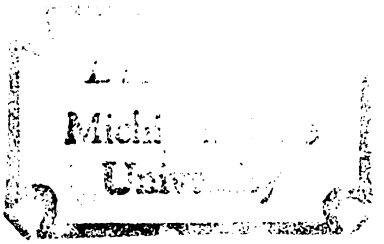

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PARTIAL CHARACTERIZATION OF THE PYROGEN FACTOR

IN PLASMODIUM FALCIPARUM

presented by

Lenka Dimanina

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of the requirements for

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Major professor

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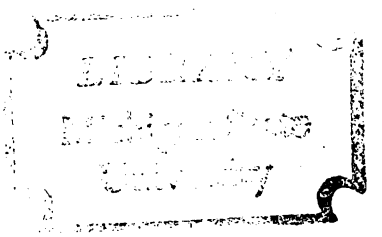

PARTIAL CHARACTERIZATION OF THE PYROGEN FACTOR IN PLASMODIUM FALCIPARUM

By

Lenka Dimanina

A THESIS

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ABSTRACT

PARTIAL CHARACTERIZATION OF THE PYROGEN FACTOR

IN PLASMODIUM FALCIPARUM

By

Lenka Dimanina

In an effort to clarify the genesis of fever in falciparum malaria, parasites were cultured in vitro and fractions assayed for pyrogenicity in rabbits or for "endotoxin-like" activity in the Limulus amebocyte lysate assay. Parasite post-schizont residual material, rich in the malarial pigment hemozoin, was found to have minimal activity in the Limulus assay. Extracts from schizont-infected red cells were positive in the assay and thus an "endotoxin-like" substance may be responsible for the febrile response in falciparum malaria infections. Treatment with pancreatic protease altered but did not significantly decrease the "endotoxin-like" activity in the parasite extract. Post-ribosomal supernatant, by ultracentrifugation of the parasite extract, as well as the subcelluler and membrane pellet, produced strong febrile responses in rabbits.

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Most importantly, I would like to express my love, gratitude and appreciation to my son, Sashe, for being so supportive, patient and most important, believing in me which made it all possible.

To my son,

Sashe Dimitroff

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INTRODUCTION

Hippocrates, writing two millenia ago, categorized certain common fevers according to their periodicity. We now recognize these "quotidian", "tertian" and "quartan" fevers as the different sorts of malaria, caused by organisms of the genus Plasmodium. Our basic knowledge has come from the work of Laveran and Golgi in the late 19th Century. Working independently, Laveran correlated the disease malaria with the presence of organisms in the blood, and Golgi attributed the periodicity of the fevers to the cyclic life cycle of the parasite within the host.

But while the correlation of clinical fever spikes with the waves of parasites released into the bloodstream was a great step forward in rationalizing this ancient affliction, the physiology associated with this correlation remains largely obscure. In this study, we will seek to examine some possible mediators between fever and parasite.

That the connection should still be unclear is not surprising, for it is only in the past 40 years that what now seem the most basic processes of fever, have begun to be elucidated. The exact mechanisms, the mediators involved and even the physiological utility of fever all remain areas of intense research.[5] Moreover, malaria parasites, unlike the more intensively studied bacterial pathogens, pose burdensome and sometimes insuperable obstacles to their exact observation and study. It is only in the past five years that methods have become available for routine, in vitro cultivation of P. falciparum, deadliest of the marlarial parasites.[45]

The new methods permit, to a degree unknown before, the study of the physiological, immunological and pharmaceutical interactions between host, parasite and chemotherapeutic agent. They also permit the isolation and study of substantial populations of the parasites at different stages of their life cycle.[17, 25, 39, 41, 45]

We used these culture and separation techniques to isolate fractions of cultivated P. falciparum and subsequently tested their ability to produce fever in rabbits. The rabbit has been previously established as an appropriate test subject with similar, if somewhat less sensitive, reactions to a number of common fever-producing agents.[23]

Before discussing the febrile response of rabbits to our various Plasmodium fractions, we will review briefly both fever and the parasite life cycle.

Fever

Fever is the result of a complex process by which the thermoregulatory center of the central nervous system (CNS) raises its "set point". That is, there is an elevation of the temperature which the body actively maintains by increasing or reducing heat production and heat dissipation. That is in contrast to non-CNS-mediated causes of hyperthermia, such as exercise or heat stroke, in which there appears to be a simple insufficiency of cooling capacity in the face of increased heat production. In these cases, once cooling capacity has caught up, the body will equilibrate at its previous set point temperature.[43]

In fevers, the body may both increase heat generation, as by shivering, and decrease heat loss, as by vasoconstriction. In addition, at the onset of fever, the subject will feel cold, as his "thermopreferendum" rises. He may then seek out an extra blanket or turn up the furnace.

