Autoradiographic Studies of Leukocyte Formation.* (24462)

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Results of recent investigations by Hamilton(1), Ottesen(2), Osgood(3), and Resegotti(4) have shown a considerable difference between life spans of granulocytes and lymphocytes in mammals. Their studies indicate a life on the order of several days for granulocytes and several weeks for the majority of lymphocytes. The classical technic of thoracic lymph duct cannulation(5), however, has shown in several mammals that enough lymphocytes enter the circulation from thoracic lymph alone to replace the entire blood lymphocyte pool several times daily. Comparable to this is a life span for the granulocyte of 23 minutes from parabiotic rat experiments of Van Dyke and Huff(6). Classical transfusion experiments as well as recent transfusion experiments with tagged cells have also indicated rapid disappearance of injected leukocytes(7). The apparent conflict may be resolved by employing a label for newly formed leukocytes permanent for the life of the cells, and simultaneously studying cells in hematopoietic tissues and circulation. The autoradiographic technic used here enables a measurement to be made of the activity of individual cells which have incorporated a radioactive DNA precursor. We report data based on analyses of labeling seen in different classes of white cells in blood and blood-forming tissues and analysis of their separate life spans. This is not possible by the biochemical methods employed in the past.

Methods. Adenine C^{14} is a convenient label for cells synthesizing new DNA during an interval at start of experiment(8). Adenine is quickly taken up in cells in the form of nucleotides and at some later time is synthesized into both RNA and DNA. It is now generally agreed that once DNA has been synthesized from nucleotides, it remains and is stable for the life of the cell, or until it is segregated at the next division. Active DNA in a cell is then a permanent label for the cell. 8.5 μC adenine 8-C^{14} (2.2 mC/mm) were injected intraperitoneally into 10-11 week-old male A strain mice weighing 23 g. The animals were sacrificed at regular intervals following injection and smears were made from various tissues—tail blood, thymus, lymph nodes, and femoral marrow. Smears were fixed in absolute methanol for 1 hour, followed by distilled water 1 hour to remove unpolymerized nucleotides. Duplicate smears were also digested with ribonuclease to remove RNA. Then essentially the only remaining activity is due to adenine incorporated into DNA. Smears were next prepared with English Kodak Ltd. AR-10 autoradiographic stripping film(9) and exposed 30 days. Then the slides were photographically processed and subsequently stained through the film with Giemsa stain. Grain counts were made over individual cells and results recorded for a minimum of 1000 cells/slide. A cell was considered labeled if it had more than 2 grains, which was above the background. Lymphocytes of diameter <8 μ were classified as small lymphocytes, 8 to 10 μ as medium lymphocytes, and >10 μ as large lymphocytes. Band neutrophils in the marrow were distinguished from mature forms by the smooth ring shaped nucleus. When the nucleus showed some folding it was considered to be a mature neutrophil.

Results. Fig. 1 shows the strikingly different courses of adenine incorporation into DNA in the 3 major classes of white cells in peripheral blood. Labeled neutrophils rise sharply from the second to fourth day after injection, by which time more than half the cells are labeled. This is followed by a rapid decline. Labeled small lymphocytes, on the other hand, form a plateau at 2-3% that lasts at least 8 days. This indicates that most of the small lymphocytes were formed before start of experiment in contrast to neutrophils. Correspondingly, the life of the small lymphocyte is long compared to the neutrophil. In contrast to the small lymphocytes a high per-

