Phagocytosis of *Borrelia recurrentis* by Blood Polymorphonuclear Leukocytes Is Enhanced by Antibiotic Treatment

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The removal of *Borrelia* spirochetes from the blood in relapsing fever was studied by examining patients' blood phagocytic cells with the Dieterle silver stain. Polymorphonuclear leukocytes ingested *Borrelia* at increased rates for several hours after antibiotic treatment, during which time the total numbers of circulating plasma spirochetes were decreasing. Incubation of infected blood at 37°C for 2 h resulted in a progressive increase in phagocytosis. Addition of penicillin G and tetracycline to infected blood caused a further enhancement of phagocytosis. Electron microscopy of polymorphonuclear leukocytes revealed spirochetes in phagosomes. These results indicated that blood polymorphonuclear leukocytes have a prominent role in removing *Borrelia* from the plasma and suggested that antibiotics act by altering the surface of spirochetes to render them more susceptible to phagocytosis.

Louse-borne relapsing fever is an epidemic febrile and sometimes fatal illness caused by the spirochete *Borrelia recurrentis*. The spirochetes circulate in plasma in high densities of about 10,000 to 100,000/mm³, allowing the diagnosis of this disease by microscopic examination of peripheral blood smears stained with aniline dyes. Antibiotic treatment is effective in clearing the blood of spirochetes within several hours. It also provokes a Jarisch-Herxheimer-like reaction characterized by a rigor, a rise in temperature, and a drop in blood pressure (4). The fate of spirochetes after antibiotic treatment is unknown. Phagocytosis of organisms by blood polymorphonuclear leukocytes was suggested by Schofield et al. (16) because of the leukopenia and cell vacuolation observed in patients after therapy, but blood smears stained with aniline dyes have not consistently revealed intracellular spirochetes. The observation by Faine et al. (8) that phagocytosed leptospire were demonstrable intracellularly with a silver stain led us to apply the Dieterle silver stain (6) to blood smears of patients with relapsing fever. Organisms were frequently seen in polymorphonuclear leukocytes, and the numbers of cells containing the organisms increased after antibiotic treatment.

MATERIALS AND METHODS

Patients. Eleven Ethiopian adult males with fever and blood smears showing spirochetes in the plasma

with Wright stain were selected. None had received antibiotics before the study. Single-dose antibiotic regimens were administered: erythromycin stearate, 500 mg orally to three patients; tetracycline hydrochloride, 500 mg orally to one and 250 mg intravenously to three patients; and procaine penicillin G, 600,000 U intramuscularly to four patients.

Blood smears. Repeated blood smears were obtained hourly until negative for spirochetes. Blood smears, which had been taken before treatment, during treatment nearest to the time of onset of rigors, and at the time that plasma spirochetes stainable with Wright's stain had been cleared, were selected for restaining by Dieterle's method (6). The rate of phagocytosis was measured by recording the number of polymorphonuclear leukocytes containing spirochetes per 100 cells counted.

In vitro phagocytosis. Blood from two patients with blood smears positive for spirochetes was drawn into heparin (final concentration, 75 U/ml) and cooled to 4°C. Several hours later, the blood was pipetted into sterile plastic tubes containing potassium penicillin G (final concentration, 100 U/ml) or tetracycline hydrochloride (final concentration, 100 µg/ml) or no antibiotic. The tubes were incubated at 37°C and inverted every 15 min. Specimens were removed at intervals of 15, 30, 60, and 120 min after the start of incubation for counting spirochetes.

Electron microscopy. After 120 min, incubation was stopped and a leukocyte-rich fraction was separated by centrifuging the blood at 100 × g for 5 min and then centrifuging the supernatant at 500 × g for 15 min. Glutaraldehyde (2%) in 0.05 M phosphate buffer at pH 7.4 was added to the pellet. After 48 h, the glutaraldehyde was poured off and replaced by phosphate buffer. The specimens were stored at 4°C until prepared for electron microscopy.

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RESULTS

The Dieterle stain of blood smears gave good definition of both extracellular spirochetes and spirochetes that were within polymorphonuclear leukocytes (Fig. 1). The organisms appeared intracellular by their planes of focus and were occasionally coiled or compacted within cytoplasmic vacuoles. Leukocytes containing spirochetes were seen more often in smears obtained after than before antibiotic treatment (Table 1). At a time when blood smears stained with Wright stain were read as negative for plasma spirochetes, approximately one-quarter of polymorphonuclear leukocytes contained spirochetes, although these organisms had not been discernible within cells stained with Wright stain.

