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Granulocyte Function in Experimental Human Endotoxemia

By Mary C. Territo and David W. Golde

The effects of endotoxin administration on in vitro granulocyte function were studied in normal man. Four healthy volunteers received an intravenous injection of *Pseudomonas* endotoxin, 0.1 μg/kg. Endotoxemia resulted in transient neutropenia followed by a rebound neutrophilia. The nadir of the granulocyte count occurred at about 1 hr and maximal neutrophilia 2–4 hr after endotoxin administration. Throughout this time period, neutrophil phagocytosis and killing of *Candida albicans* were normal, as were resting and postphagocytic glucose metabolism and leukocyte random migration. However, postendotoxin neutrophils demonstrated a markedly decreased chemotactic response in Boyden chambers. The defect was maximal 1 hr after endotoxin administration and persisted 3–4 hr. These observations suggest that, in addition to neutropenia, endotoxin can transiently cause a chemotactic defect or select for a population of circulating neutrophils with an impairment of chemotactic activity.

THE LIPOPOLYSACCHARIDE (LPS) component of endotoxin is responsible for some of the manifestations of gram-negative bacteremia. Although LPS has potent effects on many biologic systems, its interaction with granulocytes is perhaps most directly related to host defense mechanisms against microorganisms. Administration of endotoxin causes a rapid and pronounced granulocytopenia due to margination and nonrecirculation of neutrophils.[1,2] The granulocytopenia is followed by a rebound neutrophilia and release of immature granulocytes from the bone marrow into the peripheral circulation.[3,4]

There is considerable information available regarding the direct effects of endotoxin on granulocyte function in vitro and in vivo in animal models.[1,2,5-7] The effects of endotoxin are known to be highly species specific. Most of the information regarding the in vivo effects of endotoxin in humans derives from studies on patients with gram-negative septicemia in which a number of variables may be operating.[8] The present study was undertaken to evaluate the effects of intravenous administration of purified endotoxin on certain aspects of granulocyte function in normal man. The studies indicate that experimental human endotoxemia is associated with a pronounced defect in granulocyte chemotaxis.

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MATERIALS AND METHODS

Subjects

Four healthy adult volunteers gave informed consent for endotoxin administration. Piromen, a purified *Pseudomonas* endotoxin LPS (Flint Laboratories), was injected intravenously in a dose of 0.1 μg/kg. Studies were carried out in the UCLA Clinical Research Center with the subjects fasting and at rest. Blood samples were collected before and at intervals after endotoxin injection for total and differential cell counts and for tests of granulocyte function.

Cell Function Studies

Leukocytes were separated from heparinized whole blood by dextran sedimentation and differential centrifugation. The leukocyte-rich plasma was centrifuged at 150 *g* for 10 min and erythrocytes in the pellet were removed by ammonium chloride lysis.[9] The cells were washed in phosphate-buffered saline solution containing 10% fetal calf serum and resuspended in Hanks' balanced salt solution with 10% fetal calf serum at a concentration of 1×10^7 granulocytes/ml.

Candida killing activity was evaluated by the method of Lehrer and Cline[10] in which the per cent of *C. albicans* killed by leukocytes was determined by the inability of the dead yeast to exclude the methylene blue dye. Postphagocytic leukocyte metabolism was measured by the conversion of glucose-1-^{14}C to $^{14}CO_2$.[11] Neutrophils (10^7) were incubated in siliconized center-well flasks. Methyl benzethonium hydroxide (Hyamine-OH, Sigma Chemical Co., St. Louis, Mo.) was placed in the center well to adsorb the CO_2 produced. The flasks were sealed with serum stoppers and 0.1 μCi of glucose-1-^{14}C was injected into the leukocyte suspension. Into half of the flasks, a phagocytic stimulus of 10^7 heat-killed *C. albicans* was injected ("stimulated" cells). The flasks were incubated at 37°C in a Dubnoff Metabolic Shaking Incubator (Precision Scientific Co., Chicago, Ill.). After 1 hr, the reaction was stopped and CO_2 released by the injection of 1 ml perchloric acid (10%). The Hyamine-OH with adsorbed CO_2 was transferred into vials containing scintillation fluid (Permafluor, Packard, Downers Grove, Ill.) and counted in a liquid scintillation spectrometer (Model 3375, Packard, Downers Grove, Ill.). The radioactivity present represented the amount of $^{14}CO_2$ produced by the metabolizing leukocytes, and the results were expressed as nanomoles of CO_2 produced per hour per 10^7 neutrophils. "Resting" and "stimulated" cells were compared, and the increase due to the phagocytic stimulus was determined.

