

Functional Capacity of Marginated and Bone Marrow Reserve Granulocytes

RUSSELL W. STEELE,* CHRISTOPHER R. STEELE, NEYLON S. PILKINGTON, JR.,
AND RONALD K. CHARLTON

Division of Infectious Disease-Immunology, Department of Pediatrics, University of Arkansas for Medical Sciences and Arkansas Children's Hospital, Little Rock, Arkansas 72202

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Marginated and bone marrow reserve granulocytes were obtained from young healthy volunteers after subcutaneous administration of aqueous epinephrine (0.4 ml/m²) or intravenous administration of hydrocortisone sodium succinate (250 mg), respectively. These leukocytes were compared with circulating granulocytes for the ability to adhere to surfaces, migrate in a random fashion, respond to chemoattractants, interact with autologous serum opsonins, and phagocytize and kill five common bacterial pathogens. As contrasted with circulating neutrophils, marginated cells had enhanced phagocytic and killing capacity for some pathogens, whereas adherence, random migration, chemotaxis, and chemiluminescence for the two cell populations were equivalent. Bone marrow reserve cells demonstrated increased activity for three functional mechanisms; chemotaxis for these cells averaged 21% higher than that for circulating cells, and phagocytosis was 32% higher, with 6 to 17% greater killing of the five bacterial species studied. All of these differences were statistically significant ($P < 0.05$). Random migration and interaction with serum opsonins were unchanged in bone marrow granulocytes. These enhanced functional properties of neutrophils which are outside of the circulating pool may represent important host defense mechanisms during episodes of bacterial infection.

It has long been recognized in the clinical setting that bacterial infections are accompanied by a peripheral blood leukocytosis consisting predominately of granulocytes. This increase in the absolute granulocyte count has often been used as an indicator of bacterial as opposed to viral infection in febrile patients (11, 18). These additional leukocytes represent the mobilization of marginated cells and, to some extent, bone marrow reserves, and it is speculated that this mobilization reflects a host response to microbial invasion which results in a more efficient elimination of pathogens (6).

The functional capacity of granulocyte reserves has been evaluated indirectly by examining cells from infected neonates (1, 16), children (8), and adults (3, 7, 10). Researchers have observed some differences from normal circulating cells and concluded these were evidence of impaired function (3, 16).

The present study was designed to comprehensively evaluate functional aspects of marginated and bone marrow reserve granulocytes to delineate the potential contribution of these responses to host defense against bacterial pathogens. Assays included quantitation of marginated cells, adherence, random motility, response to chemoattractants, interaction with serum opsonins, phagocytosis, and killing of common bacterial pathogens.

MATERIALS AND METHODS

Subjects. Leukocytes were obtained from 10 normal healthy adults, who received aqueous epinephrine or hydrocortisone on 5 to 12 occasions during the 12-month experiment. Informed consent was obtained, and the study was approved by the Human Research Advisory Committee of the University of Arkansas for Medical Sciences.

Demargination of granulocytes. On each study day, peripheral venous blood was first obtained by venipuncture, each

volunteer was then given 0.4 ml of aqueous epinephrine (1:1,000) per m² of body surface area by subcutaneous injection, and an additional sample of venous blood was obtained 18 min after the injection. This optimal timing for obtaining demarginated-leukocyte samples was determined in earlier experiments in which blood was obtained every 3 min after epinephrine administration.

Circulating granulocytes (before epinephrine treatment) and combined circulating and marginated granulocytes (after epinephrine treatment) were isolated by a dual Ficoll-Hypaque gradient separation technique. The double-layer gradient was prepared by placing 2 ml of Mono-Poly Resolving Medium (specific gravity, 1.114; Flow Laboratories, Inc.) in a 16-by-100-mm plastic centrifuge tube and carefully layering 2 ml of Histopaque (specific gravity, 1.077; Sigma Chemical Co.) over the first layer. Blood (4 ml) was gently layered on top of the gradients and centrifuged at $250 \times g$ for 30 min in a swinging-bucket rotor at 20°C. The top fraction was discarded, and the lower fraction was collected and washed once with Hanks balanced salt solution by centrifugation at $400 \times g$ for 10 min. To remove contaminating erythrocytes, the cell pellet was suspended in 0.84% ammonium chloride and incubated for 5 min in a 37°C water bath. The granulocytes were washed three more times before they were adjusted to the desired concentration. Such processing did not alter the viability of polymorphonuclear leukocytes (PMNs) from the circulating or marginated pool, as judged by trypan blue dye exclusion.