To determine whether leukocytes phagocytose plasma spirochetes in vitro, we incubated heparinized infected blood at 37°C. We observed a progressive increase in the rates of phagocytosis during 2 h of incubation, and this increase was accelerated by the addition of penicillin G

FIG. 1. Blood smear of a 25-year-old male patient who had been treated with erythromycin stearate 500 mg orally 6 h previously. The smear, stained with Dieterle silver stain, reveals extracellular spirochetes and spirochetes within a polymorphonuclear leukocyte, 42% of which contained stainable spirochetal forms. A smear obtained 1 h later was negative for extracellular spirochetes. Bar equals 5.0 μ m.

TABLE 1. Rates of phagocytosis of *B. recurrentis* by blood polymorphonuclear leukocytes in 11 infected patients as measured by the proportion of polymorphonuclear leukocytes in blood smears containing silver-stained spirochetes

Time of examination	Mean h after antibiotic treatment	No. of polymorphonuclear leukocytes (per 100 counted) containing spirochetes	
		Range	Mean
Before antibiotic treatment ^a	0	0-22	5.3
Jarisch-Herxheimer-like reaction ^b	2.1	8-52	22.7 ^c
Clearance of extracellular spirochetes in Wright-stained smears	5.8	0-55	24.6 ^c

^a Antibiotic treatment was erythromycin stearate, 500 mg orally in three patients; tetracycline hydrochloride, 250 mg intravenously in three patients; tetracycline hydrochloride, 500 mg orally in one patient; and procaine penicillin G, 600,000 units intramuscularly in four patients.

^b A Jarisch-Herxheimer-like reaction, heralded by rigor and temperature rise, occurred in nine patients.

^c Mean values were significantly greater than the mean number of polymorphonuclear leukocytes containing spirochetes before antibiotic treatment by Student's *t* test ($P < 0.05$).

and tetracycline (Table 2). After 60 and 120 min of incubation in patient 1 and after 30 and 60 min in patient 2, the rates of phagocytosis were increased significantly by the addition of antibiotic ($P < 0.05$). Blood incubated with antibiotics at 4°C showed no increase in phagocytosis. Spirochetes that were not phagocytosed exhibited no signs of lysis or fragmentation in the presence of the antibiotics, and examination of these organisms with phase-contrast microscopy showed an active pattern of normal rotational motility.

To ascertain the intracellular locations of spirochetes within polymorphonuclear leukocytes, we examined leukocyte-rich fractions of blood by electron microscopy. When blood was examined both from patients after therapy and after incubation of blood with antibiotics, intact spirochetes had been phagocytosed and were located within membrane-bound phagosomes (Fig. 2). Digestion of spirochetes within the vacuoles was observed after 2 h of incubation in the presence of antibiotic.

DISCUSSION

These studies showed that the rapid removal of large numbers of plasma spirochetes from the blood of patients with relapsing fever is accom-

TABLE 2. Effect of *in vitro* incubation of heparinized whole blood from two patients for 2 h at 37°C and the addition of antibiotics^a to the blood on the rate of phagocytosis of *B. recurrentis* by polymorphonuclear leukocytes

Patient	Time after start of incubation (min)	No. of polymorphonuclear leukocytes containing silver-stained spirochetes ^b		
		No antibiotic	Penicillin G	Tetracycline
1 ^c	0	5.3 ± 0.7		
	15	3.7 ± 0.9	5.3 ± 0.9	4.3 ± 1.5
	30	7.0 ± 3.0	13.0 ± 5.3	11.3 ± 2.3
	60	11.3 ± 0.9	19.0 ± 1.5 ^d	22.7 ± 2.3 ^d
	120	12.7 ± 2.9	31.7 ± 0.7 ^d	26.7 ± 4.1 ^d
2 ^c	0	8.7 ± 0.7		
	15	13.0 ± 4.4	20.7 ± 4.7	22.7 ± 3.9
	30	31.0 ± 4.9	35.3 ± 2.9	46.7 ± 3.9 ^d
	60	27.3 ± 1.7	32.7 ± 3.3	40.0 ± 2.3 ^d
	120	34.0 ± 4.9	43.7 ± 3.2	39.0 ± 1.2

^a Antibiotics were added in 0.9% NaCl in a volume 1/10 that of the blood to give final concentrations of potassium penicillin G of 100 U/ml and tetracycline hydrochloride of 100 µg/ml.

^b Values are the means and standard errors of three independent countings of 100 polymorphonuclear leukocytes.

^c The blood of patient 1 contained approximately 1 spirochete per polymorphonuclear leukocyte and the blood of patient 2 contained approximately 13 spirochetes per polymorphonuclear leukocyte.