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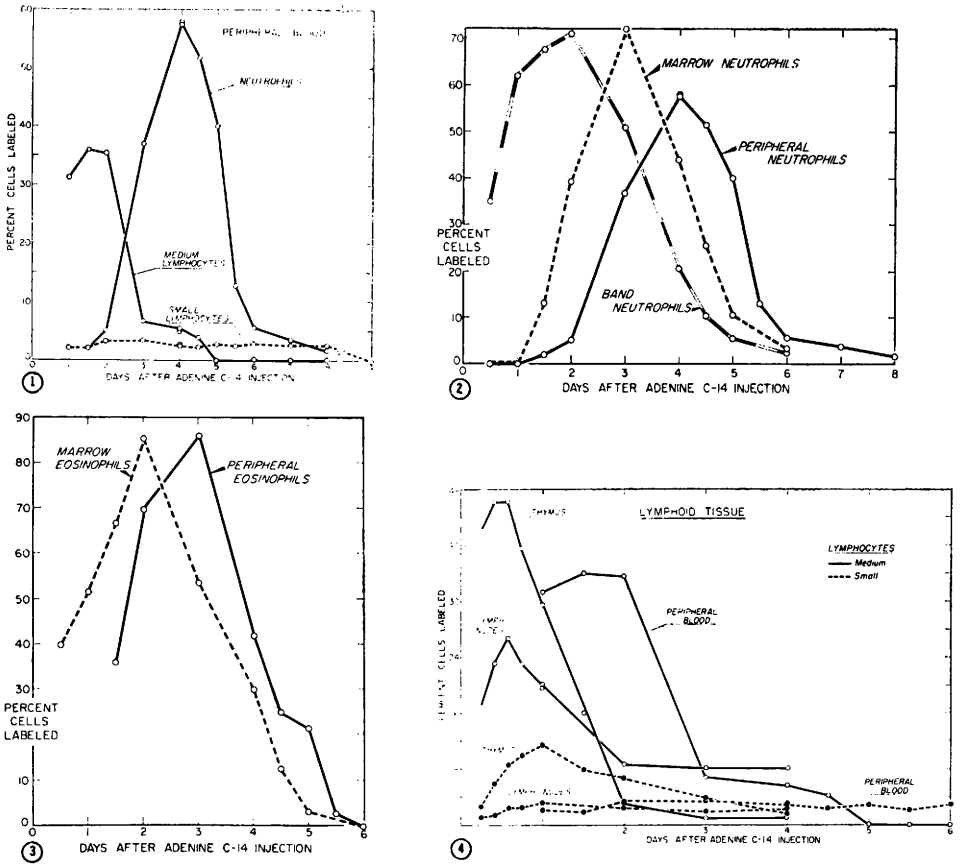

FIG. 1. Percent of circulating leukocytes with labeled DNA after a single inj. of adenine C-14.

FIG. 2. Percent labeled neutrophils seen in marrow and peripheral blood after a single inj. of adenine C-14.

FIG. 3. Percent eosinophils labeled after a single inj. of adenine C-14.

FIG. 4. Percent DNA labeled lymphocytes seen in lymphoid tissues and peripheral blood after a single inj. of adenine C-14.

centage of labeled medium and large lymphocytes were found at 1 and 2 days and disappeared by fifth day.

To interpret the curve for neutrophils in peripheral blood, data must be known on the immediate precursors in marrow. Fig. 2 shows percentage labeled cells for the last 2 maturation stages of neutrophils—the band and mature neutrophils in the marrow—together with the peripheral neutrophils. It should be noted that these values are from bone marrow smears not extracted with ribonuclease since this treatment would make marrow cell identification impossible. Peripheral neutrophils contain little RNA label, however, since RNase treatment does not measurably lower the activity of this class. This may be true of mature marrow neutrophils since they have lost the staining property for RNA in the cytoplasm.

Data of Fig. 2 show that labeled cells do not enter the mature marrow neutrophil pool until about 1 day, suggesting that this is the minimum time required for differentiation of mature neutrophils from the last division stage. The preponderance of band cell forms over earlier stages of differentiation as well as the initial portion of the band neutrophil curve, indicate that almost a whole day is spent in the band cell stage. This may be taken as a maximum for band cell maturation time.

These observations may be used to approximate relative pool sizes and turnover times

of marrow and peripheral neutrophils. Consideration of the time relation between labeling, seen in band and mature marrow neutrophil classes, indicates that release of mature neutrophils to circulation tends to be orderly rather than random. Similar consideration of the relation between labeling seen in mature marrow neutrophils and peripheral neutrophils indicates that neutrophils disappear from the periphery at the end of a finite life span rather than randomly. The relation between the 2 classes has been approached by a comparison of experimental blood values with theoretically predicted values derived from labeling, in mature marrow neutrophils which are the immediate precursors to the peripheral neutrophils. The best fit of predicted values to experimental values has been obtained with a finite time of ⅔ day for duration of mature cells in marrow, followed by 1 to 1⅓ day life span in the periphery.

Such computations for levels of labeled cells agree closely with the experimental, whether we choose population of all cells labeled, or autoradiographically more definitive population of cells having more than 10 grains. These 2 independent calculations do not exclude the probability of a small degree of randomness in release of cells from each pool. The presence of a few band cells in the circulation indicates that some cells are randomly released from the marrow.