Substances which cause an elevation in the thermal set point are called pyrogens. They comprise a diverse group, including fungi, viruses, bacterial cell wall material, antigen-antibody complexes, certain steroids and a number of inorganic substances. In the last group, it has been found that the sodium:calcium ratio in the cerebrospinal fluid appears to change during fevers in cats, with calcium increases being associated with defervescence.[5]

One of the most intensively studied of the pyrogens is the lipopolysaccharide cell wall material of Gram negative bacteria. Injected intravenously in rabbits or humans, this substance is capable of producing a measurable fever in doses as low as 1 ng. per kilogram of body weight. The active portion appears to be the lipid A moiety, a beta 1-6 linked diglucosamine backbone containing both ester- and amide-linked long chain fatty acids, as well as 2-keto-deoxyoctonate and pyrophosphate groups.[5,26]

The thermoregulatory center is located largely or entirely in the preoptic anterior hypothalamus. Due to the blood-brain permeability barrier and the kinetics of pyrogen action, it is believed that the great majority of pyrogens cannot reach the hypothalamus directly and must operate through a mediator. This mediator is called "endogenous pyrogen" (EP). It is a low molecular weight protein (13,000-15,000 daltons) produced by neutrophils, eosinophils and certain monocytes. It is not produced by lymphocytes.[15] (Figure 1) It does not appear to be stored in active form by these inflammatory cells but is an inducible protein only synthesized upon receipt of the appropriate chemical signal.[7] In addition to blood monocytes and alveolar macrophages, certain fixed-cell macrophages, notably the hepatic Kupffer cells and the splenic sinusoidal cells, are capable of producing EP.

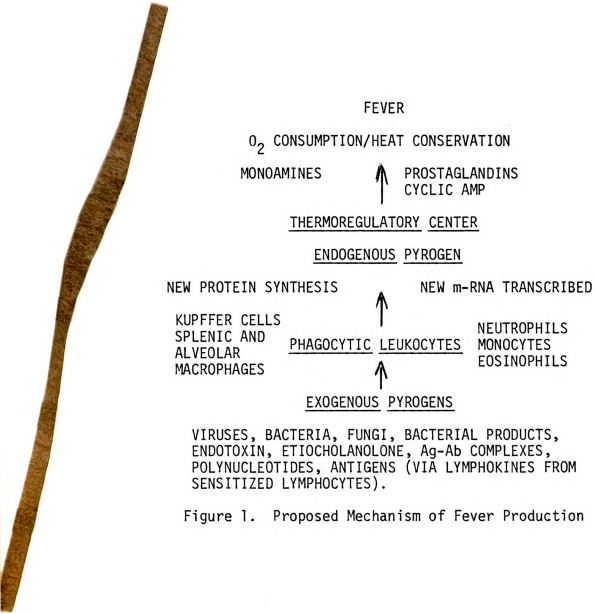

Figure 1. Proposed Mechanism of Fever Production

Repeated doses of exogenous pyrogens induce a state of tolerance, in which increasingly large doses are needed to produce a febrile response.[4]

Endogenous pyrogen may act directly on the hypothalamus, or it may need to be mediated by prostaglandins or vasoactive monoamines. The prostaglandin level in the CNS fluid does not appear to rise and fall with febrile spikes, and the evidence seems to be leaning away from a mandatory role for prostaglandin mediators.[3,28] Endogenous pyrogens (EP) are capable of producing fevers almost immediately upon injection into the experimental subjects, while exogenous pyrogens may take up to three hours or more to produce an unambiguous fever. The quantity of EP needed to produce fevers in both humans and rabbits has been estimated as between 30 and 50 nanograms, but the issue is clouded by the question of how well the EP has been purified.

Atkins et al[2] outlined three general routes by which leukocytes appear to release EP. First is the release of EP upon phagocytosis of a stimulatory agent, such as bacterial cell walls. Phagocytosis does not necessarily result in EP production, as has been shown by experiments in which the leukocytes phagocytize latex balls without concomitant EP release.[5] The phagocytic mechanism is invoked to explain the fevers of septicemia and infection of deep tissues.

The second general route is stimulation of the leukocytes by factors which induce other aspects of inflammation, including chemotactic factors and/or complement. The pyrogenicity of most inflammatory factors and the inflammatory capabilities of most pyrogens remain to be explored. The third basic mechanism of EP production cited by Atkins is its spontaneous release by certain tumor and Hela cells. The EP-releasing leukocytes have receptors for both complement and Fc portions of antibodies, and antigen-antibody complexes have been shown by Bernheim, Francis and Atkins

to stimulate EP production.[6] Interestingly, 10:1 excesses of either antigen or antibody, working by weight from equivalence zone, induced EP production, but the precipitated complexes themselves did not, even though they were phagocytized by the leukocytes. Cell-mediated immune responses are also believed to play a part in fever production, as T-cell products such as lymphokines are able to activate leukocyte EP production.[33]

The Parasite Life Cycle

The life cycle of the malarial parasite spans two hosts;the anopheline mosquito and either an avian or a mammalian host.

Human infection by P. falciparum usually begins with the bite of an infected anopheline mosquito, which, as it takes its blood meal, injects the malarial sporozoite into the bloodstream. The sporozoite circulates through the blood for approximately 30 minutes before it absorbs to and invades a hepatic parenchymal cell. Within the hepatic cell, the parasite grows and its nucleus repeatedly divides until approximately 40,000 merozoites are produced, each about 0.7 microns in diameter. These are then released into the bloodstream.

The prepatent period, which is the interval between injection of the sporozoite and the appearance of parasites in the blood, ranges between six days to a month, most commonly two weeks or a little less.[10] The first fever above 101 F usually occurs within one to two days after the end of the prepatent period.

The merozoites released by the liver cell are quickly taken up by the red blood cells. There is evidence for a specific protein or glycoprotein recognition site on the erythrocyte surface, a site which is sensitive to trypsin treatment but not chymotrypsin.(34)

One hypothesis for the mechanism of parasite uptake is that a highly basic (70% histidine) protein, carried by the merozoite in terminal structures called rhoptries and micronemes, may cause a local disturbance in the erythrocyte membrane.(29)

During its uptake by the erythrocyte, the parasite comes to be enclosed by a second membrane just overlaying its own plasma membrane. This second membrane, called the parasitiphorous vacuolar membrane (PVM), is erythrocyte-derived, but appears to come under increasing control by the parasite within.