^d Values that were significantly greater by Student's *t* test (*P* < 0.05) in the antibiotic-treated specimens than in the specimens without antibiotic examined at the same time.

panied by an increased rate of phagocytosis of the spirochetes by circulating polymorphonuclear leukocytes. Fixed phagocytic cells of the reticuloendothelial system in the liver, spleen, and bone marrow may also participate in the removal of spirochetes, but this was not studied in these patients. The site of this accelerated phagocytosis is presumably intravascular and likely resembles the intravascular surface phagocytosis of other bacteria by granulocytes described by Wood et al. (17). This mechanism of phagocytosis did not require specific antibody and consisted of adherence of granulocytes to vascular endothelium with entrapment of bacteria between granulocytes and in fibrin meshworks. In the blood of patients with acute relapsing fever that was examined in our study, there was also probably no specific antibody present because any antibody being produced at this stage of the illness would have been removed by the excess of antigen. Further studies are required to determine the roles of antibody and complement in the opsonic requirements of borreliae for phagocytosis.

Our observation of accelerated intravascular phagocytosis of organisms in infected patients after antimicrobial treatment has not been described before in any bacterial infection. The

unique feature of relapsing fever that permitted this observation is the high density of circulating extracellular organisms which is rapidly reduced during antimicrobial treatment. A similar enhancement of phagocytosis of bacteria by antibiotic treatment probably occurs in many other bacterial infections because this action of antibiotics has been observed during *in vitro* studies with *Staphylococcus aureus* (10), *Listeria monocytogenes* (1), and *Pseudomonas aeruginosa* (14). Synergistic killing of bacteria by leukocytes and antibiotics has been documented (2, 15), and studies with subinhibitory concentrations of antibiotics have suggested that antibiotics can exert effects on bacteria to enhance their killing by leukocytes at concentrations of antibiotic considerably below those required to kill bacteria *in vitro* (10, 12).

The mechanisms by which antibiotics render bacteria more susceptible to killing by leukocytes have been recently elucidated by *in vitro* studies of Root and others (R. E. Isturiz, V. T. Andriole, and R. K. Root, Clin. Res. 27:347A, 1979; A. Molavi, J. Metcalf, and R. Root, Abstr. 18th Intersci. Conf. Antimicrob. Agents Chemother. 1978, Abstr. no. 203; and R. K. Root and J. A. Metcalf, Abstr. 18th Intersci. Conf. Antimicrob. Agents Chemother. 1978, Abstr. no. 204). Only antibiotics active against cell wall synthesis produced enhanced killing, which required attachment of bacteria to leukocytes. Increased leukocytic killing of group B streptococci by penicillin has been related to a loss of antiphagocytic capsular material from bacteria (D. Horne and A. Tomasz, Abstr. 11th Int. Congr. Chemother. and 19th Intersci. Conf. Antimicrob. Agents Chemother. 1979, Abstr. no. 314). These studies suggested that an alteration in the surface of bacteria renders them more susceptible to interactions with the leukocytic membrane that lead to killing or phagocytosis or both.

The concentrations of penicillin and tetracycline used in this study did not impair motility of the spirochetes during the 2-h period of observation. We did not assess viability of the borreliae after exposure to antibiotic and, thus, do not know whether they were viable at the time of phagocytosis. Eagle and Musselman showed that penicillin renders treponemes nonviable at concentrations that did not impair motility (7). Our studies, which included the ribosomally active antibiotic tetracycline and which showed no impaired motility during the observation period of increased phagocytosis, suggest a novel action of tetracycline on borreliae, other than alteration of cell wall or motility, to render the organisms more susceptible to phagocytosis.

Another possible mechanism of increased phagocytosis is a nonspecific action of antibiotics

FIG. 2. (a) Polymorphonuclear leukocytes of a patient with relapsing fever 2 h after incubation of whole blood with penicillin G, 100 U/ml. A phagosome (arrow) contains borreliae in cross-section. Bar equals 1.0 μ m. (b) Inset shows higher magnification of the spirochete-containing phagosome. Bar equals 0.5 μ m.

on the phagocytes. Although this was not evaluated in this study, it is an unlikely mechanism because tetracyclines have been actually demonstrated to have an inhibitory effect on both

phagocytosis and chemotaxis of polymorphonuclear leukocytes (9, 13).