Bone marrow reserve granulocytes. On other study days, the same volunteers were given 250 mg of hydrocortisone sodium succinate by rapid intravenous infusion, and peripheral blood was obtained 4 h later. Processing of granulocytes was done as described above.

Adherence. Adherence was measured with nylon fiber columns as previously described (9). Briefly, 50 mg of nylon fiber was carefully packed into a Pasteur pipette to a length of 15 mm. Blood (1 ml) was dispensed into the column and

* Corresponding author.

TABLE 1. Characteristics of circulating and marginated granulocytes^a

Granulocyte ^b	Adherence (%)	Random migration ^c	Chemotaxis ^c	Peak chemiluminescence response (cpm) with:		
				Zymosan	<i>S. pneumoniae</i>	<i>S. aureus</i>
Pre	58 ± 4	473 ± 58	1,262 ± 139	364,228 ± 50,507	53,945 ± 7,195	92,018 ± 4,041
Post	60 ± 5	514 ± 39	1,347 ± 80	394,801 ± 58,711	53,124 ± 7,314	95,687 ± 7,024

^a Values are expressed as the mean of 12 samples ± the standard error of the mean. *P* values (Pre and Post) were not significant.

^b Pre, Circulating granulocytes isolated before epinephrine treatment; Post, marginated granulocytes isolated after epinephrine treatment.

^c Number of migrated cells in a single plane.

allowed to filter through the nylon fiber by gravity. The precolumn granulocyte count and differential were compared with the effluent blood count to ascertain the percentage of granulocytes adhering to the column. The percentage of adherent granulocytes was calculated as follows: % adherence = 100 - {[(granulocytes per milliliter in effluent sample)/(granulocytes per milliliter in original sample)] × 100}.

Random migration and chemotaxis. Granulocyte random migration and chemotaxis were assessed by a modification of the chemotaxis-under-agarose technique (4). A series of three wells, 4 mm in diameter and 2 mm apart, were cut into agarose plates by using a stainless steel template. The concentration of granulocytes was adjusted in RPMI 1640 containing 10% heat-inactivated fetal calf serum so that 10⁶ leukocytes were added to the middle well of the plate. Chemotactic factors were generated from fresh autologous serum by incubation with zymosan and pipetted into one peripheral well. The other peripheral well contained tissue culture medium alone. After incubation of the plates for 4 h at 37°C in an atmosphere of 5% CO₂, the cells were fixed with methanol and formaldehyde, the agar was removed from the plate, and the plate was air dried. The agarose was discarded, and the granulocytes were stained with Wright solution. Cells were quantitated by using a 1-by-1-mm microscope eyepiece grid. This was positioned between the center and peripheral wells and moved in a vertical direction until all cells within the grid were counted. Cell migration was measured by counting all cells between the middle well, which contained the granulocytes, and the well containing the chemotactic factors or the control well, which contained tissue culture medium. Results were expressed in terms of the total number of PMNs counted.

Chemotaxis data were examined by measuring both absolute cell counts and linear migrating distance. Both methods yielded similar results; however, because absolute counts more clearly reflected activity in this assay, these data were chosen for presentation.

All assays were done daily in triplicate for 12 days. Experimental day-to-day variation was similar to that previously reported (4).

Bacterial pathogens. The following laboratory strains of bacteria were used in the assays of chemiluminescence, phagocytosis, and killing described below: *Streptococcus pneumoniae* type 12F, *Escherichia coli*, *Staphylococcus aureus* 502A, *Haemophilus influenzae*, and *Pseudomonas aeruginosa*. Organisms were inoculated into several tubes of Todd-Hewitt broth and incubated at 37°C overnight. The suspensions were centrifuged at 1,000 × *g* for 10 min at 25°C and then washed and suspended in Geys balanced salt solution (pH 7.2) to a concentration of 5 × 10⁸ to 10⁹/ml. The bacterial suspension was divided into samples and stored at -70°C until they were used.

Chemiluminescence. The interaction of granulocytes with pooled human sera and two bacterial pathogens, i.e., *S.*

aureus and *S. pneumoniae*, or with zymosan particles was tested by chemiluminescence methods described in detail previously (17). On the day of each assay, bacteria were opsonized with 40% test serum (0.5 ml) for 30 min at 37°C. After incubation, 0.5 ml of Geys balanced salt solution was added to each opsonized bacterial suspension. This 1-ml volume was added to the reaction vial for a final bacterium/PMN ratio of 100:1.