Inside the erythrocyte, the merozoite dedifferentiates, first appearing as a "ring" or "applique" in which the lumen of the ring is a large vacuole containing cytoplasmic material of the host, and the "stone" of the ring is chromatin. As the trophozoite grows, "knobs" develop which appear to be beneath the surface of the erythrocyte membrane, and seem to cause its increasing deformation as the parasites mature. These knobs have been found to form focal connections with endothelial cells and may participate in the sequestration in deep tissue of the later stages of parasite development.(1) The younger trophozoites appear to have smaller and more numerous knobs while older, segmented forms appear to have larger, fewer knobs. There appears to be some shedding of knob material during the growth of the parasite within the red blood cell.(29)

Antigenically, these knobs have been found to be distinct from the surrounding erythrocyte surface. Experiments in which parasite cultures were incubated with 32S-labeled methionine showed greater radioactivity in membrane fractions derived from knobby erythrocytes than in non-knobby

fractions. In addition, knobby fractions showed altered binding characteristics with the glycoprotein probe, concanavalin A.[28]

During its growth phase, the parasite utilizes the hemoglobin of its host as a source of amino acids. In addition, a substantial part of the hemoglobin is converted to the characteristic golden malarial pigment, hemozoin. Hemozoin has been found to consist largely of monomers and dimers of hematin. Some of it consists of ferriprotoporphyrin coupled to a plasmodial protein and some methemoglobin is also present. Hemozoin is inert insofar as respiration is concerned and otherwise nontoxic according to Maegraith, who studied the pigment in great detail during the 1950's.[32]

The mature trophozoite nucleus undergoes a series of divisions which lead to the production of a schizont in which the several nuclei are each surrounded by a little bit of cytoplasm, to form merozoites. The usual number is about 16. When fully mature, the merozoites are released to infect more erythrocytes. The mechanism of release is unknown. After one or more generations of merozoite formation some of the parasite population will develop into the sexual stage within the infected red blood cell. The micro- or macrogametocyte formed may then be taken up by an anopheline mosquito to begin the sporogonic part of its life cycle.

In the gut of the mosquito the hametocytes develop into gametes, fuse to form a zygote and then migrate through the gut wall to form an oocyst on its outer surface. The oocyst then ruptures, releasing sporozoites into the insect's hemocel. Subsequently the sporozoites migrates into the mosquito's salivary glands and thus come to the injected into a mammalian host during feeding, starting a new cycle.

Malaria and Fever

It has been long recognized that many of the signs and symptoms of malaria resemble those of an acute general inflammation.[8] These include increased permeability of the vascular endothelium, permitting the loss of fluids and the ultimate blockage of circulation. The signs also include profound vasomotor disturbances and, of course, fever.

Maegraith analyzed the blood of animals infected with P. knowlesi and found decreased levels of bradykininogen and kallidinogen, precursor forms of the vasoactive peptides bradykinin and kallidin, respectively.[37] He also found a rise in serun kinin and kallikrein levels. The latter enzyme, which converts kininogen to kinin, has considerable kinin-type activity. These findings were significant in helping to flesh out a model for the pathogenesis of malaria in which vascular collapse plays a central role.

In addition, however, it is of interest because of the multiple feedback loops between the kinin system and Hageman Factor XII, and thence, to the fibrinolytic, clotting and complement systems. As mentioned earlier, complement may stimulate endogenous pyrogen production.[42] Complement levels have been shown to be lowered within a few hours of schizont rupture and peak fever in P. vivax infection, apparently due to activation of the classical complement pathway.[36]

Maegraith, as had others before him, pointed out the similarity between the malarial "inflammation" and the febrile paroxysm produced by bacterial infections. The gram-negative bacterial cell wall, and particularly the lipid A portion, in addition to being a good pyrogen, is also the cause of gram-negative toxicity, the symptoms of which are those of acute general inflammation.

The malarial parasite, a eukaryote, is very different from the bacterial prokaryotes -- the outer surface which it presents to the host immune system being fundamentally different. The bioactive part of the gram-negative bacterial cell wall, the lipopolysaccharide, and particularly its lipid A backbone, is without an obvious structural parallel in the membrane-enclosed parasite. This is true whether the membrane components of the parasite's outer surface are host-derived or produced by the parasite. It is possible, however, that there may be inserted in the parasite's outer membrane some component, whether lipid, protein, carbohydrate or some hybrid structural type, which mimics the biologic activity of the lipopolysaccharide (LPS). To date, however, no such component has been found; LPS still appears to be uniquely a component of bacterial cells. There is tantalizing evidence that, however different they may be chemically, LPS and the malarial parasite's outer surface share at least some patterns of bioactivity.

In 1973 Orsay and Heath[37] injected Plasmodium berghei into the preoptic hypothalamic region of rabbits, testing for pyrogenicity. The test was positive and the rabbits showed a 1.5 to 3.0 C rise in temperature within four hours of injection. As the authors pointed out, it was impossible to tell whether the effect was due to some substance released by lysed erythrocytes or to a substance produced or stimulated by the parasites. However, the shape and timing of the febrile responses closely resembled those induced by Piromen who used a derivative of Pseudomonas LPS, and not those expected from endogenous pyrogen.[37]

In the late 1960's and early 1970's, Levin et al[30,31] published reports on the remarkable sensitivity of an extract of blood cells from the horseshoe crab, Limulus polyphemus, to the presence of lipopolysaccharide. The horseshoe crab has only one sort of blood cell, the amebocyte. A

protein extract of this cell will clot in the presence of as little as 0.5 ng. of chloroform-extracted LPS.

