Evaluation of Marrow Granulocytic Reserves in Normal and Disease States

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THIS REPORT presents a summarization of recent findings with respect to the kinetics of granulocyte physiology, and an attempt to interpret changes in granulocyte levels in light of these concepts.

The principles upon which the granulocyte data reported are based are as follows:

1. In the normal steady state granulocytopoiesis is confined to the marrow. There is general agreement that cell division in the granulocytic series does not occur after the myelocyte stage.

2. In the normal human in the steady state the granulocyte remains in the marrow for a period of several days, probably five to six days, after the last division of granulocyte precursors. After maturation occurs, the majority of granulocytes are released into the blood in an orderly fashion. This mass of granulocytic cells (which we have called the marrow granulocyte reserve, or MGR, and which is incapable of proliferation) comprises about five-sixths of the marrow granulocytic cell population. It is evident that this intramedullary mass of maturing cells is many times larger than the circulating mass of granulocytes. This was shown in leukopheresis experiments in normal and heavily irradiated dogs.1,2

Yoffey estimates the ratio of marrow to blood granulocytes at about one hundred to one in the guinea pig.3 Recently, Donohue et al.4 have estimated a ratio of 25 to 30 to 1 in the human, employing an ingenious method for quantitating the marrow erythroid elements with Fe59. Osgood arrived at a comparable relationship on the basis of his calculations.6

3. The rise in circulating granulocyte number in response to such stimuli as leukopheresis or the intravenous injection of bacterial endotoxin is due to the accelerated entry of granulocytes into the blood from the marrow granulocyte reserve.3,7 This was established by labeling granulocyte desoxyribose nucleic acid (DNA) and taking advantage of the time interval when the marrow granulocyte population was highly labeled relative to the circulating cells. Induction of a demand for increased granulocytes in the periphery at a time when the MGR is completely labeled will cause an acceleration in the rise in circulating granulocyte DNA-P32 specific activity. Accordingly, the majority of cells contributing to the peripheral demand came from the highly labeled marrow population and not from the unlabeled cells in various peripheral organs.

4. In the normal steady state, granulocytes which enter the peripheral blood from the

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marrow may circulate for a variable time. The average time granulocytes spend in the circulation is about 10 hours, as will be discussed subsequently. The peripheral circulation of granulocytes may be divided into two main compartments: (a) those freely circulating in the larger vessels, and (b) those "sequestered" or "trapped" in the capillary networks of various organs. The lung seems to play a role of major importance in this trapping of granulocytes. There is an interchange of cells between these two main peripheral compartments. The work of Ambrus and Ambrus has demonstrated the re-trapping of granulocytes. There is an interchange of cells between these two main networks of various organs. The lung seems to play a role of major importance in this interchange between the freely circulating compartment and cells in capillary beds has led to the belief that granulocytes leave and re-enter the blood stream many times during their total life span.

6. Granulocytes which have moved across the endothelial barrier between blood and tissues do not re-enter the circulation, at least not in numbers that approach those leaving the blood stream. No evidence has been presented showing a free interchange of granulocytes between blood and tissues. The evidence against such an interchange is considerable: (a) Leukopheresis experiments in irradiated dogs. These failed to show re-entry of granulocytes which had migrated into areas of infection. (b) Experiments such as that shown in figure 1. Here the preponderance of DNA-P labeled cells had left the blood stream prior to the time of stimulation, and these cells failed to re-enter the blood to cause a rise in granulocyte DNA-P. (c) Experiments such as those in figure 2. Here the granulocytes were "pulled" into a peritoneal exudate and not removed from the body. The DNA-P specific activity of the granulocytes in the peripheral blood does not correspond with that in the peritoneal exudate, indicating a lack of rapid interchange between the granulocytes in these two compartments.

Findings such as this do not preclude the re-entry of some granulocytes from certain extravascular areas. They do indicate, however, that the primary direction of granulocyte movement is from the marrow into the circulating blood, into capillary beds, and then into the tissues. This would not seem incompatible with the known functions of these cells. The work of Hollingsworth et al. indicates the importance of granulocytes sequestered in capillary beds in regard to bacterial phagocytosis and that significant numbers may be in capillary beds despite circulating agranulocytosis.

The fact that the granulocytes circulating in the blood do not freely exchange with tissues outside means that the circulating cells do not represent the average age of all granulocytes in the periphery. No data exist as to the size of the "extravascular pool" of granulocytes or as to the time which the granulocyte survives in the tissues. (The term "tissues" includes all areas, secretions, body cavities, etcetera where granulocytes may enter.) It is reasonable to believe this time period will vary enormously depending upon the environmental conditions at the site at which the granulocyte moves across the capillary wall. It is illogical to assume that the granulocytes in bronchial secretions are of the same average age as those just entering the blood from the marrow. Therefore, the disappearance rate of DNA-labeled cells is not a reflection of the time granulocytes survive in the blood and tissues.

