Neutrophil Kinetics after Acute Hemorrhage* (34099)

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An increase in blood neutrophils is commonly observed after acute hemorrhage (1). An increase in the blood neutrophil concentration may develop by any of the three kinetic mechanisms discussed below or by combinations of these mechanisms (2).

In the blood of man (3) or dog (4) approximately one half of the neutrophils are marginated on walls of small blood vessels rather than circulating freely. Such marginated neutrophils are not enumerated in venous blood samples. However, a neutrophilia, resulting from demargination of neutrophils, does occur after the injection of epinephrine or during exercise (3). This shift of neutrophils from the marginal to the circulating pool increases the concentration of circulating neutrophils without changing the total number within the vascular system and therefore may be considered a pseudoneutrophilia.

The intravascular sojourn of neutrophils is brief, averaging 7 hr in man (2), and in the presence of steady-state kinetics the influx of new neutrophils from the bone marrow is balanced by an outflow of blood neutrophils to tissues and body cavities. The marrow contains a large storage pool of band and segmented neutrophils which can be released to the blood upon demand. Therefore, neutrophilia may develop by accelerating the rate of release of neutrophils from the bone marrow, increasing both the marginal and circulating blood neutrophil pools (5).

Finally, neutrophilia may be produced by reducing the rate at which neutrophils leave the blood as has been observed after the administration of adrenal glucocorticosteroids (6).

In order to determine which of these mechanisms or combinations of mechanisms is responsible for the neutrophilia after acute hemorrhage the following study was performed. Mice were bled and at frequent intervals thereafter, blood neutrophil concentration, the blood band to segmented neutrophil ratio, and the total number of neutrophils in the bone marrow (humerus) were determined.

Materials and Methods. Mice were F_1 (C57BL female $\times$ DBA male) bred in our laboratory from stock purchased from Jackson Laboratories, Bar Harbor, Maine. Male mice, 8–10 weeks of age and weighing from 24–30 g were used in all experiments. In each experiment a control group matched for age and weight was studied concurrently with the experimental group.

Mice were bled by inserting a heparinized, microhematocrit tube (Abbott Laboratores) into the orbital sinus (7). In order to remove a constant amount of blood from each animal, nine consecutive tubes were removed each containing an average of 0.08 ml of blood.

The method for determining the total number of neutrophils in the humerus of a mouse and the relation of this marrow neutrophil sample to the size of the total bone marrow neutrophil compartment have been reported in detail (7). In brief, mice are killed, the humerus is dissected free, the epiphyseal cap is pulled off, and the extreme distal tip of each epicondyle is removed. Cells are flushed from the bone by passing a measured volume of 1% ethylenediamine tetraacetic acid–saline solution through the humerus several

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times, a procedure found to remove at least 90% of the cells (7). A single cell suspension is obtained by passing the cell suspension in and out through a syringe and needle several times. The nucleated cell concentration is determined electronically (Coulter Counter, Model B, Coulter Electronics, Hialeah, Florida). This method for determining total nucleated cells in the humerus is reasonably accurate, with cell counts from the right and left humerus of the same mouse varying by an average of less than 10% (7). Smears for determining cell type are made from the femur and stained for peroxidase or with Wright's stain. From the percentage of neutrophils and the total nucleated cell count in the humerus the absolute number of neutrophils per humerus is calculated.

Total and differential leukocyte counts were determined from the first sample of blood (first microhematocrit tube) removed from the orbital sinus of nonanesthesized mice, and hematocrits were determined from the second microhematocrit tube. Circulating blood neutrophil concentration was calculated from the percentage of neutrophils in 200 cell differential counts from Wright's stained coverslip smears and the total leukocyte count. Band-to-segmented-neutrophil ratios were determined on the same smears. Segmented neutrophils were defined as those cells with a clearly demonstrable filament between at least two lobulations in the nucleus.

In order to determine the rate of erythropoiesis, 0.1 μc of radioactive iron (^{59}Fe) was injected subcutaneously. Six hours later mice were killed, the spleen and a femur removed, and the total radioactivity in spleen and femur was determined in a well-type gamma counter. The amount of ^{59}Fe taken up by the spleen and marrow is proportional to the rate of erythropoiesis in these organs (8, 9).

