MASTER

THE HUNGRY GRANULOCYTE
ITS FATE AND REGULATION OF PRODUCTION *

E.P. Cronkite

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Brookhaven National Laboratory
Medical Department
Upton, N.Y. 11973/USA

present address:
Nuclear Research Center Jülich
Institute of Medicine
5170 Jülich

Alexander von Humboldt USA
Senior Scientist Awardee

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Any discussion about granulocytes is incomplete without an initial reference to the work of Ilya Metchnikoff, Professor of Anatomy University of Odessa. As a political dissident with czarist policies he was obliged to leave Russia. He took his family and microscope to Messina, Sicily. His enquiring mind could not rest. One evening he commenced a simple study on defensive mechanisms by placing a rose thorn into a starfish embryo. In the morning he found the thorn surrounded by cells apparently in the process expelling it. This commenced a life long study of cellular defense mechanisms. In 1882 Pasteur invited him to Paris. He discovered two phagocytic cells in mammals - the macrophage and the granulocyte. In addition he described phagocytosis, the killing of bacteria and observed chemotaxis along with other mechanisms of cellular defenses against infection. In 1908 he shared the Nobel Prize with Paul Ehrlich and used his share of the prize money to support research at the Pasteur Institute. It was easy in those days to make discoveries - so little was known and high technology had not arrived.

Today as a result of electron microscopy, identification and separation of subcellular constituents by homogenization and density gradient centrifugation with biochemical degradation of granules into their constituent enzymes and antibacterial agents we know the granulocyte is an end cell laden with potent hydrolytic enzymes and antibacterial agents, packaged in

granules and ready to be injected into the phagocytic vacuole to kill and digest invading bacteria.

The essentials of phagocytosis and killing of bacteria are:

1. Recognition and phagocytosis - usually through opsonins the bacteria are recognized. Cytoplasmic processes surround the bacterium enclosing it within the granulocyte but in a vacuole that is lined by cell membrane.
2. Degranulation and injection of granule contents - granules line up on the phagocytic vacuole and inject their potent enzymes.
3. Metabolic events include increased oxygen consumption, production of peroxide and free radicals, a drop in pH etc.
4. Killing of bacteria is accomplished by a combination of peroxide and free radicals, myeloperoxidase and the activation of the antibacterial cationic proteins by a falling pH amongst a whole series of biochemical events still under study.

The preceding capsule summary is a condensation of 40 years of work by a large number of individuals notable Hirsch, Karnofsky, Janoff, Bainton, Zucker-Franklin to mention a few.

Thus the mature granulocyte is a non-dividing cell laden with potent enzymes and antibacterial agents, packaged in granules and waiting to engage bacterial invaders in battle. One granulocyte can phagocytize an average of 50 bacteria before its granules are exhausted.

To fight infection effectively there must be a continuous supply of large numbers of granulocytes. In their absence or seriously diminished supply as after large amounts of whole body irradiation the subject succumbs to overwhelming bacterial infection if the bone marrow does not renew the supply.

The granulocyte is short lived and disappears randomly from the blood with an average life span of about 11 hours. A large number are produced in man of about 6.79×10^7 per kg per hour or 1.5×10^{11} per day in the average man (Cartwright et al.1). They are produced in the bone marrow. My associates and I have been concerned with the kinetics of production and its regulation since the techniques for following cell proliferation were developed in 1956. Prominent in these studies were T.M.Fliedner now Prof. Clinical Physiology, Univ. Ulm and L.E.Feinendegen now Prof. Medicine Univ. Düsseldorf and Director Institute of Medicine KFA Jülich.

The key to these studies was the synthesis of tritiated thymidine, a specific DNA precursor that allowed one to label pro-

liferating cells *in vivo* and then follow their movement by Hughes (2) in US at Brookhaven and by Verly (3) in Belgium almost simultaneously. It allows high resolution autoradiography because of its low energy beta emission.