Since the threshold for detectable fever production by E. coli LPS was shown to be in the range of 1 ng., the Limulus lysate test marked a great step forward in the early identification of infection by gram-negative organisms.[29,30] The test was used by Glew and Levin in 1974 to test for endotoxin activity in plasma from volunteers infected with either P. vivax or P. falciparum.

The plasma samples used were taken from volunteer patients during periods of schizont rupture, i.e. rupture of erythrocytes infected by mature schizonts with release of merozoites. This event corresponds to the malarial paroxysm, which is the clinical response to schizont rupture in humans.[21] All samples obtained from volunteers infected with P. vivax or P. falciparum produced negative Limulus tests. The patients' temperatures had ranged as high as 40.7 C and the parasitemias as high as 7,000/mm^3. In 1980 Tubbs repeated the Limulus assay, using plasma samples from mice infected with P. berghei and from children hospitalized with falciparum malaria. He obtained positive results in both tests, but found that the strength of the positive reaction was not directly related to the degree of parasitemia.[46] He noted that the levels of endotoxin observed were lower than those normally associated with endotoxin-related disease in man. Tubbs also speculated that perhaps his test equipment was more sensitive than that of Glew and Levin. He also suggested that the endotoxin was derived from either the parasites or the endogenous bacteria in the gut.

Felton, Prior, Spagna and Kreier pointed out that, regardless of the results of plasma tests of infected individuals, the parasite should itself be studied with the Limulus assay.[46] They used an ultrasonic device to free P. berghei cells from their erythrocyte hosts in the method developed

earlier by Prior and Kreier.[40] The results were positive for endotoxic activity, using a system with minimum sensitivity for an E. coli standard of 0.06 ng/ml. of LPS. The level of activity corresponded with approximately 0.5 ng. equivalent of E. coli endotoxin per ml. of packed parasites. This was approximately three log units below the threshold level (1 ng/kg) required for fever induction. The authors concluded that some other factor besides the parasites' endotoxin activity must be producing the malarial fever, and that the endotoxin detected by others in plasma from malarious patients is probably derived from endogenous bacteria in the gut.

While it is extraordinarily sensitive to the presence of bacterial endotoxin, the Limulus assay is not uniquely specific for bacterial endotoxin. Elin and Wolff showed in 1973 that the test would give a positive result for such diverse compounds as thrombin, thromboplastin, ribonuclease and the polynucleotides, polyriboadenylic-polyribouridylic acid (poly A/poly U) and polyriboinosinic-polyribocytidylic acid (poly I/poly C).[19] Guarding against the possibility that these positive results were due to the contamination of samples by bacteria, Elin and Wolff showed the test compounds had very different biochemical and physiological properties than the endotoxin samples.

For example, thrombin (but not thromboplastin) showed a 100-fold drop in Limulus amebocyte lysate (LAL) activity upon extraction with chloroform and usual work-up for LAL test samples[31] while the bacterial standards showed no such drop. Ribonuclease showed a positive LAL reaction before extraction but not after (at a concentration of 64 Kunitz units/ml). The polynucleotides showed drops in LAL activity after extraction. In addition, the ratio of the minimum unextracted concentration of a compound needed to produce a positive LAL test to the amount needed to produce a 0.5 C fever

in rabbits was characteristic for the bacterial reference standards, and differed from the ratio of LAL activity/pyrogenic dose shown by the other samples. In view of the ability of such diverse substances to produce positive LAL results, the authors warn that it is essential to take the nonspecificity of the reaction into account when interpreting positive LAL tests of unknown solutions.

In 1978 Clark offered the hypothesis that perhaps plasmodial parasites did in fact produce LPS at a very low level, below the levels of Limulus detection and that, in a very susceptible host, this may be enough to produce endotoxin shock.(9) He theorized that non-antibody mediators, released during endotoxin shock, would also be released during malarial attacks and that this would explain a number of puzzling findings. These included the fact that exposure to BCG or Corynebacterium parvum offers some protection against malaria, and the fact that, during malarial infection, susceptibility is increased to bacterial LPS. Using tests with mice he found that after injection with any of four protozoa (P. vinckei petteri, P. berghei, B. microti and B. rodhaini) the effect of LPS was greatly enhanced. All of the mice which became ill or died appeared to be affected in the same way as normal mice given a large dose of endotoxin, though often more severely. The changes were pathognomonic for endotoxin shock in mice. He wrote that, if there is a corresponding increase in sensitivity of LPS in humans infected with malaria, there would be pyrexia at plasma concentrations several hundred times lower than that detected by the Limulus test.(9) The increased permeability of the vascular endothelium previously noted could be both cause and effect of a heightened endotoxin response.

Another possible route of pyrogenicity, for the most part unexplored, is the non-specific stimulation of B cells noted in the crisis of malarial infection. As previously noted, antigen-antibody (Ag-Ab) complexes have

pyrogenic activity.[22] The mitogenic activity of malaria, however, is not unique; indeed, it has been found to be a property of LPS as well,[13] leading us back to the preceding discussion.