Statistical comparisons between experimental and control groups were performed using Student's t test.

Results. Time course of neutrophil changes after bleeding (Fig. 1). At 1, 2, 3, 6, 12, 24, 36, and 48 hr after removing 0.72 ml of blood, blood neutrophil concentration, blood band to segmented neutrophil ratio (band/seg) and total number of neutrophils per humerus were determined.

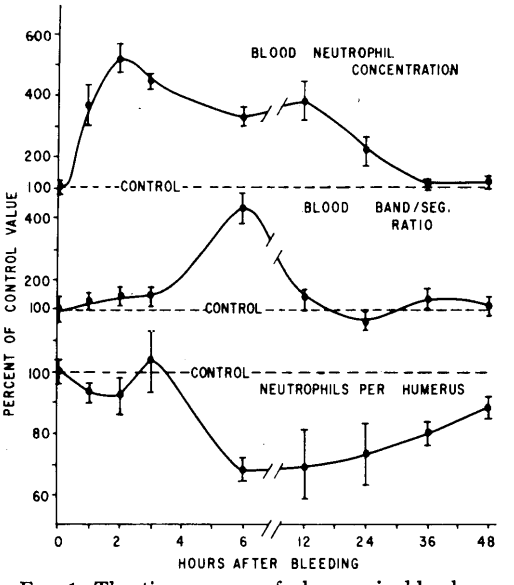

FIG. 1. The time course of changes in blood neutrophil concentration, blood band/segmented neutrophil ratio and total number of neutrophils per humerus in mice subjected to acute hemorrhage. Whole blood (0.72 ml) was removed from the orbital sinus of mice (approximating 40% of their blood volume) and at intervals thereafter, the above determinations were made. Each point on each curve represents a group of at least four mice (eight humeri), the bars surrounding the point indicate the standard error of the mean.

Within an hour after bleeding there was a significant increase in the concentration of blood neutrophils reaching a peak value at 2 hr and then gradually decreasing to normal levels by 36 hr. However, no significant change occurred in either the blood band/seg ratio or in the total number of neutrophils within the humerus during the first 3 hr after the acute bleed. By 6 hr highly significant changes occurred in these two values; the blood band/seg ratio increased to more than 400% of control and the neutrophils in the humerus decreased to 68% of controls. Blood band/seg ratio then promptly returned to normal and the number of neutrophils in the humerus gradually increased toward normal during the next 42 hr.

Changes in hematocrit and blood lymphocytes after acute hemorrhage. A decrease in

hematocrit was apparent within 1 hr and reached a minimum value (56% of control), (mean hematocrit of 29.7%) within 12 hr. A significant increase in hematocrit (from 56–70% of control) occurred between 24 and 48 hr after the acute bleed. Blood lymphocyte concentration decreased within 1 hr after hemorrhage, reached a minimum of 30% of control values within 6 hr and was returning toward normal levels by 48 hr.

Relationship between degree of bleeding and the number of neutrophils flushed from the humerus 6 hr after bleeding (Fig. 2). Groups of 8–12 mice were bled either 0.16 ml; 0.32 ml; 0.48 ml; or 0.72 ml of whole blood and 6 hr later the total number of neutrophils in the humerus was determined. A linear, arithmetic relationship between the amount of whole blood removed and the number of neutrophils flushed from the humerus was observed for the first three increments of blood removed. With removal of a larger volume of blood (0.72 ml), no further increase in the number of neutrophils flushed from the humerus was observed (Fig. 2A).