In figure 1 is shown a schematic notion of a cellular self renewal system. The stem cell has the capability of self renewal and of feeding cells into a multiplication compartment in which the cells have a few amplification mitoses along with continuing maturation. Cell division is terminated presumably as cells lose receptor sites for stimulatory factors. Note that the stem cell in steady state retains one cell and supplies one cell for continuing differentiation. In the multiplication compartment the cell number doubles with each mitosis and is thus expanding exponentially.

In figure 2 are shown means by which one can study kinetics of cell proliferation. The top row shows the cell cycle - mitosis, rest, DNA synthesis, rest mitosis. During this sequence one can make measurements that allows one to determine the stage a cell is in and the rate which it progresses through the cell cycle. The middle row shows the changes in DNA content. DNA content is measured easily by microspectrometrical measurement of DNA content after staining DNA with Feulgen reagent. Cells with contents of DNA between $2n$ and $4n$ are in active DNA synthesis. Cells

with 4 n DNA are either in the pre-mitotic rest or in mitosis. The bottom row shows the cells in DNA synthesis that are labeled by the specific precursor, tritiated thymidine. With time one can measure the flow of this labeled cohort of cells through premitotic rest, mitosis and through another cycle when conditions allow.

In figure 3 is shown the movement of the labeled cohort through premitotic rest and mitosis. A highly idealized situation is shown at the top for a self renewal population without any variance in the phases of the cell cycle. Note that the cells proceed repeatedly through the cycle giving labeled mitosis curves of same shape with each cycle but of course with a halving of the intensity of label since the label, 3-H thymidine, is only available for a short time. The middle panel shows what happens when variance is introduced. The labeled mitosis curve is rounded off and the ratio of labeled mitoses falls to the ratio of the time of average DNA synthesis to the average generation time. The bottom panel shows the labeled mitosis curve of granulocytic precursors of human bone marrow after a single injection of 3-H thymidine. There is no labeling of mitoses as the cells that were in mitosis and premitotic rest move onwards. As the leading edge of labeled cells enter mitosis the fraction of labeled mitoses increases sharply to near 100 % and remains at this level until the unlabeled cells that were in G₁ enter mitosis and then the fraction of labeled mitoses falls

sharply. The distance between the 50% labeling of mitoses on the ascending and descending limbs approximates the average DNA synthesis time. In a series of studies Stryckmans et al. (4) have shown the DNA synthesis time of erythroid and granulocytic precursors to be 12 - 13 hours.

Figure 4 shows an example of the increase in labeling of metamyelocytes after 3-H thymidine injection into man from Cronkite et al. (5). It takes a minimum of 3 hours for cells to mature to metamyelocytes after the terminal myelocyte mitosis. Thereafter the labeling curve increases in this instance at about 3.3% per hour. If only labeled cells are migrating the turnover time is then 33 hours. If labeled and unlabeled were migrating in parallel the turnover time will be shorter.

Labeled cells proceed through the metamyelocytes, band and segmented neutrophils in an orderly manner. Since the turnover time of the first compartment of the non-dividing pool is measured the average turnover times of the other two is calculated on the basis of their compartment size (Cronkite et al. 5; Cronkite and Vincent 6).

Labeled neutrophils first emerge into the peripheral blood after about 96 - 120 hours (Fliedner et al. 7, 8). Granulocytes have two modes of exit from the blood one random and a senescent process that truncates the random loss after about 30 hours. This is shown in figure 5. In the steady state granulocytes enter the blood at the same rate as they are lost. Fliedner et al. (8) has shown that it takes on the average 30 hours for the label on normal granulocytes to appear on pyknotic dying cells. Thus the random loss from the blood irrespective of time after entrance into the blood is truncated by a senescent process 30 hours after entrance into blood. It is further assumed that the senescent cells are lost by a rapid random process.