Chemiluminescence was measured at room temperature in dark-adapted polypropylene scintillation vials, with the photomultiplier tube of a Beckman LS 9000 liquid scintillation spectrophotometer set in the out-of-coincidence mode. The vials contained 5 × 10⁶ PMNs in 4 ml of Geys balanced salt solution and were equilibrated in the scintillation counter until a stable background was reached. Chemiluminescence was initiated by adding 1 ml of previously opsonized bacteria (5 × 10⁸ to 10⁹) to each vial. All vials were counted for 15 s at approximately 7-min intervals for 90 min. Chemiluminescence was expressed as peak activity throughout these studies, and maximum or peak counts per minute usually occurred between 15 and 45 min. Absorbed and hypogammaglobulinemic sera were used to measure nonspecific opsonization (i.e., complement and nonantibody serum proteins), and these background counts were subtracted from the total counts per minute of the experimental samples.

Phagocytosis and bacterial killing. The killing of bacteria was measured first by using the colony count bactericidal method (15). Briefly, each 1-ml incubation mixture included 5 × 10⁶ CFU of bacteria and 10% autologous serum. The 2-h incubation was done at 37°C in plastic test tubes with horizontal rotation on a shaker at a rate of 120 rpm. Immediately after incubation, neutrophils were lysed by adding 9 ml of sterile distilled water to the reaction mixtures, serial dilutions of the bacteria were made in 0.9% NaCl, and 100 μl of the dilutions was spread on Trypticase soy agar (BBL Microbiology Systems) plates to quantitate viable bacteria.

After preliminary studies with the colony count assay, a fluorochrome microassay with acridine orange as an indicator of viable cellular material (14) was used to directly evaluate phagocytic and microbicidal functions of granulocytes. Blood was drawn in a syringe with no anticoagulant, and a leukocyte monolayer was prepared by placing 0.2-ml samples of blood on sterile circular cover slips. All cover slips were incubated at 37°C in a humidified 5% CO₂ incubator for 60 min. After incubation, the clot and serum were gently washed from the cover slip, and the cover slip was gently rinsed with Hanks balanced salt solution to remove nonadherent cells. A 0.2-ml amount of the bacterial suspension was added to the monolayer, and the cover slip was incubated at 37°C for 90 min. Each cover slip was then carefully rinsed and stained with 0.14% acridine orange for 45 s. The cover slips were mounted on glass slides with the monolayer side down, and the edges were sealed with clear

TABLE 2. Bacterial phagocytosis and killing by circulating and marginated granulocytes^a

Bacterium	Phagocytosis ^b			% Killing		
	Pre	Post	P ^c	Pre	Post	P ^c
<i>E. coli</i>	3.29 ± 0.82	4.21 ± 1.11	NS	44 ± 9	63 ± 4	<0.02
<i>S. aureus</i>	5.57 ± 2.42	6.56 ± 1.89	NS	66 ± 12	81 ± 13	NS
<i>S. pneumoniae</i>	5.58 ± 1.55	7.82 ± 1.14	NS	60 ± 9	85 ± 6	<0.01
<i>P. aeruginosa</i>	7.30 ± 0.85	9.33 ± 1.87	NS	69 ± 3	85 ± 4	<0.01
<i>H. influenzae</i>	8.62 ± 1.27	13.24 ± 3.03	<0.02	69 ± 2	80 ± 4	<0.05

^a Values are expressed as the mean of five samples ± the standard error of the mean. Pre and Post are as defined in Table 1, footnote b.

^b Expressed as a phagocytic index, i.e., the average number of bacteria ingested per granulocyte in 100 cells counted.

^c NS, Not significant.

nail polish. The cells were examined with an immersion objective. To quantitate phagocytic capacity, the total numbers of dead (red) and live (green) intracellular bacteria in 100 leukocytes were counted on each of the three cover slips. Killing and phagocytic capacity were determined by the following formulas: % killing = [(number of red organisms)/(number of red and green organisms per 100 leukocytes)] × 100; phagocytic capacity = (number of red and green organisms)/(100 leukocytes).

Statistics. Statistical analyses were done with the Student *t* test.

RESULTS

Marginated leukocytes. Peripheral blood obtained 18 min after subcutaneous epinephrine treatment contained absolute neutrophil counts averaging 7,197 ± 2,047/mm³ as compared to 3,332 ± 958/mm³ before epinephrine treatment. This finding represented an increase of 116%, with a range of 72 to 225%. Although blood samples taken after epinephrine treatment contained both circulating and marginated leukocytes, these samples are referred to as marginated cells for clarity in presentation. It was assumed that any observed differences in functional capacity were attributable to the marginated pool.

