Evaluation of Non-Steady-State Neutrophil Kinetics During Endotoxin-Induced Granulocytosis[1] (35661)

R. E. OSTLUND, C. R. BISHOP, AND J. W. ATHENS
(Introduced by G. E. Cartwright)

Department of Medicine, University of Utah College of Medicine and Salt Lake Veterans Administration Hospital, Salt Lake City, Utah 84112

By means of the $DF^{32}P$ labeling technique the total blood granulocyte pool (TBGP), circulating granulocyte pool (CGP), and marginal granulocyte pool (MGP) can be measured and blood granulocytes can be shown to leave the blood of normal human subjects in a random manner with a half-disappearance time (T ½) of about 7 hr (1).

In contrast to fairly extensive studies of steady-state granulocyte kinetics, relatively little is known about granulocyte kinetics in perturbed, nonequilibrium situations. A recent report described changes in blood granulocyte inflow and outflow rates before, during, and after cortisol-induced granulocytosis (2). In that study, a computer was used to derive the rate of flow into (RI) and outflow (RO) from the blood by matching model curves to experimentally observed changes in blood granulocyte concentration and blood granulocyte radioactivity curves. In the present study, a simple method more directly calculating the RI and RO is described. In addition, changes in RI and RO after bacterial endotoxin administration are reported.

Methods. Subjects. Eighteen normal men from the Utah State Prison between the ages of 20 and 36 volunteered to cooperate in the studies.

Kinetic studies. Granulocytes from the subjects were labeled *in vitro* with $DF^{32}P$ as previously described (3). The blood was then returned to the donor. Hourly samples, and occasionally half-hourly samples, of 22 ml of venous blood were drawn for measurement of granulocyte count and specific activity. The determination of granulocyte specific activity has been described in detail (1, 3). After 4–7 hr, when the disappearance curve of the $DF^{32}P$-labeled granulocytes was established, bacterial endotoxin was given intravenously to each of 12 subjects. The granulocyte count and specific activity were followed for at least 6 hr thereafter. The remaining six subjects served as controls and received no endotoxin.

Endotoxin dosages were 0.1 μg of *Salmonella abortis equi* lipopolysaccharide[2] or between 8 and 16 μg of *Pseudomonas* lipopolysaccharide.[3] Except for the 12- and 16-μg doses of *Pseudomonas* lipopolysaccharide, these doses of endotoxin did not produce symptoms in the subjects. Two of four subjects given these larger doses had headache and fever up to 101° F.

Calculations. Blood granulocyte inflow and outflow rates were calculated as follows and expressed as granulocytes/kg/hr either entering or leaving the CGP.

The outflow equation is based on the fact that the number of granulocytes leaving the CGP is related to the amount of granulocyte radioactivity leaving during a given time interval, thus:

$$RO_{(t_1 t_2)} = \frac{[GSAI_{(t_1)} \times CGP_{(t_1)}] - [GSAI_{(t_2)} \times CGP_{(t_2)}]}{\{[GSAI_{(t_1)} + GSAI_{(t_2)}]/2\} \times T}$$

[1] This investigation was supported by Research Grant AM 04489 and Graduate Training Grant AM 5098 from the National Institute of Arthritis and Metabolic Diseases, and Grants FR-00012 and FR-00064 from the National Institutes of Health, Division of Research Facilities and Resources, Bethesda, Maryland.

[2] Lipexal, A. Wander, S. A. Berne, Switzerland.

[3] Piromen, Travenol Laboratories, Inc., Morton Grove, Illinois.

where t_1t_2 is the time interval from t_1 to t_2 in hours, GSAI is the granulocyte specific activity index or the cpm per mg granulocyte nitrogen, CGP is the circulating granulocyte pool size (cells/kg), and T is the time from t_1 to t_2.