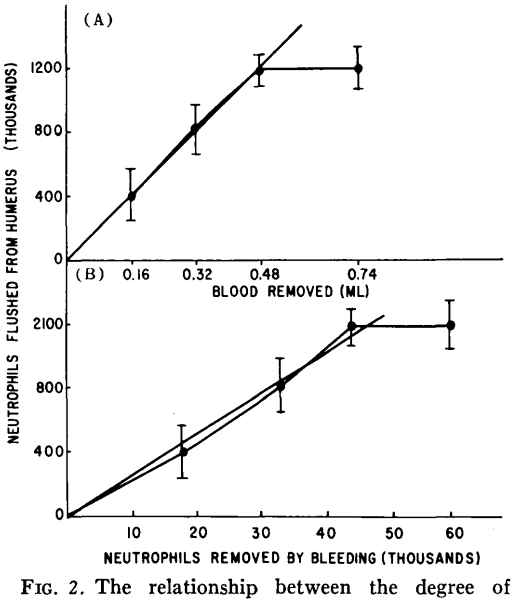

FIG. 2. The relationship between the degree of bleeding and the number of neutrophils flushed from the humerus. In Fig. 2A the amount of whole blood (milliliters) removed acutely by orbital sinus puncture is plotted against the absolute number of neutrophils which were flushed from the bone marrow 6 hr after the bleeding. In Fig. 2B, the number of neutrophils removed by bleeding is plotted against the number flushed from the bone marrow. Each point represents the mean of at least eight mice (16 humeri) and the bars surrounding each point represent the standard error for the group.

The number of neutrophils removed in successive microhematocrit tubes decreases since the neutrophil concentration decreases in consecutive samples of blood removed from the orbital sinus (7). Therefore, the total volume of whole blood removed does not exactly reflect the total number of neutrophils removed by bleeding. The total number of neutrophils removed with different volumes of bleeding was calculated from the known concentration of neutrophils in successive blood samples from the orbital sinus of a mouse (7), and plotted against the total number of neutrophils flushed at 6 hr (Fig. 2B). Again there was a near linear relationship between the number of neutrophils removed by bleeding and the number of neutrophils flushed from the marrow.

Uptake of radioactive iron in the spleen and marrow of bled mice. Within 12 hr after an acute bleed (0.72 ml blood removed) there was a significant increase in the amount of radioactive iron taken up by spleen and femur. By 48 hr radioactive iron uptake by the spleen was continuing to increase while at the same time, marrow radioactivity decreased to slighly below control values (Fig. 3). Marrow neutrophils began to increase while spleen ^{59}Fe uptake was still elevated (Fig. 3).

Discussion. The preceding data suggest that a complex series of changes in neutrophil kinetics produces neutrophilia after acute hemorrhage.

A maximal increase in blood neutrophil concentration was observed within 2 hr after acute hemorrhage. However, during this period of time, there was no significant change in the total number of neutrophils within the bone marrow (humerus) or in the ratio of band-to-segmented neutrophils in blood. This combination of events suggests that the initial increase in blood neutrophil concentration is a pseudoneutrophilia resulting from the demargination of intravascular neutrophils. The marginal pool of the mouse may

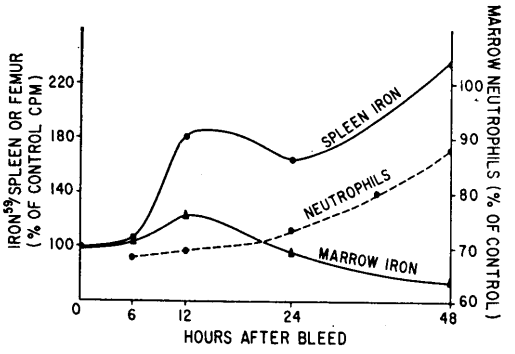

FIG. 3. Amount of radioactive iron (^{59}Fe) taken up by the spleen and femur 6 hr after ^{59}Fe injection in mice bled 6, 12, 24, and 48 hr before being killed. The labeled lines represent spleen and femur uptake of radioactive iron and the dotted line in the figure represents the neutrophil number in bone marrow (same line as in Fig. 1).

be much larger than the circulating pool (7), so that the degree of neutrophilia observed is achievable by demargination.

Between the third and sixth hour after acute hemorrhage there was a marked decrease in the total number of marrow neutrophils. Therefore, during this time interval a marked acceleration in the rate of release of marrow neutrophils to the blood must have occurred. The change in band/seg ratio observed during this time also reflects the accelerated release of neutrophils from the marrow. The available segmented neutrophils are released from the marrow storage pool to the blood with some preference over bands (10). However, as release suddenly accelerates the proportion of bands released increases and therefore, the band/seg ratio increases.

