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Failure to Demonstrate Circulating Endotoxin in Malaria[1] (38572)

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(Introduced by F. B. Bang)

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The distinctive clinical hallmark of human malaria is the febrile paroxysm, which is comprised of three successive stages: Chill or rigor; febrile phase; and sweating phase (defervescence and diaphoresis) (1). This clinical picture is similar to that seen in human volunteers following the intravenous injection of purified endotoxin (2). Furthermore, tolerance to the febrile effect of endotoxin has been demonstrated to occur in humans experimentally infected with malaria (3, 4). In order to evaluate the possibility that endotoxin-like material, derived from malarial parasites, could be the agent which precipitates the malarial paroxysm, we attempted to demonstrate the presence of endotoxin in the blood of humans and monkeys infected with malaria, using the Limulus amebocyte lysate test.

Materials and Methods. Infections. Studies of human infections were performed at the Human Malaria Unit of the National Institutes of Health, NIAID-LPD, located in the U.S. Penitentiary, Atlanta, GA. Healthy, adult male prisoner volunteers were informed fully of the dangers of the project; no coercion or excessive inducements were used. These investigations were performed on volunteers with malaria infections induced for purposes of other studies.

Each volunteer was infected by the bites of one or more heavily infected *Anopheles* mosquitoes previously fed on *Aotus trivirgatus* monkeys, infected with either *Plasmodium vivax* or *P. falciparum*. The infection in each volunteer was monitored with daily peripheral blood smears, stained with Giemsa. In each malarial paroxysm studied, the patient's rectal temperature and thick blood smears were taken at least hourly, beginning prior to the onset of the paroxysm. Parasitemia, expressed as the number of asexual parasites per mm^3, was determined from thick smears. The relationships of plasma samples for endotoxin assay to clinical events of the paroxysm are indicated in *Results*.

In order to minimize the risks of malaria in in the human volunteers, antimalarial therapy was instituted at low levels of parasitemia. Therefore, as a means of studying malarial infections during high levels of parasitemia, two rhesus monkeys (*Macaca mulatta*) were infected simultaneously by the intravenous injection of heparinized, whole blood from another rhesus monkey infected with *P. coatneyi*. Each animal received 10^5 parasites. *Plasmodium coatneyi* is a monkey malarial parasite which resembles human *P. falciparum* in its asexual morphology and periodicity (tertian) (5). Parasite counts in the monkeys were obtained from thick blood smears, stained with Giemsa, according to the method of Earle and Perez (6). Plasma samples for endotoxin assay were obtained during periods of schizont rupture (i.e., rupture of red cells infected by mature schizonts, with subsequent release of merozoites), since this process corresponds to the human malarial paroxysm, which is the clinical response to the events of schizont rupture in human infections (1).

Limulus Test. Endotoxin causes gelation

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of lysates derived from the blood cells (amebocytes) of the horseshoe crab (*Limulus*) (7, 8), and this assay has been used to detect endotoxin in the blood of patients with a variety of gram-negative bacterial infections (9, 10). In the current studies, venous blood, from human volunteers or monkeys, was drawn into sterile, pyrogen-free plastic syringes containing 500 units of heparin, and plasma was separated by centrifugation. To permit recovery of endotoxin, plasma was extracted with chloroform (one part chloroform:four parts plasma) for 4 hr. The assay was performed as described previously (8–10). A definite increase in viscosity and turbidity was interpreted as indicating the presence of endotoxin. The ameboyte lysate used in these experiments was capable of detecting 0.0005 μg/ml of *E. coli* endotoxin (Difco Laboratories, Detroit, MI) (8).

Results. All samples obtained from volunteers infected with *P. vivax* or *P. falciparum* produced negative Limulus tests. Of the 15 samples from four volunteers infected with *P. vivax*, two were drawn during periods of rigorous chilling, three during periods of rapidly rising fever, seven at the stage of peak fever, one during the period of defervescence, and two during afebrile periods. Parasite counts at the time the samples were drawn ranged from 200/mm^3, to 7400/mm^3 and the patients' temperatures were as high as 40.7°. In two volunteers infected with *P. falciparum*, four malarial paroxysms were studied. Two samples were drawn while the patients were undergoing chills at the onset of paroxysms, three samples during periods of peak fever, and one sample was drawn during a chill which occurred while the patient was experiencing his peak fever. At the time the samples were obtained, parasite densities were between 60/mm^3 and 2000/mm^3, and the patients' temperatures were as high as 40.1°.

Nine plasma samples, obtained from two rhesus monkeys infected with *P. coatneyi*, during three episodes of schizont rupture, produced negative Limulus tests. Four samples were drawn 1–7 hr before the onset of schizont rupture, three during the phase of rapidly increasing numbers of ring forms in the peripheral blood (about 2 hr after the onset of schizont rupture), and two samples at the time of peak ring form count. Peak parasite counts during the sampling periods ranged from 136,000/mm^3 to 376,000/mm^3.

Discussion. In a review of recent developments in the field of malaria, Neva suggested that the malarial paroxysm might have pathophysiologic features in common with the pyrogenic reaction to endotoxins (11). Firstly, there is clinical similarity between the two phenomena. Secondly, the malarial paroxysm, like the endotoxin reaction, seems to be preceded by a latent period, since the onset of the malarial paroxysm is preceded by the rupture of red cells infected by mature schizonts, and release of merozoites and parasite by-products from infected red blood cells (1, 12). Therefore, if an endotoxin-like substance were responsible for the malarial paroxysm, it should be present in the blood at the time of schizont rupture. However, a recent review of the biochemistry of plasmodia makes no mention of lipopolysaccharide constituents (13).

In order to determine whether malarial parasites release endotoxin-like material into the blood during some stages of the infection, we attempted to demonstrate the presence of endotoxin in the plasma of seven humans infected with malaria. A total of 21 samples, drawn at various times in the malarial paroxysm, were negative for endotoxin or endotoxin-like activity, when tested with Limulus amebocyte lysate. In addition, nine plasma samples drawn from two rhesus monkeys heavily infected with *P. coatneyi* were negative using the Limulus test. Therefore, it seems unlikely that significant concentrations of an endotoxin-like substance are present in the blood during infection with plasmodia. Although it is possible that, in some instances, the Limulus assay is not sensitive enough to detect concentrations of endotoxin sufficient to produce fever, this seems unlikely since the Limulus test has been shown to be ten times as sensitive as the standard U.S.P. rabbit test for pyrogenic activity (i.e., for endotoxin) (14). The latter, less sensitive test, apparently is adequate to detect concentrations of endotoxin that are pyrogenic in humans.

Additional studies will be required to determine the mechanism by which malarial parasitemia produces the acute, febrile

paroxysm which is so prominent a feature of human malaria.

Summary. The possibility that endotoxin or an endotoxin-like substance plays a role in malaria has been suggested by the clinical similarity between human malaria and the febrile reaction to endotoxins, as well as the occurrence of endotoxin tolerance in humans infected with malaria. However, endotoxin or endotoxin-like activity was not demonstrable, using the Limulus test, in the plasma of humans or monkeys infected with plasmodia. The data indicate that the febrile paroxysm of malarial infection is not associated with detectable levels of endotoxin in the blood.

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