The enhancement of phagocytosis after antibiotic treatment in relapsing fever may aid in

explaining the Jarisch-Herxheimer-like reaction. The onsets of the Jarisch-Herxheimer-like reaction and the increased rate of phagocytosis of spirochetes both occur within 1 to 4 h after institution of antibiotic treatment. Polymorphonuclear leukocytes are known to release endogenous pyrogen after phagocytosis of other bacteria (3), and leukocytic pyrogen may be one of the mediators of the rigor and temperature rise in the Jarisch-Herxheimer reaction (16). Although borreliae do not possess bacterial endotoxin, the spirochetes contain a nonendotoxin particulate pyrogen, which might exert its effects by stimulating production of leukocytic pyrogen after phagocytosis (5). Release of other biologically active molecules by phagocytic cells might also participate in the mediation of the intravascular coagulation and hypotension (11) that accompany the Jarisch-Herxheimer-like reaction in patients with relapsing fever.

LITERATURE CITED

1. Adam, D., W. Schaffert, and W. Marget. 1974. Enhanced in vitro phagocytosis of *Listeria monocytogenes* by human monocytes in the presence of ampicillin, tetracycline, and chloramphenicol. *Infect. Immun.* 9: 811-814.
2. Alexander, J. W., and R. A. Good. 1968. Effect of antibiotics on the bactericidal activity of human leukocytes. *J. Lab. Clin. Med.* 71:971-983.
3. Berlin, R. D., and W. B. Wood. 1964. Studies on the pathogenesis of fever. XII. The effect of phagocytosis on the release of endogenous pyrogen by polymorphonuclear leukocytes. *J. Exp. Med.* 119:715-726.
4. Bryceson, A. D. M., E. H. O. Parry, P. L. Perine, D. A. Warrell, D. Vukotich, and C. S. Leithead. 1970. A clinical and laboratory study of 62 cases in Ethiopia and a reconsideration of the literature. *Quart. J. Med.* 39: 129-170.
5. Butler, T., P. Hazen, C. K. Wallace, and S. Awoke. 1979. Infection with *Borrelia recurrentis*: pathogenesis of fever and petechiae. *J. Infect. Dis.* 140:665-675.
6. Dieterle, R. R. 1927. Method for the demonstration of *Spirochaeta pallida* in single microscopic sections. *Arch. Neurol. Psychol.* 18:73-80.
7. Eagle, H., and A. D. Musselman. 1944. The spirocheticidal action of penicillin in vitro and its temperature coefficient. *J. Exp. Med.* 80:493-505.
8. Faine, S., A. Shahar, and M. Aronson. 1964. Phagocytosis and its significance in leptospiral infection. *Aust. J. Exp. Biol. Med. Sci.* 42:579-588.
9. Forsgren, A., D. Schmeling, and P. G. Quie. 1974. Effect of tetracycline on the phagocytic function of human leukocytes. *J. Infect. Dis.* 130:412-415.
10. Friedman, H., and G. H. Warren. 1974. Enhanced susceptibility of penicillin-resistant staphylococci to phagocytosis after in vitro incubation with low doses of nafcillin. *Proc. Soc. Exp. Biol. Med.* 146:707-711.
11. Galloway, R. E., J. Levin, T. Butler, G. B. Naff, G. H. Goldsmith, H. Saito, S. Awoke, and C. K. Wallace. 1977. Activation of protein mediators of inflammation and evidence for endotoxemia in *Borrelia recurrentis* infection. *Am. J. Med.* 63:933-938.
12. Lorian, V. 1975. Some effects of subinhibitory concentrations of antibiotics on bacteria. *Bull. N.Y. Acad. Sci.* 51:1046-1055.
13. Majeski, J. A., and J. W. Alexander. 1977. Evaluation of tetracycline in the neutrophil chemotactic response. *J. Lab. Clin. Med.* 90:259-265.
14. Nishida, M., Y. Mine, S. Nonoyama, and Y. Yokota. 1976. Effect of antibiotics on the phagocytosis and killing of *Pseudomonas aeruginosa* by rabbit polymorphonuclear leukocytes. *Chemotherapy* 22:203-210.
15. Pruul, H., and P. J. McDonald. 1979. Enhancement of leukocyte activity against *Escherichia coli* after brief exposure to chloramphenicol. *Antimicrob. Agents Chemother.* 16:695-700.
16. Schofield, T. P. C., J. M. Talbot, A. D. M. Bryceson, and E. H. O. Parry. 1968. Leucopenia and fever in the "Jarisch-Herxheimer" reaction of louse-borne relapsing fever. *Lancet* i:58-62.
17. Wood, W. B., M. R. Smith, W. D. Perry, and J. W. Berry. 1951. Studies on the cellular immunology of acute bacteremia. I. Intravascular leukocytic reaction and surface phagocytosis. *J. Exp. Med.* 94:521-533.