Leukocyte random migration was measured by the capillary tube method.[12] Isolated leukocytes were centrifuged in hematocrit tubes, and the tubes were then placed in a vertical position. The distance of cell migration from the cell pellet was measured optically at the end of a 3-hr period using a microscale (1 unit = 0.025 inches).

Chemotaxis was determined by a modification of the Boyden chamber technique,[13] using a 3-μ-pore size nucleopore chemotaxis filter (Wallabs, Inc., San Rafael, Calif.); 7×10^5 neutrophils were placed in the upper chamber. AB serum was zymosan activated (incubated for 30 min at 37°C with 0.5 mg zymosan/ml serum) and then heat inactivated at 56°C for 30 min to stop complement activation and eliminate inhibitors. The activated AB serum was diluted to 20% with minimal essential medium (MEM) and used as the standard chemotactic solution for measuring chemotaxis of leukocytes obtained at various times after endotoxin administration. The number of cells migrating to the bottom of the filter was counted in 20 high-power fields, using a microgrid. The mean number of cells per field was determined and expressed as per cent of the 0 time value.

Serum Assays

Sera obtained before and at intervals after endotoxin administration were spectrophotometrically assayed for lysozyme by measuring the dissolution of a suspension of *Micrococcus lysodeikticus* (Lysozyme Reagent Set, Worthington Biochemical Corp., Freehold, N.J.) and expressed as micrograms per milliliter.

RESULTS

After endotoxin injection, blood leukocyte counts became maximally depressed at about 1 hr. Leukopenia was followed by a rebound leukocytosis last-

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Table 1. Blood Cell Counts After Endotoxin Injection

	Time (hr)						
	0	0.5	1	2	3	4	24
WBC/cu mm*	6,825 ±1,146	6,400 ±623	3,925 ±918	10,850 ±2,266	12,267 ±1,438	10,050 ±50	6,467 ±578
PMN/cu mm*	4,436 ±1,281	4,464 ±769	2,677 ±871	9,386 ±2,201	10,402 ±1,318	8,038 ±462	4,113 ±584
Lymph/cu mm*	2,044 ±400	1,577 ±191	989 ±127	958 ±102	1,060 ±213	1,356 ±157	1,760 ±48
Mono/cu mm*	446 ±152	286 ±67	173 ±109	224 ±73	578 ±252	503 ±203	482 ±58
Platelets/cu mm†	295,000 ±80,000	290,000 ±71,000	295,000 ±86,000	277,000 ±71,000	290,000 ±88,000	290,000 ±60,000	310,000 ±89,000
Hct (%)†	38 ±1	36 ±1	36 ±1	36 ±2	37 ±2	37 ±3	36 ±2

*Mean of 4 ± SE.
†Mean of 3 ± SE.

ing several hours and returning to baseline by 24 hr. These changes in total leukocyte counts primarily reflected changes in neutrophil numbers, but absolute monocyte and lymphocyte counts showed a similar pattern (Table 1). No consistent alterations in platelet count or packed red cell volumes were observed in these studies (Table 1).

Serum lysozyme levels and leukocyte random migration remained relatively constant throughout the study (Table 2). Leukocyte *Candida* killing activity increased slightly during the 24 hr, but there was no statistically significant difference ($p > 0.1$) between the pre- and postendotoxin samples. The baseline (resting) glucose metabolism, as measured by $^{14}CO_2$ production, did not significantly change except for a slight increase ($p > 0.05$) seen at 24 hr. The in-

Table 2. Leukocyte Function After Endotoxin Injection

	Normal* ± SD	Time (hr)					
		0	0.5	1	2	4	24
Serum lysozyme† (μg/ml)	2 − 11	9 ± 1	10 ± 1	11	11 ± 2	11 ± 1	10
Candida killing‡ (%)	35 ± 14	36 ± 6	39 ± 6	41 ± 7	41 ± 7	41 ± 8	49 ± 6
Random migration‡ (U/3 hr)	9 ± 4	8 ± 2	8 ± 1	12 ± 3	9 ± 2	10 ± 2	7 ± 2
Glucose metabolism							
Baseline† (nmoles CO_2/hr/10^7 PMN)	208 ± 87	164 ± 19	166 ± 55	145 ± 23	145 ± 20	130 ± 4	225 ± 23
Stimulation† (fold increase)	12 ± 3	13.8 ± 3.1	13.4 ± 1.9	14.1 ± 3.6	12.5 ± 4.3	13.9 ± 8.6	10.6 ± 1.1
Chemotaxis‡ (% 0 hr)	99 ± 27	100 ± 15	105 ± 11	48 ± 10	74 ± 14	114 ± 33	130 ± 15

*Mean of 20 normal individuals.
†Mean of two patients ± SE.
‡Mean of four patients ± SE.