From all of the above studies Cronkite and Vincent (6) constructed a deterministic model for granulopoiesis. This is shown in figure 6. The model states that there must be an average influx from the stem cells of 0.4×10^7 /kg/hour into granulopoiesis. Note that the vertical axis is flux (cells/kg/hour) and time is the horizontal axis. Accordingly the area under the curve represents absolute cellularity (cells/kg). The stepwise function plotted arises from plotting average fluxes and time. There is one mitosis at the myeloblast level after an average of 18 hours.

Another mitosis at the promyelocyte level with a generation time of about 24 hours and then two successive myelocytic mitoses with an average time of 54 hours for each generation. This is followed by a minimum of 3 hours of maturation before labeled cells appear as metamyelocytes with an average time as metamyelocytes of 33 hours, 57 hours as band neutrophils and nearly 70 hours as marrow segmented neutrophils or a total of 166 hours on the average before appearance in the blood.

Since DNA synthesis time is 12 hours and G_2 plus mitosis 4 hours the G_1 phase is 38 hours. G_1 cells are diploid hence 38/54 or 0.7 of the myelocytes must be diploid or the model is not correct. Boecker et al (9) determined the DNA content of human myelocytes.

About 70% of the cells are within the diploid range of DNA content. In separate studies it has varied from 68 - 72%. Thus one condition required for the model has been met. From the model one can also predict that certain things might happen when infection requires an increase in granulocyte production. For example a means of increasing granulocyte production might be a shortening of the myelocyte generation time allowing more mitoses at the terminal end of the production line. If this were to happen the ratio of myelocytic to erythrocytic mitoses would have to increase and a greater fraction of myelocytes would be labeled in

addition to fewer diploid myelocytes. For ethical reasons this study could not be performed in man and accordingly was studied in the dog (10).

Animals were given tritiated thymidine and the bone marrow and blood were studied to determine the flow of cells and fraction in DNA synthesis and the ratio of myeloid to erythroid mitotic figures. The animals were rested for a month and then sterile inflammation was induced and the studies repeated.

Inflammation was initiated 24 hours before injection of ^3H -thymidine and simultaneously with ^3H -thymidine. The inflammation was induced by turpentine injection into the uterus.

As expected the inflammation produced a striking and prompt increase in the granulocyte count in the blood of about 4 times the control level. The changes in the bone marrow are shown in figure 7.

On the top of this figure is shown the ratio of myeloid to erythroid mitotic figures. The solid line is the control ratio. The dashed line shows the increase within 24 hours by a factor of three meeting the first requirement for our hypothesis. The lower three panels on the left show the changes in the fraction of granulocytic precursors labeled by thymidine. The solid black line shows the control levels. The middle dashed line shows the influence of giving thymidine simultaneously with inflammation and the upper line when inflammation

is initiated 24 hours before administration of thymidine. The results are a clear cut increase in the fraction of myelocytes labeled (M_{3+4}) fulfilling the second requirement of the hypothesis. What was not anticipated is the expansion of proliferation into the normally non-dividing metamyelocytes the M_5 panel. The lower left panel shows the more rapid transit through the non-dividing pools as anticipated. The right panels show that there is an increased intensity of labeling in the myelocytes after inflammation. This may result from a decreased duration of DNA synthesis and/or an increased efficiency in utilizing tritiated thymidine in the salvage pathway.

The changes in the periphered blood are shown in figure 8. The upper and middle panels shows the very rapid emergence in the peripheral blood of cells labeled at the terminal myelocyte level. The usual emergence time is 48 - 52 hours. Inflammation and ^3H TdR simultaneously shortens the emergence time to 16 - 20 hours. When inflammation precedes ^3H TdR by 24 hours the emergence time is decreased to 8 - 16 hours. The bottom panel shows the change in the peripheral blood. One will note that the trauma of repeated anesthesia and bone marrow puncture produces a small increase in granulocyte count. Since the dogs became quite ill a hysterectomy was performed. In each of the 5 dogs there was an increase in the granulocyte count suggesting that removal of an inflammatory sink for granulocytes allowed the granulocyte count to increase since the bone marrow production rate had been set higher in response to the

inflammation. For the remainder of the study (88 hours) the count remained elevated.

