infections than in nonlethal infections.

It is remarkable how little is known about the course and state of activation of macrophages in malaria. The enhancement of activity observed in studies on carbon clearance (10, 16) may largely reflect the great increases in the numbers of macrophages in the spleen (15, 32) and liver (Dockrell, unpublished data). Although activation was demonstrated in the spleens of mice infected with *P. berghei* (29), no comparison was made either with infections resolving spontaneously or in vaccinated mice. Among the products of macrophages that have been demonstrated to change during infection are lymphocyte-activating factor (33) and tumor necrosis factor. Recently, Clark and co-workers (6a) have shown that endotoxin-induced sera from mice infected with *Plasmodium vinckei* subsp. *petteri* contain tumor necrosis factor, and they have proposed that various macrophage-derived mediators are involved in the pathogenesis of acute malaria, as well as in the destruction of the parasite. We have now shown that TNS can indeed kill parasites and thus have confirmed the suggestion that nonspecific serum factors may also play a role in protection.

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