The inflow equation is based on the fact that changes in CGP size reflect the differences between inflow and outflow during a given interval; thus:

$$CGP_{(t_2)} = CGP_{(t_1)} + [RI_{(t_1t_2)} - RO_{(t_1t_2)}] \times T.$$

Rearrangement gives

$$RI_{(t_1t_2)} = \frac{CGP_{(t_2)} - CGP_{(t_1)} + [T \times RO_{(t_1t_2)}]}{T}.$$

The CGP size at the beginning of the experiment was calculated from the granulocyte count and the blood volume (1). Changes in CGP size were assumed to be proportional to changes in the blood granulocyte count, and hence the CGP is some multiple of the granulocyte count, (*i.e.*, we assume there is insignificant change in blood volume during the study).

Each experiment was divided into a steady-state period consisting of several hours after the labeling of blood granulocytes and a non-steady-state period lasting from 1 until 5 hr after intravenous endotoxin (Fig. 1).

In all subjects, the average granulocyte concentration, and mean RI and RO were computed during the steady-state period. In addition, the percentage of change in the granulocyte concentration, RI and RO from the steady-state average were determined for each time interval between data points during the non-steady-state period. The average of these changes was recorded as the deviation from normal due to endotoxin injection.

Endotoxin effect on neutrophilic cells in vitro. $DF^{32}P$-labeled whole blood (3) was incubated at 37° with .03 and with .003 μg of *Piromen* per ml for 4 hr. The lower concentration corresponds to 16 μg diluted in 5000 ml. These experimental conditions are known to release endogenous pyrogen from granulocytes (4). The specific activity of the granulocytes was determined in triplicate before and after the incubation, and the results were compared with control granulocytes incubated with equal volumes of pyrogen-free isotonic saline.

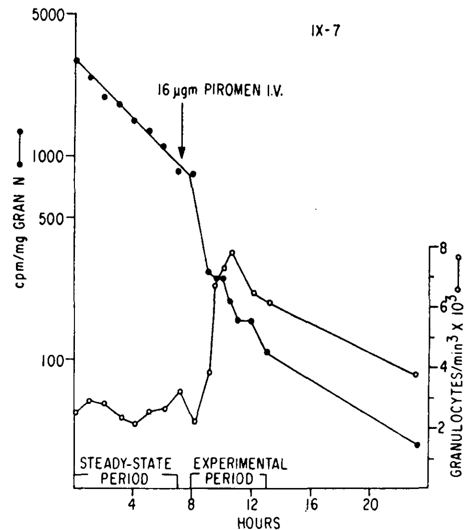

Fig. 1. Blood granulocyte specific activity (solid circles) and granulocyte counts (open circles) in normal subjects given endotoxin intravenously.

In similar studies, ^3HDFP-labeled blood was incubated with the same two concentrations of endotoxin. Autoradiographs were prepared from blood smears before and after the incubation (5). The mean number of grains per cell was determined by counting 60 neutrophils for each incubation mixture.

Results. Variation in RO and RI in control subjects and those given endotoxin. The percentage of change in granulocyte concentration, RI and RO, which occurred in control subjects and in subjects given endotoxin is shown for each subject in Table I.

There were six subjects in whom a significant granulocytosis (mean increase 105% ± 6.4, 1 SE) developed after endotoxin injection (Table IA). In these subjects, the RI increased an average of 180% during the period from 1 to 5 hr after endotoxin injection (range 58–383%) and RO increased 150% (range 24–420%). These changes in RI and RO are significantly different from control subjects studied at the same time after $DF^{32}P$ labeling but not given endotoxin ($p < .01$).

TABLE I. Percentage of Change In Mean Granulocyte Count, Inflow (RI) and Outflow (RO) of Neutrophils During the Period Beginning 1 Hour and Ending 5 Hours After Endotoxin Injection.