Granulopoiesis has also been studied in plasma clot diffusion chambers (PCDC) inserted into the peritoneum of the irradiated mouse and grown for 8 days. The diffusion chamber is Lucite one cm in diameter and 2 mm in thickness. Millipore filters 0.45 μ m pore size are cemented to the ring. The sterile chambers are filled with 190 μ l suspension of cells in plasma which is allowed to clot before implantation. When one places 200 cells in the diffusion chamber, let's it clot and then examines microscopically there are no pairs or triplets of cells. With time one can see the emergence of pairs of cells by 24 - 36 hours, groups of cells up to 30 or so by 48 - 72 hours and larger colonies at later intervals. As the cells proliferate they also migrate and produce small satellite groups of cells in some instances.

The quantitative aspects of growth in the PCDC are shown in figure 9. In this instance 2000 bone marrow cells were introduced into the PCDC obtained from regenerating bone marrow in order to have a high fraction of cells that are further to the left as illustrated in figure 11 and thus are more immature and able to produce in principle larger colonies. In figure 9 one sees colonies that are unidentifiable appearing as early as 24 hours increasing in number and size and peaking at 36 - 48 hours and then declining in number although some are still present throughout the culture period. By 36 - 48 hours granulocytic colonies appear and increase in

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number until 4th day and then decline in number as colonies degenerate. However, some colonies continue to grow and become enormous by the 8th day. The dimensions of some of these colonies are shown in figure 10.

When one assumes a domain of 10^3 cubic μm for a single cell and estimates the volume of the macroscopic colonies one "guesstimates" that these colonies have 0.25 to 1.0×10^6 cells. To produce this number of cells from a single precursor requires 18 - 20 mitoses. Since cells are dying, migrating out of the colonies and also becoming non-mitotable granulocytes, the actual number of mitoses is greater than the estimate. This number of mitoses is substantially in excess of what one would predict in a model that has an influx from a self-renewing stem cell pool followed by exponential amplifying mitoses as illustrated in figure 1 and figure 11. At this stage one must consider the possibility of substantial self renewal of the precursor cells that produce these huge colonies.

The differences between in vitro and in vivo studies are illustrated in figure 11. This figure is based on studies in man and mouse. At the top our estimates of the numbers of mitoses from the stem cell to the non-dividing pool of cells in vivo are shown to be 9 - 11 mitoses with a minimum transit time of about 4.5 - 5.5 days. At the bottom are shown observations on the in vitro and diffusion chamber culture. One would expect cells close to the border of the non-dividing maturation pool to produce smaller colonies and those close to the stem cell to produce larger colonies but in exponentially

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decreasing numbers and an increasing time to produce the large colonies. Data are not sufficiently extensive as yet to test the model that assumes exponential expansion of the non-recognizable pool of cells. At this stage one must consider the possibilities of extensive self-renewal and also that cells may at any time interval after starting down the granulopoietic pathway enter the identifiable pool of granulocytes without going through a specified number of mitoses. In other words the system may not be a pipeline with exponential expansion.

Experimental data on regulation of granulopoiesis are accumulating rapidly and considerable insight into regulation is now available. This is shown schematically in figure 1.

The flow of cells from the stem cell pool on the left to the blood on the right is shown. Between the stem cells and the maturation and storage pool there is exponential growth.

Regulatory feedback loops involving humoral factors have been established (see review Cronkite 1979). Inflammation and/or granulocyte decrease results in liberation of an alpha-1 glycoprotein-leukocytosis inducing factor LIF which promptly discharges storage leukocytes into the blood. Concomitantly the concentration of serum colony stimulating factor, CSF increases. CSF is a mandatory factor for proliferation of granulocyte in tissue culture and is

presumed to be an in vivo regulator of the early progenitor cells although rigid experimental proof has not been obtained. Inflammation as discussed earlier also increases the terminal number of myelocyte divisions either through an as yet undisclosed humoral factor or some population density sensing system.