The steady state requires the flux of cells through each pool to be constant and equal. Therefore, if V_1 and V_2 are sizes of band and mature marrow neutrophil pools respectively $\frac{V_1}{T_1} = \frac{V_2}{⅔ \text{ day}}$ where T_1 is maturation time of band cells. The marrow differential indicates that $V_2/V_1 = 0.8$. T_1 is then about 20 hours in good agreement with the value derived from labeling.

If V_3 is the size of the peripheral neutrophil pool, then applying the steady state condition $\frac{V_2}{⅔ \text{ day}} = \frac{V_3}{1 \text{ to } 1⅓ \text{ day}}$. Therefore, V_3/V_2, the ratio of peripheral to marrow neutrophils is between 1.5 and 2. Since the circulating vascular pool is known to be quite small compared to the mature marrow pool, it must be concluded that peripheral neutrophils spend a large part of their time extravascularly. This agrees with conclusions published recently by several authors (for review see Patt, 10).

Approximate data on eosinophils are presented in Fig. 3. Eosinophils regularly comprised only 7-10% of granulocytes so that each point represents only 30 to 60 cells counted. This has precluded any quantitation such as was attempted with neutrophils; however, it is clear that labeled eosinophils appeared and disappeared more rapidly than did neutrophils in the circulation.

Data on events in lymphoid tissues are presented in Fig. 4. The most striking finding is the high activity of mouse thymus compared to lymph nodes. By the third day, however, essentially all active cells have disappeared from the thymus, indicating that turnover time for thymus cells is in the range of 2 to 3 days.

Lymph nodes reach a much shallower peak and retain a comparatively large proportion of active cells for at least 4 days. This suggests that lymph nodes have a long turnover time compared to thymus. These turnover times are in good agreement with our biochemical estimates from P^{32} incorporation into DNA—2 days for thymus and 1 week for lymph nodes in A strain mice.

Labeled medium lymphocytes of nodes, thymus, and blood reach a higher peak earlier in time than that of small lymphocytes in these tissues. This is consistent with the hypothesis of a precursor-product relation between these 2 classes of cells. In addition, early label in the small lymphocyte class could be due to a few small lymphocytes preparing for division in accordance with the scheme of Sainte-Marie and Leblond(11).

In peripheral blood labeled small lymphocytes plateau at 2-3% for the first 8 days and disappeared by the fourteenth day. Small lymphocytes therefore live for at least 8 days. A large part of this time is probably spent in the organized or diffuse lymphoid tissue rather than in the circulation. The simplest interpretation of the data appears to be that cells released from the nodes into the circulation represent a random sample of cells pres-

ent in the nodes at any time. This would reconcile the apparent discrepancy between the short circulating life calculated from thoracic duct outputs and the long life obtained from DNA labeling. To clarify this further the present experiments are being repeated using tritiated thymidine as DNA precursor. To date excellent agreement has been obtained between adenine and thymidine data. Thoracic duct lymphocytes are labeled to a degree consistent with the above concept (Schooley, Bryant, Kelly, to be published).

Summary. 1. Twenty-four hours are required for maturation of the neutrophil from the last division stage in the marrow. 2. The data are best explained by a finite life of ⅔ day for mature neutrophils in marrow, followed by a peripheral life span of 1 to 1⅓ days. 3. Lymphocytes fall into two classes—medium and large cells, which are rapidly renewed, and small cells, which live at least a week.

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Effect of Endotoxin on Vascular Reactivity to Epinephrine in the Perfused Dog Forelimb and Lung.* (24463)

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Recent publications have indicated that endotoxin may have a direct effect on blood vessels, altering their reactivity to epinephrine (1-4). Changes in reactivity to epinephrine were reported to occur in blood vessels of the isolated perfused rabbit ear and in the mesoappendix of the rat following administration of small doses of endotoxin. Injections of larger doses resulted in vascular hyperreactivity to epinephrine followed by hyporeactivity. Arterioles and venules became completely refractory to epinephrine, while increased reactivity persisted in larger arteries and veins. The work of Zweifach, Nagler and Thomas(1) suggested very large changes in vascular reactivity after endotoxin in the rat mesentery, ranging from 100 times less, to 700 times greater than the pre-endotoxin response. It was postulated that changes in reactivity to epinephrine might be responsible for damaging effects of endotoxin on the vascular systems of intact animals and could account for certain resemblances between endotoxin reactions and "intoxication" by epinephrine(2). It was suggested that the end result of altered reactivity to epinephrine was pooling of blood in distended capillaries and venules(1). A number of vascular responses to endotoxin have been reported from this laboratory(5-8). Constrictions of blood vessels in a variety of organs have been observed

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