MATERIALS AND METHODS

Limulus Amebocyte Lysate Assay (LALA)

The Limulus amebocyte assay was run according to the methods of Levin et al[30] in pyrogen-free glassware baked at 180 C overnight. One-tenth milliliter of Limulus lysate was thoroughly mixed with 0.1 ml. of sample dilution to be assayed and placed in a waterbath at 37 C for one hour. Samples were examined after one hour and monitored for the next 24 hours. All samples were run in duplicate. The criterion for a positive test was the presence of solid gelation. The bacterial endotoxin standard was cell wall material from the organism Salmonella typhimurium, obtained from Difco Laboratories, Detroit, Michigan.

The RPMI 1640 powder (Grand Island) media was prepared according to label instructions, with 10.4 mg. per liter of solution to which was added 5.94 g N-2Hydroxy-ethylpiperozine-N^{y}-2-ethanesulfonic acid (HEPES). The RPMI 1640 media was tested for endotoxin activity before and after adding 5% sodium bicarbonate and 0.1 ml. gentamycin (GM). The media was also tested after adding 5% pooled serum obtained from the American Red Cross, Lansing, Michigan.

Since blood usually gives false-positive reactions in the LAL assay the blood and sera used to grow the malarial parasites were tested before and after heat treatment at 100 C for 10 minutes. All components of the parasite culture medium, i.e., distilled water, sodium bicarbonate, culture

medium stock solutions, were tested for endotoxin in the LAL assay before and after medium preparation.

Plasmodium falciparum, FCR_3TC strain were grown using the candle-jar method according to Jensen and Trager.[26] The parasite cultures were synchronized by washing them with 5% aqueous sorbitol which lyses all but the youngest parasite stages. The synchronized parasites were returned to cultures until they matured to segmenting schizonts (13-21% parasitemia). They were then concentrated using the gelatin-flotation technique described by Jensen.[25] Trophozoites were collected using the same procedures. The parasites obtained from the gelatin suspensions were diluted with equal amounts of RPMI 1640 medium (without serum) and stored frozen at -70 C until used in the LAL assay or rabbit fever experiments.

Parasite Extracts in LAL Assay

Experiment 1

Extracts obtained from schizont-infected red cells were tested using the LAL assay. In the first experiment an extract was prepared by repeated freezing and thawing of the schizont-infected red cells obtained by the gelatin concentration method. The parasite extract was serially diluted from 1:10 through 1:640, mixed with the Limulus amebocytes and incubated under the appropriate test conditions. Lipopolysaccharide from S. typhimurium (LPS) was tested at 10-fold dilutions from 12.5 mg/ml down 0.00125 mg/ml.

Experiment 2

This experiment was conducted to determine whether the substance in the parasite extract responsible for the positive LAL reaction was susceptible to protease digestion. Accordingly, a second portion of the schizont extract was treated with pancreatic protease (Type 1, Sigma Chemical Co., St. Louis, Missouri)

Seven milliliters of extract were mixed with phosphate buffer (pH 7.5) and to this was added 10 mg. of pancreatic protease followed by thorough mixing. The mixture was incubated at 37 C in a water bath for 60 minutes. After incubation the mixture was diluted as in Experiment 1 and tested for activity in the LAL assay. Salmonella typhimurium LPS was used as an endotoxin standard, as before.

Experiment 3

Experiment 3 was conducted to determine whether the parasite residual debris, rich in the malarial pigment, hemozoin, was a source of LAL activity and malarial pyrogen. The hemozoin-rich debris was collected from the culture medium aspirated from parasite cultures where the parasitemia was near 20%. This material was washed several times in pyrogen-free water and tested in the LAL assay for properties. All tests were run in duplicate.

Fever-Testing of Parasite Extracts in Rabbits

Since rabbits are routinely used in pyrogen testing. New Zealand white rabbits, weighing approximately 3 kg. were injected intravenously in the lateral ear vein with varying concentrations of parasite extract to determine their fever-producing properties. The general procedure was to take a rectal temperature reading every 30 minutes using a digital thermometer (YSI Model 49 TA, Yellow Springs Inst. Co., Three or more temperature readings were taken on each rabbit until the rabbits became accustomed to being handled and their body temperatures stabilized. In each case, the rabbits were injected with a quantity of human red cells suspended in the same culture medium used to grow the parasite cultures. Several temperature readings were taken at 30 minute intervals until the temperature stabilized. These procedures were then

followed by injection of the parasite extract and again the body temperature monitored until stable.

Experiment 1a

Different amounts of parasite extracts were injected until it was determined that 1.6 ml. consistently produced a fever in each rabbit tested.

Experiment 2a

In order to determine whether the pyrogenic substances in the parasite extract were soluble, 1.6 ml. of the parasite extract was diluted 1:5 in RPMI 1640 medium (to reduce the hemoglobin concentration and to allow the sedimentation of the subcellular components) and centrifuged at 100,000 X G for 90 minutes. This produced a post-ribosomal supernatant solution containing the soluble components of the extract and a pellet of the non-soluble subcellular particles and membranes. The supernatant fluid (8 ml) was injected ultravenously into one rabbit and the resuspended pellet into another. The changes in body temperatures of the rabbits were then monitored. A few days later this experiemnt was repeated on the same rabbits, reversing the order of injection.

RESULTS

As can be seen in Table 1, all components of the culture system, except the parasite extract, were negative for the presence of endotoxin. The LAL assay detected endotoxin activity in the S. typhimurium LPS suspension down to a concentration of 0.125 ng/ml. Extracts from schizont-infected erythrocytes showed a positive LAL reaction down to a 1:320 dilution, which was equivalent to 50-100 ng/ml of S. typhimurium LPS (Table 2).