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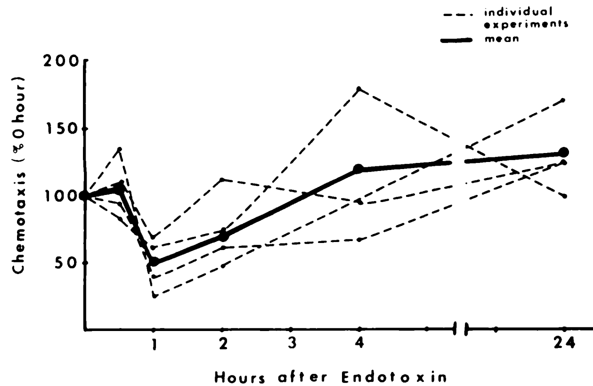

Fig. 1. Leukocyte chemotactic activity after in vivo exposure to endotoxin.

crease in metabolic activity with phagocytic stimulation likewise showed no change during the study (Table 2).

A substantial decrease in granulocyte chemotactic activity was noted. Figure 1 depicts the individual experiments in broken lines and the means of the values in the solid line. The maximal depression in chemotactic activity occurred at 1 hr after LPS administration ($p < 0.001$). The defect was transient and was corrected in most cases by 4 hr. A modest increase in chemotactic response ($p > 0.05$) was observed by 24 hr after endotoxin (Fig. 1).

DISCUSSION

Endotoxin injection into mice and rabbits leads to a biphasic effect on both the peripheral blood leukocyte count and host susceptibility to infection. The first phase occurs shortly after administration and is associated with neutropenia, increased "stickiness" of leukocytes, impaired leukocyte migration into areas of inflammation, and with increased mortality from bacterial infections.[1,5,14,15] Subsequently, there follows a phase of marked neutrophilia and normal or improved host defense status.[1] The neutrophilia is due to release of granulocytes from bone marrow stores.[3] Endotoxin has therefore been used as a test for adequacy of marrow granulocyte reserve.[16,17] The cause of the first-phase neutropenia appears to be primarily due to margination of granulocytes along the walls of blood vessels with intravascular sequestration within the lungs, spleen, liver, and other capillary beds.[1,2] In rabbits, the increased susceptibility to infection in this phase is not thought to be caused by cellular phagocytic or bactericidal defects but rather to be due to reduced accumulation of leukocytes in inflammatory sites.[18]

In the present study, the phases of neutropenia and neutrophilia were clearly demarcated. In agreement with previous investigations in animals, we found no defect in microbicidal activity of these cells.[18] Assays of human and guinea pig granulocytes exposed to high concentrations of LPS in vitro have shown increased glucose metabolism and lysozyme release[6,7] and decreased random migration.[19] We were unable to demonstrate these changes in isolated leukocytes subsequent to in vivo administration of much smaller doses of endotoxin. Dose and species variations may account for these differences. We did not study the effect of higher doses of endotoxin because the clinical response (fever,

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chills, etc.) was unpredictable, and occasionally the symptoms were quite prominent using the standard dose.

We observed a marked and consistent impairment in chemotactic activity of peripheral blood neutrophils occurring maximally 1 hr after LPS injection. This time also corresponded to the nadir of blood neutrophil counts. The chemotactic defect in experimental human endotoxemia may relate to the impaired mobilization of granulocytes into inflammatory locations that has been described during the neutropenic phase of endotoxemia in animals.[1]

The mechanism of the endotoxin-induced chemotactic defect in man is not clear. Endotoxin may have a direct inhibitory effect on the neutrophils or may stimulate other plasma factors which, in turn, inhibit the neutrophils. Endotoxin is known to stimulate the complement system with the subsequent formation of chemotactic factors (C3a, C5a, C567). In rabbits, at least one of these factors (C567), has been shown to activate a serine esterase on the neutrophil surface; this is related to its chemotactic activity.[20] Exhaustion of the esterase activity (i.e., by increased amounts of C567) leads to decreased chemotactic response. It is possible that a similar situation occurs in these human cells with an exhaustion or utilization of surface enzymes or receptors necessary for chemotaxis. Another consideration may relate to the increased leukocyte "stickiness" that has been reported in animals during the neutropenic phase.[15] The adherence of these leukocytes to one another may decrease their ability to migrate through the chemotaxis filters. An alternative explanation may be that granulocyte margination or sequestration leaves a population of cells remaining in the circulating blood that are less chemotactically competent.

The findings on granulocyte function in experimental human endotoxemia may have relevance to host defense function during gram-negative bacterial infection. The data indicate that, at levels of endotoxemia which cause neutropenia but no appreciable alterations in granulocyte glucose metabolism or fungicidal capacity, endotoxin can transiently cause a chemotactic defect or select for a population of circulating neutrophils with an impairment of chemotactic activity.

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