Results for experiments which examined adherence, random migration, and chemotaxis of granulocytes are summarized in Table 1. No significant differences between circulating and marginated granulocytes were observed in these assays.

Although peak chemiluminescence activity was similar for the two populations of granulocytes, the response kinetics differed in that the peak responses were achieved more rapidly with the marginated pool (data not shown). This result was evident for both bacterial strains, i.e., *S. aureus* and *S. pneumoniae*, as well as for opsonized zymosan particles.

Phagocytosis, as measured by a phagocytic index (ingested bacteria per granulocyte), was greater for marginated neutrophils than for circulating cells, but this finding reached statistical significance only for phagocytosis of *H. influenzae*

(Table 2). Data for bactericidal capacity, as measured by both the acridine orange fluorochrome microassay and the colony count method, were similar; those for the microassay are presented in Table 2. Killing capacity was greater for marginated granulocytes, with increased function statistically significant for four of the five bacterial strains tested.

Bone marrow reserve granulocytes. Ten experiments in which the number of granulocytes was almost tripled after intravenous hydrocortisone administration were analyzed (Table 3). The adherence of bone marrow reserve granulocytes was increased by an average of 8% above that of control neutrophils, but this difference was not statistically significant. Random migration was unchanged in bone marrow granulocytes, as was interaction with serum opsonins. Chemotaxis averaged 21% above that of circulating cells (*P* < 0.05), while peak chemiluminescence responses to zymosan, *S. pneumoniae*, and *S. aureus* were similar for the two populations of granulocytes.

Bone marrow reserve granulocytes showed significantly higher phagocytic and killing responses than circulating cells did with both assays, and data for the fluorochrome microassay are summarized in Table 4. These differences were consistent for all five bacterial strains studied.

DISCUSSION

The leukocytosis which accompanies systemic bacterial infection is generally interpreted as a host response which augments the immune defense. Early in the course of the disease, the additional circulating neutrophils come primarily from the marginated pool, whereas bone marrow reserves account for the majority of cells during continued infection or during convalescence (6). The granulocytes in bone marrow reserves represent a younger population of cells with functional capacities different from those of peripheral circulating PMNs; the more consistently observed differences have included decreased adherence (7, 9), increased chemiluminescence, and increased release of myeloperoxidase (7).

There is disagreement as to whether the marginated neutrophils constitute a population which possesses properties

TABLE 3. Characteristics of circulating and bone marrow reserve granulocytes^a

Granulocyte	No. of granulocytes/mm ³	Adherence	Random migration ^b	Chemotaxis ^b	Peak chemiluminescence response (cpm) with:		
					Zymosan	<i>S. pneumoniae</i>	<i>S. aureus</i>
Pre	2,464 ± 535	59 ± 4	473 ± 216	1,466 ± 293	317,964 ± 63,238	48,350 ± 9,211	85,439 ± 6,509
Post	7,317 ± 1,648	63 ± 6	531 ± 189	2,071 ± 214	364,052 ± 81,735	46,873 ± 7,752	89,031 ± 8,625

^a Values are expressed as the mean of 10 samples ± the standard error of the mean. Pre, Circulating granulocytes isolated before hydrocortisone treatment; Post, bone marrow reserve granulocytes isolated after hydrocortisone treatment. *P* values (Pre and Post) were as follows: number of granulocytes per cubic millimeter, <0.001; chemotaxis, <0.05. All other *P* values were not significant.

^b Number of migrated cells in a single plane.

TABLE 4. Bacterial phagocytosis and killing by circulating and bone marrow reserve granulocytes^a

Bacterium	Phagocytosis ^b			% Killing		
	Pre	Post	P	Pre	Post	P
<i>E. coli</i>	3.3 ± 0.8	4.6 ± 0.5	<0.05	44 ± 9	61 ± 5	<0.05
<i>S. aureus</i>	5.6 ± 1.4	7.3 ± 0.6	<0.05	66 ± 12	76 ± 4	<0.05
<i>S. pneumoniae</i>	5.6 ± 1.6	8.4 ± 1.0	<0.05	60 ± 9	75 ± 6	<0.05
<i>P. aeruginosa</i>	7.3 ± 0.8	9.1 ± 0.9	<0.05	69 ± 3	85 ± 3	<0.02
<i>H. influenzae</i>	8.6 ± 1.3	11.2 ± 1.2	<0.05	69 ± 2	75 ± 4	<0.05

^a Values are expressed as the mean of 10 samples ± the standard error of the mean. Pre and Post are as defined in Table 3, footnote a.