Kline and Clifton, and subsequently several other investigators, have interpreted the disappearance rate of labeled granulocytes from the blood as indicating a "life span" of the granulocyte of 13 to 20 days. Similar figures have been obtained with Cr and S labeling. Ottesen concluded on the basis of his experiments that the average age of the circulating granulocyte was 9 days, implying a much longer total "life span." All of these experimenters assumed that the circulating granulocytes were in "equilibrium" with those in the tissues, and all ignored the very large intramedullary mass of maturing granulocytes which was also labeled by these technics.

An additional observation which indicates the error in interpreting granulocyte DNA labeling in terms of survival time of granulocytes is that the disappearance rate of DNA-
Fig. 1.—In dogs rendered leukopenic by irradiation, the administration of typhoid after most of the P32-labeled leukocytes have left the peripheral blood does not result in an increase in leukocyte DNA-P32 activity.

Fig. 2.—Creation of peritoneal exudate with removal of granulocytes into the exudate. This was done at a time when the DNA-labeled granulocytes were first entering the blood from the marrow. Note that the blood granulocyte specific activity rises much faster than that in the peritoneal cavity. Also note the fall in blood leukocyte number despite the movement of cells into the exudate.
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labeled cells is not greatly accelerated in conditions known to be associated with increased granulocyte destruction. In patients with pyogenic infections, the fall-off of DNA-labeled granulocytes is not markedly altered. The rise and fall of blood granulocyte DNA-P32 is primarily a reflection of the turnover of the MGR. The labeling of the MGR DNA with $^{32}$P follows a definite pattern and depends upon the period of time which labeled precursors are available and upon the dilution of the DNA label by cell division. The granulocytes which first enter the MGR after labeling are the most highly labeled, and as they move through this maturing pool they are succeeded by cells whose DNA is less highly labeled as a result of division of myelocytes which are in turn, less highly labeled. This process may be depicted diagrammatically as shown in figure 3.

The size of the MGR is so much greater than that of the circulating mass of granulocytes (fig. 4.) that acute fluctuations in the latter cause very little alteration in the turnover of the MGR. The turnover time of the MGR in the normal human is five to six days. Estimating the size of the MGR at about $140 \times 10^{10}$ granulocytes in the 70 Kg. man, as others have done, this would mean that sufficient numbers of cells are entering the peripheral blood from the marrow (1 $\times 10^{10}$ per hour) to replace those circulating every 6 to 12 hours. Since the evidence previously cited does not indicate recirculation of granulocytes, it appears that the average time a granulocyte spends in the bloodstream is of this order of magnitude. It should be re-emphasized that this is an average figure and that some granulocytes may be held in capillary beds much longer and that others may go into the tissues soon after entering the blood. This randomness of granulocyte removal from the blood agrees with the data of Athens et al. using DFP32 labeling.

This concept of granulocytopoiesis envisions the blood granulocytes as the "top layer" of a larger intramedullary mass of granulocytes which are proliferating and growing in an orderly and predictable manner, in the normal steady state. The granulocytes which leave the marrow and enter the blood and then the tissues are "expendable" cells, in the sense that their function is to be completed shortly after this time. An acute demand for granulocytes is met by the release of cells from the "maturing" marrow granulocyte pool (MGR) at a rate exceeding the basal rate. Since the size of the MGR is so much larger than the mass of granulocytes in the peripheral circulation, the latter can be doubled or tripled in size by a slight acceleration of the release of cells from the MGR.

The nature of the stimulus to the marrow and the mechanisms involved in the release of granulocytes are beyond the scope of this paper. It would seem, as stated in an earlier publication, that the fundamental stimulus involves the removal of cells. Leukopheresis, or the removal of white cells without the introduction of known toxic factors, seems to be a sufficient stimulus. The possible humoral mediation remains to be demonstrated convincingly.

With these concepts in mind, an attempt has been made to obtain a clearer view of blood granulocyte alterations. The acute leukocytosis observed after intravenous injection of bacterial endotoxin will not occur unless the marrow contains granulocytes (3). The material employed here, Pyrexal, is a lipopolysaccharide from Salmonella abortus equi.

This material in doses of 0.1 to 0.2 gamma intravenously causes very little in the way of systemic symptoms, except, rarely, slight transient chilliness.