Whether changes in the rate of neutrophil egress from the blood occurred as a result of hemorrhage cannot be determined from the present study. However, neutrophilia occurring as a result of decreased egress alone might be expected to develop more gradually than was observed in the present study. This mechanism should result in a decrease in the band/seg ratio as an increasing proportion of bands are given time to mature into segmented neutrophils within the blood.

We would suggest that the following sequence of events explains the kinetic changes observed in these studies. The acute hemorrhage leads to sudden changes in the vascular tone of the capillary and postcapillary venous network where most marginated neutrophils are found. This leads to demargination of neutrophils in these sites resulting in a decrease in the total number of marginated neutrophils. In the presence of a decreased marginal pool, a feedback mechanism (10) is initiated which leads to an increased rate of release of neutrophils from the bone marrow. The initiating stimulus for this feedback loop might be a decreased concentration of neutrophils in marginal sites *per se* or it might represent a decreased outflow of neutrophils to certain tissues. Egress from the blood is presumably from the marginal pool since the only mechanism demonstrated for loss of neutrophils from the blood is by diapedesis from capillaries or postcapillary venules. There was a more exact relationship between the total amount of blood removed and number of neutrophils released from the marrow than between the total number of neutrophils removed by bleeding and the number of neutrophils released which lends some support to the above hypothesis.

One third of the total number of neutrophils and neutrophil precursors was flushed from the marrow after 0.48 ml (25% of the total blood volume) of blood was removed. The degree of flush was not increased by further bleeding. This is the same magnitude of flush which was observed after large doses of endotoxin (11), and may represent the maximum number of neutrophils which can be discharged from the marrow.

Increased neutrophil production (as judged by an increase in marrow neutrophils after the initial decrease) developed while erythrocyte production (as judged by iron uptake into spleen and bone marrow) was accelerated (Fig. 3). Harris, Harris, and Kugler (12) found a reduced concentration of neutrophils and neutrophil precursors in a fixed volume of marrow in the guinea pig 3 days after acute hemorrhage. However, whether concentration was increasing or decreasing was not determined and whether or not concentration of neutrophils in marrow reflects total neutrophils in marrow is uncertain. There is evi-

dence suggesting that if the size of the pluripotential stem cell pool (which gives rise to erythrocytes and granulocytes) is decreased that an increased demand for red cell production leads to a decreased rate of granulocyte production (13). Furthermore, Lawrence and Craddock (14) presented evidence suggesting that in guinea pigs given antineutrophil antibody the subsequent rate of neutrophil regeneration was slowed if the animals were also bled. However, the normal animal probably has a surplus of stem cells and current evidence suggests that in a normal animal most pluripotential stem cells are at rest (15). Therefore, it is not surprising to find a concurrent increase in granulocyte and erythrocyte production.

Summary. Mice were bled and at intervals thereafter, blood neutrophil concentration, blood band/segmented neutrophil ratio and total number of neutrophils per humerus were determined. Within the first 3 hr after hemorrhage maximal increase in the circulating blood neutrophil concentration was observed. However, this was not accompanied by a significant increase in band/seg ratio in the blood nor was it accompanied by a significant decrease in marrow neutrophils. Therefore, the early neutrophilia after hemorrhage probably reflects a shift of intravascular neutrophils from the marginal to the circulating pool. However, between 3 and 6 hr after hemorrhage there was a striking increase in band/seg ratio in blood and a decrease in total marrow neutrophils. Thus, following the initial pseudoneutrophilia caused by demargination, an increase in the rate of release of marrow neutrophils occurred resulting in a decrease in marrow neutrophils and an increase in the band/seg ratio in the blood. It is suggested that this increase in release rate is triggered by a decrease in the size of the marginal pool.

A dose-response relationship between the degree of hemorrhage and the number of neutrophils removed as compared to the number of neutrophils flushed from the bone marrow into the blood was constructed. The relationship between the total amount of blood removed and number of neutrophils flushed was somewhat better than was the relationship between the total number of neutrophils removed from the blood and the number of neutrophils flushed from the bone marrow. It was therefore suggested that the acceleration of neutrophil release rate from bone marrow to blood was primarily a function of changes in intravascular tone rather than a function of actual loss of neutrophils from blood vessels.

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