^b Expressed as a phagocytic index, i.e., the average number of bacteria ingested per granulocyte in 100 cells counted.

different from circulating PMNs and thereby enhance elimination of foreign antigens such as bacterial pathogens. One report concluded that marginated cells were a younger population, as suggested by differences in alkaline phosphatase content (5). However, other studies which also assayed alkaline phosphatase to determine neutrophil maturity disagreed (12, 13). There appears to be a rapid equilibration of diisopropyl fluorophosphate-labeled granulocytes between the marginated and circulating vascular compartments (2). These data make it less likely that marginated PMNs migrate from the endothelium to the circulation or into tissue as a consequence of maturation per se. Location in these compartments appears to be more likely a random event. If location is random, then the functional capacities of cells from the circulating or marginated pools should be identical.

In the present study, we harvested neutrophils after epinephrine administration at 18 min postinjection. This early sampling should eliminate potential contamination with bone marrow reserve cells, which are released much later (2). Moreover, there was a small but insignificant change in the appearance of immature neutrophils at 18 min post-epinephrine treatment, a characteristic of marginated cells. On the other hand, after infusion of hydrocortisone, 40 to 50% of the granulocytes were in immature forms, a finding which supports the idea that these cells originate as bone marrow reserve cells. These data, similar to data in previous reports, allow the interpretation that differences in functional capacities for the various populations of granulocytes exist.

Earlier studies have compared granule protein content along with some functional characteristics of marginated and circulating granulocytes; differences were generally not statistically significant (7). Marginated PMN adherence was shown to be decreased in one study (9) but unchanged in another (7). Methodologic variables may have accounted for these differences since epinephrine was given intramuscularly in the first study but subcutaneously in the latter. Hetherington and Quie (7) also examined peak chemiluminescent responses after stimulation with opsonized zymosan and phorbol myristate acetate, chemotaxis induced by zymosan-activated serum and *E. coli* factor, and PMN content of myeloperoxidase, lactoferrin, and β -glucuronidase. They concluded that granulocytes of these two pools were functionally equivalent and that granule protein contents were identical. The major difference between this earlier study and our study was that subjects were given only 0.3 ml of epinephrine (1:1,000), whereas we administered 0.4 ml/m² of body surface area. With this higher dose, marginated granulocytes accounted for 54% of the recovered cells, versus 26% (7) with the lower dose. The dilution effect of normal circulating granulocytes in assays designed to evaluate differences between blood samples taken before and after epinephrine treatment might prevent delineation of

functional changes, particularly when marginated cells represent a small minority of the total population.

The earlier events of neutrophil function, i.e., adherence, migration, chemotaxis, and chemiluminescence, appear to be unaltered in marginated granulocytes, although chemotaxis is enhanced in the bone marrow reserve population. These findings confirm previous observations with somewhat different methods. However, in marginated granulocytes the later events, i.e., phagocytosis and killing, were enhanced over those in circulating PMNs, particularly so in bone marrow reserve granulocytes. These differences for phagocytosis in the marginated pool were significant ($P < 0.02$) with *H. influenzae* but were not significant, although they were higher, with *E. coli*, *S. aureus*, *S. pneumoniae*, and *P. aeruginosa*. More discriminatory were assays of bacterial killing in which capacity was significantly enhanced ($P < 0.05$) against *E. coli*, *S. pneumoniae*, *P. aeruginosa*, and *H. influenzae*. Killing of *S. aureus* did not reach statistical significance. Bone marrow reserve cells appear to be even more active, with enhanced phagocytic and killing capacity observed for all of the bacterial pathogens studied.

Most important from these data is the consistent observation of increased phagocytosis and killing function for marginated and bone marrow reserve granulocytes. This suggests an enhancement of host defense provided by recruitment of phagocytic cells. Increased activity is probably not the result of interaction with epinephrine or hydrocortisone since in vitro incubation of PMNs with these pharmaceuticals did not alter function (data not shown). More likely is an innate difference in function based on maturation or in vivo preservation of activity during margination.

The earlier respiratory burst seen in most chemiluminescent experiments may account for some augmentation in killing efficiency. Other possible mechanisms include increases in granule enzyme content, hydroxyl-radical production, or interaction with opsonins. To date, no studies have delineated such differences between the various pools of PMNs. It is certainly possible that the many factors which eventually result in bacterial killing are all relatively more efficient in reserve cells. Slight differences for each step are of course difficult to demonstrate in vitro.

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