*We are indebted to Wander & Co. for generously supplying this agent, and especially to Dr. Fred Schultz for his cooperation.
The hematologic effects of Pyrexal are nonspecific and resemble those observed after intravenous injection of a variety of bacterial toxins and foreign proteins. The material has been employed in Europe by Heilmeyer\textsuperscript{20} and Eichenberger and associates\textsuperscript{21} for the assessment of marrow function. The present studies

Fig. 3A (top), B (middle) and C (bottom).—Schematic representation of the dilution of the DNA label by myeloid proliferation. By the time the marrow reserve of nonproliferating granulocytes is labeled, the most highly labeled cells enter the blood first.
extend the use of this agent to a variety of hematologic diseases and describe certain refinements in interpretation.

The following main points concerning the response to Pyrexal, many of which have been observed by others, are noteworthy.

In the low dosage used, there is, in the normal human, a slight fall in total count, which is inconstant, followed by a rise, which is invariable (fig. 5). The early fall, occurring immediately after injection, is due usually to lymphopenia (fig. 6), although there is often a transient fall in granulocytes at the same time. The leukocytosis, which reaches a maximum in 6 to 10 hours, involves granulocytes only. The lymphocytes usually remain low and there is an absolute eosinopenia. It is necessary to express the response to Pyrexal in terms of granulocyte numbers rather than total white cell count. Instances were encountered in which changes in total WBC count might be considered insignificant and yet the granulocyte increase was adequate, the total WBC being unchanged because of lymphopenia. This is apparent in figure 5, in which the maximum total WBC in one instance was only 125 per cent and in most instances between 140 per cent and 160 per cent. Calculating the percentage increase in terms of granulocytes yielded a maximum of 180 per cent or more in all normals, the average increase being about 200 per cent.

The normal values for absolute number of circulating segmented neutrophils are 3000 to 5800 per cu.mm. with an average of 4000 per cu.mm. With the use of these values, the normal response to Pyrexal involves an increment of at least 2400 segmented granulocytes per cu.mm. The average normal response was an increase of 4000 granulocytes per cu.mm. Another characteristic of the normal response is the absence of a “left shift” in the granulocytes, no increase in the percentage of immature forms being observed with this type of stimulation at the dosage employed. As in all values pertaining to circulating leukocyte numbers, there is considerable latitude of normality. At present, we
believe the response to Pyrexal is not significant unless it is far below the response observed in normals to date.

The data to be presented represent examples of how the Pyrexal test has been employed. The results are of interest in terms of correlation with the over-all clinical picture and with the concepts discussed previously. They are not to be interpreted as showing a predictable pattern in any given disease situation since this will require more data than are presented here.

**Examples of the Pyrexal Test in Hematologic Disorders**

Aplastic Anemia

Figures 7a and b show the results in two cases of aplastic anemia. In the first case (7a) the patient required infrequent transfusions and exhibited a mild leukopenia and thrombocytopenia. The peripheral blood picture showed a moderate "left shift" in the neutrophilic series and the marrow was markedly hypocellular in all elements. The acute response of granulocytes to Pyrexal was markedly depressed. The later slow rise by 24 hours has been noted in many instances of marrow depression and will be discussed subsequently.

The second case (7b) is an example of a patient with a comparable leukopenia associated with a drug-induced pancytopenia. At the time of the Pyrexal test the patient was recovering, though still leukopenic, and the marrow appeared normal. The response was qualitatively and quantitatively normal in every respect. In contrast with the previous case, at no time was any "left shift" observed in the granulocytic series, a finding compatible with the interpretation of adequate marrow reserves of granulocytes.

Lymphomas

The granulocyte response to Pyrexal has been entirely normal in 10 cases of Hodgkin's disease with normocellular marrow and without marked splen-
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Fig. 7a (top left).—The acute leukocyte response involves an increment of only 150 granulocytes per cu.mm., a markedly deficient response in a patient with known marrow depression.

Fig. 7b (top, right).—Leukocyte response to Pyrexal recovering from bone marrow aplasia. The increment in granulocytes was 3700 per cu.mm.

Fig. 8 (bottom, left).—Lack of acute response to Pyrexal in a patient with Hodgkin's disease receiving x-ray and mustard therapy. The elevation of count at 24 hours is frequently seen in patients with marrow depression and is not a normal response.

omegaly. Patients who have marrow depression as a result of therapy exhibit a deficient response. Figure 8 is an example of this in a patient who had received a course of nitrogen mustard two weeks previously. The delayed increase by 24 hours is again noted.