Short range or short lived humoral regulatory loops have been established (Dexter et al. and Lord et al). Regenerating bone marrow produces a factor that accelerates the proliferation of the stem cells. Steady state bone marrow produces a factor that puts stem cells out of cycle. The kinetics and the regulation of granulopoiesis are well on the way to being understood. A few uncertainties remain. Whether this knowledge can be put to use in therapy of disease is yet to be shown.

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Regulation of Granulopoiesis

Long Range Humoral Feedback Loops

Short Range Feedback Loops

BASIC FACTS ABOUT H^3 -THYMIDINE CELL LABELING.

LABEL IS METABOLICALLY STABLE BUT
CONCENTRATION IS HALVED AT EACH MITOSIS:

Fig. 3

6a

6b

Fig. 6

GRANULOPOIESIS

In Vivo

- Figure 1 Schematic presentation of granulopoiesis from pluripotent stem cell pool to the functioning cells in peripheral blood. The pluripotent stem cells at left are self-renewing for every mitosis one cell remains a stem cell and one cell progresses down a designated pathway undergoing amplifying mitoses and maturation.
- Figure 2 Presentation of the cell cycle, the changes in DNA content as the cell progresses through the cell cycle and labeling of a cohort of cells in DNA synthesis that can then be followed as they move through the cell cycle.
- Figure 3 Shows the flow of a cohort of labeled cells from DNA synthesis through mitosis indicating the changes in the fraction of labeled mitoses that will be observed with time after labeling of the cohort in DNA synthesis.
- Top - Labeled mitosis curve no variance in phases of the cell cycle.
- Middle - Labeled mitosis curve rounded out by variance in phases of cell cycle.
- Bottom - An example of labeled mitosis curve for granulocytic precursors of a human being. Time for DNA synthesis equals distance between 50 % on the ascending and descending limbs of the curve.
- Figure 4 An example of the flow of granulocytic precursors labeled at the terminal myelocytic mitosis into the first non-dividing granulocytic cell compartment at a rate of 3.3 % per hour for a replacement (turnover) time of 30 hours.
- Figure 5 Compartmentalization of the blood granulocyte pool in steady state. The number of cells leaving the blood by two processes - random and senescent equals the number entering the blood. This is shown at the top. The bottom curve shows the age of granulocytes after entry into the peripheral blood truncated by the senescent process at 30 hours. The senescent cells are assumed to be lost randomly.

- Figure 6 On the deterministic model averages for flux and time are plotted. This gives the model the biological unreal stepwise function and does not show the variance known to be present. The area under the curve represents the absolute cellularity in cells per kg body weight since time and flux in cells per kg per hour are plotted.
- Figure 7 The ratio of myeloid to erythroid mitotic figures, the fraction of myelocytes, metamyelocytes, bands and segmented granulocytes, the mean grain count for all granulocytic cell types are shown after sterile inflammation is induced either 24 hours before or simultaneously with administration of 3-H-TdR to label bone marrow granulopoietic cells in DNA synthesis.
- Figure 8 The emergence time for labeled granulocytes in the peripheral blood and the granulocytosis induced by trauma of obtaining bone marrow and from sterile inflammation are shown.
- Figure 9 The developmental sequence of unidentified immature colonies, granulocytic and erythrocytic colonies are shown for an eight day culture period in plasma clot diffusion chambers.
- Figure 10 Dimensions of the projections of selected macroscopic granulocytic colonies after 8 days of culture in plasma clot diffusion chambers.
- Figure 11 Comparison of notions about in vivo granulopoiesis and in vitro and diffusion chamber granulopoiesis showing the discrepancies between number of mitoses between the stem cell and the non-dividing granulocytic cells.