When the parasite extract was treated with pancreatic protease, higher concentrations of the extract were negative until dilutions of 1:80 and 1:60 were reached. These levels showed a positive "endotoxin-like" activity in the LAL assay.(Table 2) The pancreatic protease itself was negative for "endotoxin-like" activity, as were protease-treated red cells. (Table 2)

Hemozoin-rich preparations, obtained from the exhausted culture medium from approximately 20% parasitemia cultures, showed a very low level of "endotoxin-like" activity at 1:10 dilution, but this was not significantly unlike the reaction seen with the red cell control and was not at all similar to the reaction seen with the schizont-extract. (Table 3)

Parasite extracts prepared from trophozoite-infected red cells gave positive LAL results down to dilutions of 1:100. Although this is a lower concentration of "endotoxin-like" activity than was seen for the schizont extract, it nonetheless corresponds to a LPS concentration of 31.25-62.5 ng/ml. (Table 3)

In the pyrogen testing, uninfected, washed red blood cells produced no rise in temperature in the rabbits. The injection of 0.8 ml. of

Table 1. LIMULUS AMEBOCYTE LYSATE TEST. Limulus Lysate Sensitivity 0.134 ng/ml, Lot #L02881, Expires 8/13/82, M. A. Bioproducts

		Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Plain Media	1 hr.	- -	- -	- -						
	24 hr.	- -	- -	- -						
Wash Media NaH_2CO_3+GM RPMI	1 hr.	3+ 4+	- -	- -						
	24 hr.	3+ 4+	- -	- -						
Media with NaH_2CO_3+GM 5% Serum	1 hr.	4+ 4+	+ +	- -						
	24 hr.	4+ 4+	± ±	- -						
Blood-Not △Treated	1 hr.		-	-	-	-	-	-	-	
	24 hr.		-	-	-	-	-	-	-	
Serum △ Treated	1 hr.	4+ 4+	- -	- -	- -					
	24 hr.	4+ 4+	- -	- -	- -					
Blood △ Treated	1 hr.	- -	- -	- -						
	24 hr.	- -	- -	- -						
- Control Pyrogen Free Water	1 hr.	- -	- -	- -						
	24 hr.	- -	- -	- -						
LPS	1 hr.	4+ 4+	4+	4+	4+	4+	4+	-	-	
	24 hr.	4+ 4+	4+	4+	4+	4+	4+	-	-	
LPS Conc./ml		12.5µg	1.25µg	125ng	12.5ng	1.25ng	0.125 ng	0.0125 ng	0.00125 ng	

Table 2. LIMULUS AMEBOCYTE LYSATE TEST

Sample Dilution	1/10	1/20	1/40	1/80	1/160	1/320	1/640
Sample #1 Schizonts	4+ *5ng/ml	4+ 2.5	4+ 1.25	4+ .625	4+ .3125	$\frac{+}{+}$	-
Sample #2 Schizonts	-	-	-	3+	3+		
Protease Treated	-	-	-	3+	4+		
Sample #3 Pancreatic Protease	-	-	-	-			
RBC	-	-	-	-			
LPS Concentration ng/ml	20.0	10.0	5.0	2.5	1.25	0.625	0.3125
LPS Results	4+	4+	4+	4+	4+	4+	2+
	4+	4+	4+	4+	4+	4+	2+
Controls	-	-					
Water	-	-					

*Amount of endotoxin equivalent to given reaction

Note: Tests were done in duplicate

Table 3. LIMULUS AMEBOCYTE LYSATE TEST. Limulus Lysate Sensitivity 0.134 ng/ml, Lot #L02881, Expires 8/13/82, M.A. Bioproducts

	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Hemozoin #1	4+ 4+ 4+ 4+	$\frac{+}{-}$ $\frac{+}{-}$ $\frac{+}{-}$ $\frac{+}{-}$	- - - -	- - - -	- - - -	- - - -
Hemozoin #2	4+ 4+ 4+ 4+	$\frac{+}{-}$ $\frac{+}{-}$ $\frac{+}{-}$ $\frac{+}{-}$	- - - -	- - - -	- - - -	- - - -
RBC's with Trophozoits	4+ 4+	4+ 4+	4+ 4+	- -	- -	- -
RBC's only	4+ 4+	- -	- -			
RPMI 1640 Wash	$\frac{+}{-}$ $\frac{+}{-}$	- -	- -			
RPMT 1640 Media and 5% Serum	$\frac{+}{-}$ $\frac{+}{-}$	- -	- -			

Note: Tests were done in duplicate

pooled schizonts produced a peak fever of approximately 1.5 C in one rabbit, with that peak occurring 90 minutes after injection. In the second rabbit, the peak was approximately 1 C, occurring 150 minutes after injection.

When the amount of pooled schizonts used was doubled, to 1.6 ml, the first rabbit showed approximately a 2.5 C temperature increase, with a peak at 360 minutes after injection. The second rabbit showed roughly a 2.25 C temperature increase, with the peak 180 minutes after injection. In each case, the baseline for fever was taken as the temperature mean established after injection of washed, uninfected red blood cells. (Figures 2, 3)

The rabbits began to hyperventilate and shiver when their temperatures reached an average of 39.25 C after injection with 0.8 ml. of the schizonts, and they began to hyperventilate at 40.40 and 40.06 C respectively, when injected with 1.6 ml.