Subject	Endotoxin[a] dose (μg)	% Change in G[b]	% Change RI	% Change RO
	A. Endotoxin-treated subjects showing granulocytosis[c]			
IX-7	P 16 μg	130	275	169
IX-23	P 12 μg	117	383	420
III-72	L 0.1 pg	103	198	141
IX-3	P 8 μg	97	58	106
IX-5	P 16 μg	96	101	24
IX-25	P 12 μg	87	64	40
Mean $\pm$ 1 Standard Error		105 $\pm$ 6.4	180 $\pm$ 53.3	150 $\pm$ 58.7
Significance[c]	p	< 0.01	< 0.01	0.02
	B. Controls			
VI-124	0	49	48	—29
VI-128	0	38	—27	40
VI-132	0	—6	—41	—26
VI-130	0	—13	—56	—82
VIII-99	0	—13	0	62
VIII-101	0	—21	—36	23
Mean $\pm$ Standard Error		6 $\pm$ 12	—19 $\pm$ 15	—2 $\pm$ 22

[a] L = Lipexal; P = Piromen.
[b] Mean absolute granulocyte count during the 5-hr, post-endotoxin study period.
[c] Compared with controls by rank sum test (8).

A curve illustrative of the results obtained in these subjects given endotoxin is shown in Fig. 1. After endotoxin injection, the rate of decline of the specific-activity curve increased abruptly as the granulocyte count rose to a peak value at about 5 hr post endotoxin. Thereafter, the granulocyte concentration returned toward prestimulation values, and the rate of fall of the granulocyte radioactivity curve slowed.

There was no correlation between the dose of endotoxin given and the degree of change in the granulocyte count or in the change in RO and RI.

The six control subjects who received no endotoxin exhibited no systematic variation in the granulocyte concentration, RI and RO during the study (Table IB). The mean changes of 6% in granulocyte concentration, —19% in RI and —2% in RO, were not statistically different from zero.

There were six subjects in whom no significant granulocytosis occurred; *i.e.*, the percentage of change in granulocyte concentration did not fall outside the 99% limits corresponding to 2.58 standard deviations above the mean.

Endotoxin effect on attachment of DFP to neutrophilic cells. To ascertain whether endotoxin altered the attachment of the DFP label to neutrophils, granulocytes labeled with $DF^{32}P$ or ^{3}H-DFP were incubated with endotoxin *in vitro*. It can be seen in Table II that neither the specific activity nor the grain count of the neutrophils was significantly affected by incubation with endotoxin in the several concentrations used. In the autoradiographs, all neutrophils were labeled. Background labeling was 3 grains per cell area at a maximum and the minimum grain count over neutrophils was 23 grains.

Discussion. It has been known for some time that various endotoxins will cause a transient blood granulocytosis and it has been shown that this granulocytosis reflects an actual increase in the TBGP size and not just a demargination of cells (1). Further, it has been shown that this increase in the TBGP is due to an increased inflow of cells from the marrow (6). By inspection of the

TABLE II. Granulocyte Radioactivity After Incubation with Endotoxin for 4 Hours at 36° Granulocytes Labeled with $DF^{32}P$.

Incubation conditions	No.	Granulocyte number as % of control count (mean ± 1 SD[a])	$DF^{32}P$ label (mean specific activity ± 1 SD[a])	^3HDFP label (mean grain count ± 1 SD[a])
Control	3		15,500 ± 3350	61.3 ± 19.4
Endotoxin				
.003 μg/ml	3	100 ± 2	17,300 ± 4920	66.8 ± 17.5
.03 μg/ml	3	103 ± 4	17,700 ± 4490	61.0 ± 18.1

[a] Standard deviation.

GSAI curve during a non-steady-state kinetic study the more rapid fall in GSAI at the time of the rising granulocyte count has been interpreted to mean that there is an increased inflow of unlabeled cells from the bone marrow (1). Beyond this, details about the mechanism by which the granulocytosis is produced have not been available.

In the studies reported here an increase in neutrophil count was seen in all subjects given endotoxin. However, it was evident that control subjects exhibit some fluctuation in the neutrophil count over a period of several hours, and only half of the subjects given endotoxin demonstrated an increase in the blood neutrophil count outside the 99% limits seen in control subjects. In these six subjects there was an increase in both RI and RO during a clearly significant rise in granulocyte count (Table IA). This contrasts with the mechanism by which cortisol-induced neutrophilia is produced in that in subjects given cortisol the inflow of cells increased while egress of cells from the blood decreased (2).