Figure 9 shows the response in a patient with diffuse lymphosarcoma with marrow depression, invasion of the marrow by lymphosarcoma, splenomegaly, mild anemia and thrombocytopenia. Although there was not a marked total leukopenia, there was a relative lymphocyte increase and a definite “left shift” in the granulocytic series. The granulocytes failed to increase after Pyrexal. Subsequently the patient received a total dose of 7.5 mg. of triethylene melamine in divided doses over a three week period. The tumor was markedly sensitive to the agent but at the same time the patient became severely agranulocytic and almost expired. He later entered a state of complete remission.

This case demonstrates that extreme depletion of reserves of granulocytes can occur without profound peripheral leukopenia. The morphology of the marrow, the lack of response to Pyrexal and the “left shift” in peripheral granulocytes all indicated this. The remaining granulopoietic tissue could maintain the granulocytes in the peripheral blood but there was no marrow reserve.
of granulocytes. Suppression of granulocyte production for even a brief time resulted in severe agranulocytosis.

**Leukemia**

Figures 10, 11, 12 and 13 show responses to Pyrexal in selected cases of acute and chronic leukemia. They demonstrate the necessity of calculating the absolute increase in granulocytes in determining the adequacy of marrow granulocytic reserves. Thus, in figure 11, although the percentage rise in granulocytes is not marked, the total increment in granulocytes exceeds the normal. In figure 13 the granulocyte response appears fairly good on a percentage basis, but involves only a very small increase in total number of granulocytes. Figure 12 is of interest in that this patient with typical chronic lymphocytic leukemia shows a normal response to Pyrexal despite marked lymphocytic infiltration of the marrow. The preservation of marrow granulocytic reserves and the ability to develop a granulocytic leukocytosis on demand may be factors in the generally benign course of patients with chronic lymphatic leukemia.

**Aberrant Responses**

When splenomegaly with “hypersplenism” exists, the interpretation of the response to Pyrexal becomes more difficult. The spleen may be removing leukocytes from the circulation more rapidly than normal and may possibly exert a depressant effect on the marrow. An example of the effect of an enlarged spleen is shown in the study of a case of congestive splenomegaly associated with posthepatic cirrhosis and portal hypertension, in which hepatic function tests were normal. Ferrokinetic and Cr51 studies in this patient showed the spleen to be removing red cells from the circulation at an accelerated rate prior to surgery. The patient underwent portocaval anastomosis because of esophageal varices, the spleen being left intact. The spleen rapidly decreased in size, and repeat isotope studies showed no further removal of red cells by the spleen. Figure 14 shows the response to Pyrexal before and after portocaval anastomosis. The time interval between the two studies was 10 days. The qualitative similarity but quantitative difference of the granulocytic response
Fig. 10. — Leukocyte response to Pyrexal. The granulocyte increment was 877 cells per cu.mm., a very deficient response.

Fig. 11. — Leukocyte response to Pyrexal. The increment in segmented granulocytes was 13800 per cu.mm., indicating adequate marrow reserves of these cells.

Fig. 12.—Leukocyte response to Pyrexal. Note that the granulocytic increase was normal quantitatively as well as qualitatively, the peak at two hours involving an increment of 8293 segmented granulocytes per cu.mm.
Fig. 13.—Leukocyte response to Pyrexal. Note that the maximum rise in granulocytes involves an increment of only 735 cells per cu.mm.

Fig. 14.—Leukocyte response to Pyrexal before and after portocaval anastomosis in a patient with pancytopenia associated with congestive splenomegaly due to posthepatitic cirrhosis. Pancytopenia disappeared following surgery.

to Pyrexal is clearly shown. One interpretation of this is that the spleen no longer exerted its "parasitic" effect on red cells, and presumably white cells and platelets, as shown by the labeled red cell data. These findings could also be compatible with a humoral influence of the spleen on the marrow, and do not serve to elucidate this much debated point. The results are presented to indicate the difficulties in interpretation of hematologic data of this type in the presence of "hypersplenism." Since splenomegaly is a prominent feature of many conditions in which evaluation of marrow function is important, this represents a handicap in the application of this type of study.