In experiments to determine whether the fever-producing substance(s) were soluble, a post-ribosomal supernatant obtained by ultracentrifugation of the parasite extract produced a significant temperature rise when injected into the latural ear vein of a rabbit.(Figures 2, 3) When 8 ml. of the supernatant, representing the soluble portion of 1.6 ml. of parasite extract, was injected into rabbit #1, it produced a 3 C temperature increase, with a peak three hours after injection of the soluble fraction. In rabbit #2, the insoluble fraction produced a 2 C temperature increase that peaked at three hours after injection. Four days later the injections were reversed with rabbit #1 receiving the insoluble pellet and rabbit #2 receiving the post-ribosomal supernatant. In this experiment the sedimental preparation produced a 3.6 C temperature rise that peaked 3 hours post-injection and the soluble preparation a 1.5 C rise with a peak 2 hours post-injection. (Figures 2, 3)

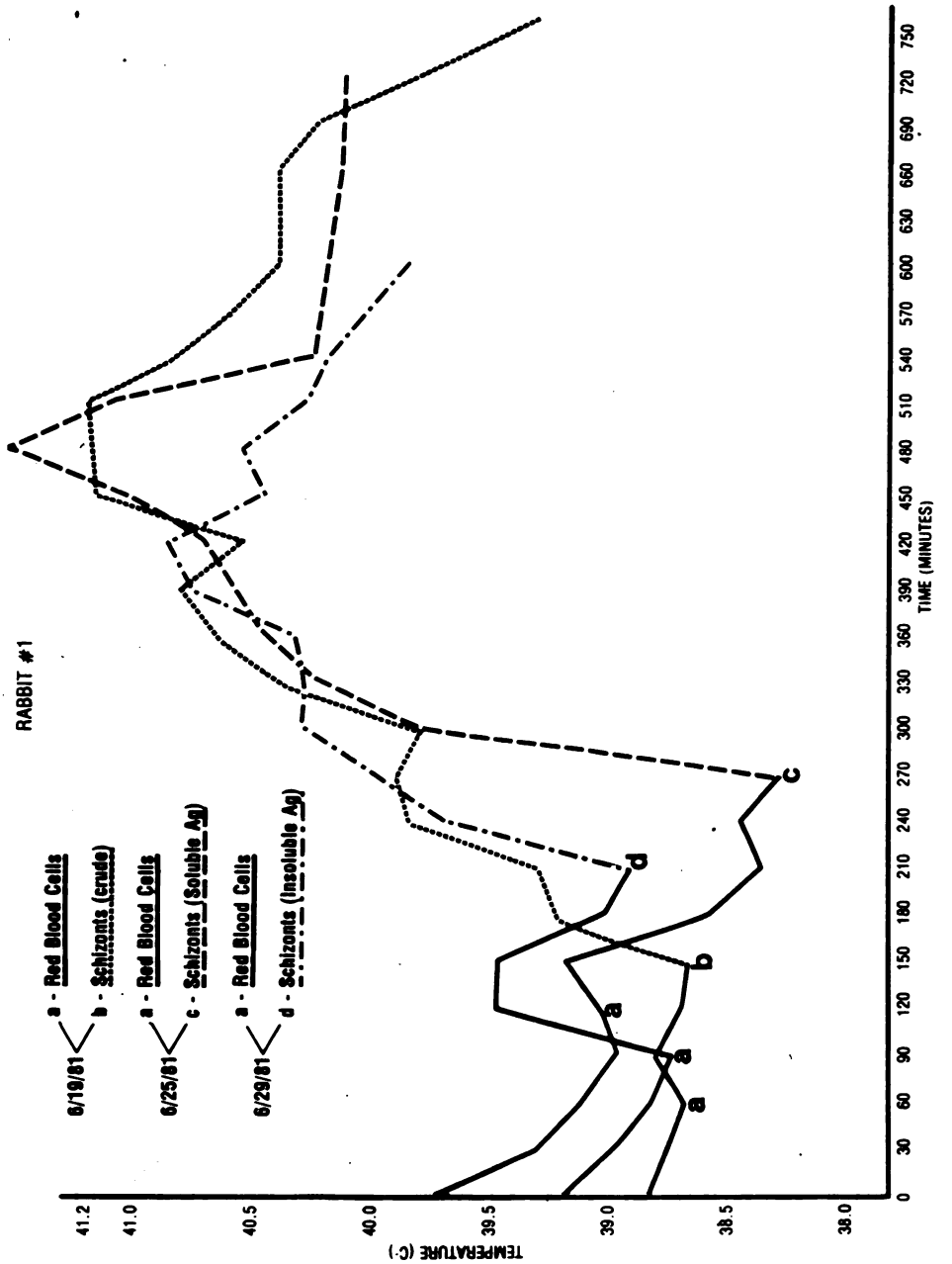

Figure 1. Temperature Changes Before and After P. falciparum Extract Injections - Rabbit #1