Since it has been demonstrated previously that 1 1/2 hr after endotoxin there is often an increase in the TBGP size before the granulocyte count increases, it may be that excessive margination of neutrophils, either in degree or in duration, accounts for the apparent lack of clearcut response in 6 of the 12 subjects given endotoxin. Usually by the fifth hour after endotoxin the normal distribution between CGP and MGP is restored (1). Because there are some subjects who do not appear to have a clearcut and consistent granulocyte response to endotoxin, cortisol appears to be a better, or at least a more predictable, agent for assessing granulocyte reserves (all of nine subjects given cortisol developed a clearly significant granulocytosis).

It has been suggested that cortisol might mediate the endotoxin-induced neutrophilia (7). However, this seems unlikely in view of the fact that the demonstrated increase in RO is not seen after cortisol injection. To the contary, there is almost always a decrease in RO after cortisol injection (2). Also, lymphopenia and eosinopenia are not seen after endotoxin as they are after cortisol injection.

The fact that endotoxin did not change the amount of radioactive label per cell as determined by autoradiography or by determining specific activity of granulocytes indicates that these doses of endotoxin have no direct effect on these measurements. Therefore, the change in radioactivity after endotoxin is given reflects a kinetic alteration rather than a direct effect on the label of the cell.

It is possible that endotoxin has an effect on the label *in vivo* that is not demonstrable in an *in vitro* system. The hour's delay, until there is a rise in the neutrophil count, would seem to be more than just coincidence, however.

Summary. The mechanism by which bacterial endotoxin induces granulocytosis in man has been investigated by labeling granulocytes with radioactive diisopropylfluorophosphate.

In man, a moderate dose of bacterial endotoxin produces a granulocytosis. By labeling circulating granulocytes with $DF^{32}P$ and later giving endotoxin intravenously, the rates of granulocyte inflow and egress from the blood were measured. Equations were

written which express these flow rates as a function of the variables derived from the experimental data. These equations were solved for several time intervals during the 24-hr experiments in each of 12 normal subjects. In the six subjects in whom there was a significant granulocytic response to endotoxin there was a mean increase of 180% (58–383) in the rate of granulocyte inflow to the blood and 150% (24–420) in the rate of egress of granulocytes from the blood during the 5-hour period immediately after endotoxin injection.

The remaining six subjects did not have a granulocyte response beyond the 99% limits of normal and there was random variation of inflow and egress of granulocytes.

In vitro studies with DFP-labeled cells incubated with endotoxin failed to demonstrate a direct effect of endotoxin on the cell label.

1. Athens, J. W., Haab, O. P., Raab, S. O., Mauer, A. M., Ashenbrucker, H., Cartwright, G. E., and Wintrobe, M. M., J. Clin. Invest. **40**, 989 (1961).

2. Bishop, C. R., Athens, J. W., Boggs, D. R., Warner, H. R., Cartwright, G. E., and Wintrobe, M. M., J. Clin. Invest. **47**, 249 (1968).

3. Mauer, A. M., Athens, J. W., Ashenbrucker, H., Cartwright, G. E., and Wintrobe, M. M., J. Clin. Invest. **39**, 1481 (1960).

4. Gerbrandy, J., Cranston, W. I., and Snell, E. S., Clin. Sci. **13**, 453 (1954).

5. Vodopick, Helen A., Athens, J. W., Warner, H. R., Boggs, D. R., Cartwright, G. E., and Wintrobe, M. M., J. Lab. Clin. Med. **68**, 47 (1966).

6. Perry, S., Weinstein, I. M., Craddock, C. G., and Lawrence, J. S., Blood **12**, 549 (1957).

7. Boggs, D. R., Chervenick, P. A., Marsh, J. C., Cartwright, G. E., and Wintrobe, M. M., J. Lab. Clin. Med. **72**, 177 (1968).

8. Goldstein, A., "Biostatistics: An Introductory Text," p. 55. MacMillan, New York (1964).

Received Feb. 11, 1971. P.S.E.B.M., 1971, Vol. 137.