Instances of Divergence of Marrow Morphology and Function

The reserves of marrow granulocytes cannot always be gauged from scrutiny of marrow morphology alone. The marrow space is only partly filled by cell precursors, and extension of the hematopoietic space can readily occur. Such must have been the case in a dog in which marked erythroid hyperplasia and
preponderance was induced by repeated phlebotomy (600 to 1175 ml. per week) for four months. This dog's nutritional condition and general health were excellent throughout this time. He was subjected to procedures producing leukocytosis on several occasions and responded normally (figs. 15 and 16). Platelet recovery was likewise normal (fig. 17). The intense erythroid hyperplasia, which might suggest encroachment upon granulocyte production on histologic examination, did not take place at the expense of marrow reserves of granulocytic tissue. This example is presented to emphasize that morphologic
Fig. 17.—Response of blood platelets to depletion by leukopheresis in a dog rendered anemic (see figures 15 and 16) as compared with normal.

examination does not always permit assessment of myelopoiesis. This discrepancy was also apparent in the case of chronic lymphocytic leukemia (fig. 12).

COMMENT

The development of an acute rise in blood granulocyte level in response to a stimulus such as that caused by Pyrexal intravenously will occur only if there exist reserves of granulocytes which can be quickly released into the blood. Normally the bone marrow contains the bulk of granulocytes which can be quickly mobilized, and whether other tissues may assume this capacity of “holding granulocytes in reserve” remains to be determined. This would not seem to be the case in agnogenic myeloid metaplasia of the spleen, in which the normal delay before the appearance of DNA-labeled cells after giving P32 is not observed (fig. 18).

Since leukopenia can exist without depression of marrow reserves of granulocytes, the response to Pyrexal would seem to offer valuable additional information in the interpretation of leukopenia. A normal response is strong evidence of adequate reserves of granulocytes. A negative or deficient response, however, must be correlated with the clinical findings as well as with blood and marrow granulocyte morphology. Failure to develop leukocytosis may be due simply to the rapid removal of cells from the blood, as in leukopheresis, or as in the experiment in figure 2, in which huge numbers of cells were entering the peritoneal cavity despite a fall in circulating concentration. An analogous situation might prevail in patients with infection or “hyper-splenism.” In the absence of such complications, a deficient response to Pyrexal
Fig. 18.—The appearance of DNA-P\textsuperscript{32}-labeled granulocytes in patient with myeloid metaplasia of the spleen and myelofibrosis as compared with normal. Note the very early appearance of labeled granulocytes.

together with a “left shift” in the peripheral blood granulocytes strongly suggests deficient intramedullary reserves of granulocytes.

**Summary**

Recent concepts of the relationship of the blood granulocyte mass to the marrow reserve of granulocytes have been reviewed. Evidence has been presented to show that the marrow is the chief area of granulocyte “reserves” or “stores.” The development of acute leukocytosis in response to a stimulus such as the intravenous injection of bacterial endotoxin depends upon release of cells from the intramedullary pool of granulocytes.

The turnover of the marrow granulocyte reserve (MGR) is an orderly process in the steady state, and determines the form of the curve of DNA-labeled granulocytes in the peripheral blood. From estimates of the turnover time of the MGR it appears that the granulocyte spends an average time of about 10 hours in the peripheral blood. Granulocytes do not appear to recirculate once they have left the peripheral blood and have entered the tissues. However, granulocytes may be sequestered within capillary beds for variable periods and may re-enter the circulating blood from such areas. Such cells are not to be considered as having re-entered the blood from the tissues.

The intravenous injection of a purified bacterial lipopolysaccharide as a stimulus to acute leukocytosis is described. The possible usefulness of this procedure in assessing the MGR is discussed.

**Summario in Interlingua**

Es presentate un revista de recente conceptos concernente le relation inter le massa del granulocytos in le sanguine e le reserva de granulocytos in le medulla ossee. Es presentate observationes que indica que le medulla es le major area pro le “immagasinage” de “reseervas” d granulocytos. Le disveloppamento de leucocytosis acute in responsa a un stimulo como per exemplo le injection intravenose de endotoxin bacterial depende del liberation de cellulas ab le fundos intramedullari de granulocytos.

Le movimento del reservas medullari de granulocytos (RMG) es un processo regulate quando le organism se trova in un stato de stabilitate e
determina le forma del curva del presentia in le sanguine peripheric de granulocytos marcate con acido disoxyribonucleic. Super le base de estimaciones del tempore de transition del RMG il pare que le granulocytos passa al media circa 10 horas in le sanguine peripheric. Apparentemente le granulocytos non re-entra in circulation post que illos ha quitate le sanguine peripheric pro entrar in le tissus. Tamen, granulocytos pote esser sequestrate durante un variabile periodo de tempore in le vasculatura capillari, e ab tal areas illos pote re-entrar in le circulation. Tal cellulas non es considerate como habente retornate ab le tissus.

Le injection intravenose de un purificate lipopolysaccharido bacterial como stimulo de leucocytosis acute es describite. Le utilitate possibile de iste manovra in le evlaulation del RMG es discutite.

REFERENCES

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