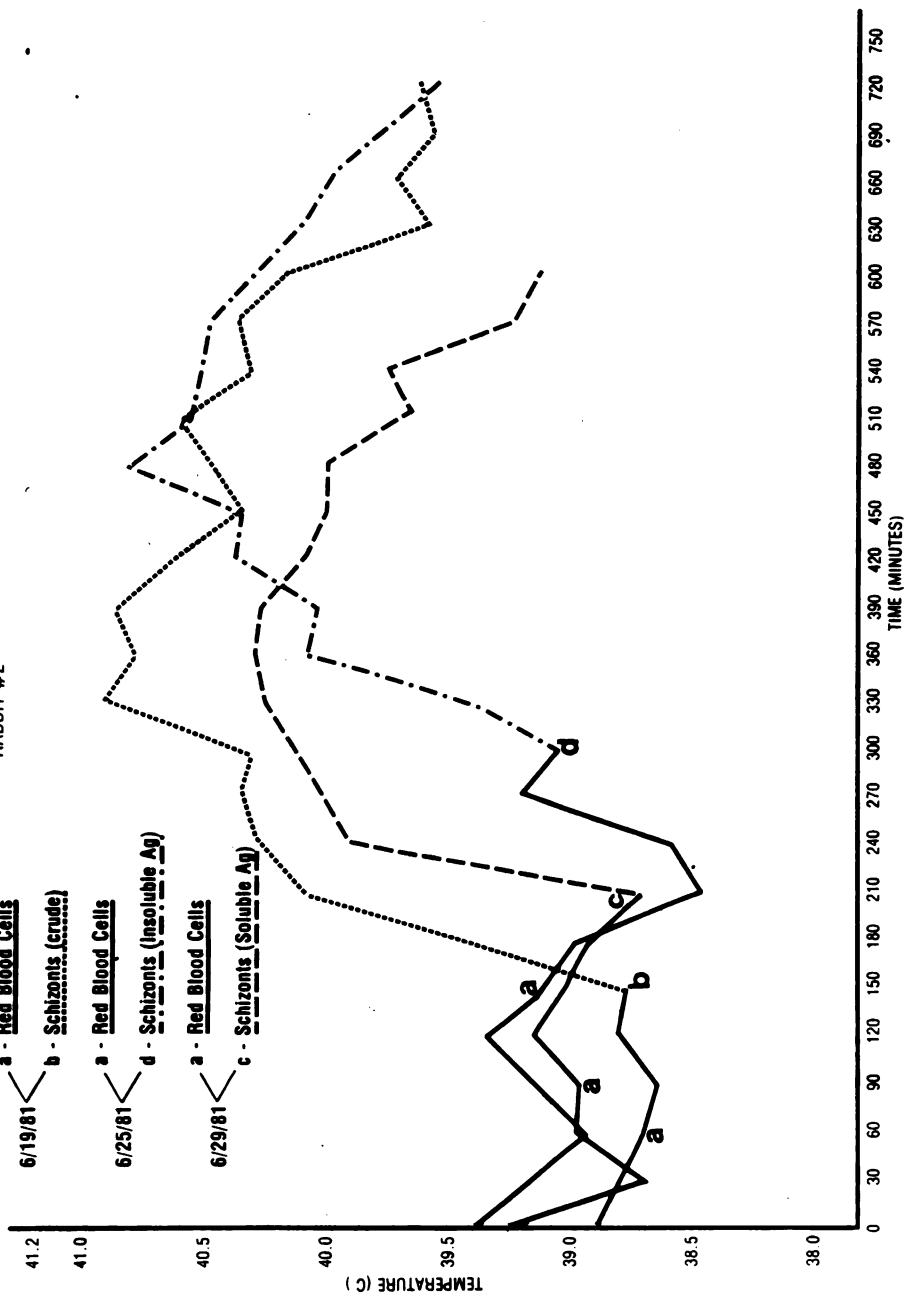

Figure 2. Temperature Changes Before and After P. falciparum Extract Injections - Rabbit #2

DISCUSSION

The level of "endotoxin-type" activity shown in the LAL assay by falciparum schizonts is sufficient, of itself, to produce measurable fevers if the mechanism of fever production is identical to that of endotoxin and if the falciparum endotoxin-activity is operating by itself without the sort of synergism suggested by Clark.[12]

The "endotoxin-like" activity of the parasite extract is apparently susceptible to protease digestion. The curious absence after proteolysis of LAL activity until the extract was diluted 1:80 was probably due to a high concentration of assay-destroying proteases which interfered with the assay until sufficiently diluted. This is supported by the observation that most LAL positive substances are lipopolysaccharides and are chloroform extractable or protease resistant. Thus it appears that the source of the "endotoxin-like" activity is not likely to be protein. Using the same reasoning, the scant LPS-like activity shown by the hemozoin-rich parasite residue also makes it unlikely to be the cause of malarial fevers.

In the rabbit-fever assay, the time-course and duration of the fevers make it appear likely that the route of fever production includes the elaboration of endogenous pyrogen and is not direct. The febrile response of the rabbits was considerably heightened and sustained as the amount of schizont-extract injected was increased from 0.8 ml. to 1.6 ml. This amount of parasite material was equivalent to a parasitemia of 0.36% in the rabbits injected. This figure was derived by approximating the total number of parasites injected (determined from the parasitemia from which the extract

was made) and the total blood volume calculated from the body weight of the rabbit (3.20 ml/kg), Biology Data Book, page 263.

The fever-producing substance(s) were found to be soluble, since the post-ribosomal supernatant produced significant pyrexia when injected into rabbits. Although the volume was large (8 ml.) we purposely did not attempt to reconcentrate it since in so doing we might have lost the low-molecular weight components.

In conclusion, extracts from schizonts of P. falciparium cultured in vitro contain "endotoxin-like" substances which produce a positive reaction in a LAL assay. The concentration of this material was equivalent to 50-100 ng/ml of bacterial endotoxin which is far in excess of the amount required to produce fevers in man. The injection of the parasite extract into rabbits produced a significant rise in body temperature accompanied by hyperventilation and shivering, similar to the common malarial chills and fever.

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