Cyclic Fluctuation in Blood Granulocyte DNA Labeling: Comparison of Tritiated Thymidine and Radiophosphorus


DNA labeling studies with $^3$HT and $^{32}$P have been done in horses. Frequent sampling has defined cyclic fluctuations in blood granulocyte DNA label content with a cycle time of around 27 hr. Simultaneous labeling studies in the same animals have produced blood granulocyte DNA labeling curves that are nearly identical indicating significant reutilization of $^3$HT.

Fluctuation in the percent of labeled cells in the blood of dogs after a single injection of tritiated thymidine ($^3$HT) has been reported by Patt and Maloney, and Fliedner et al. have reported that a single injection of $^3$HT in man is followed by fluctuations in blood granulocyte DNA label concentration as indicated by radioautographic measurements of grain counts. The fluctuations, apparent only after maximum labeling was achieved, were visible in the labeling index for all labeled cells and also in the labeling index for classes of cells defined by any particular range of grain counts. Fluctuations of blood granulocyte DNA label concentration have also been observed in orthoradiophosphate ($^{32}$P) labeling studies of rabbits.

Assessment of detailed changes in blood granulocyte DNA label content requires sampling at intervals as short as feasible with as little perturbation of the system as possible. To accomplish this studies were done in the horse, an animal from which blood can be obtained relatively easily and in which perturbations due to the volume of blood removed for sampling should be minimal. Fluctuations in blood leukocyte DNA label concentration which were cyclic were demonstrated both with tritiated thymidine and orthoradiophosphate.

Tritiated thymidine has been considered to represent a pulse or flash label with an availability of less than an hour while it has been shown that $^{32}$P as orthophosphate is available in significant concentration for several days after a single intravenous injection. These studies compare leukocyte labeling with $^3$HT and $^{32}$P simultaneously in the same animal to eliminate all variables other than the labeling characteristics of these two sub-
stances. The blood leukocyte DNA labeling curves obtained with a single simultaneous injection of these two labels were virtually identical indicating that labeling kinetics with these two substances are very similar, however achieved.

MATERIALS AND METHODS

Nine studies were performed in six different horses. Each horse received by injection into an external jugular vein either 3μCi of P3/kg or 3μCi of 32P/kg plus 5μCi of 3HT/kg. Frequent early samples for determining plasma clearance were obtained in heparinized syringes from the opposite jugular vein from an indwelling Cournand needle. Otherwise blood samples were obtained at intervals after isotope administration by individual venipuncture of either jugular vein. Leukocytes were isolated by dextran sedimentation with removal of platelets by differential centrifugation and lysis of residual red cells by exposure to hypotonic saline. Lymphocytes were isolated by incubating heparinized blood on cotton columns to which granulocytes and monocytes adhere. Cover slip smears of the leukocytes in the final isolates were prepared from a portion of the cells resuspended in horse serum and 200 cell differential counts made from Wright's stained smears. Total and differential white cell counts and the volume of packed red cells were determined daily on whole blood. The DNA of the isolated leukocytes was obtained by a modification of the Schmidt-Thannhauser procedure and DNA phosphorus quantitated by the method of Berenblum and Chain. In these studies DNA was hydrolyzed by heating in 0.5 N HClO4 at 100°C for 3 hr. Radioactivity of DNA samples was determined by liquid scintillation counting in a Nuclear of Chicago Mark I Liquid Scintillation Spectrometer. The scintillation mixture consisted of reagent grade toluene containing by weight 0.5% 2,5 dephenyloxazole (PPO) and 0.01% p-bis-[2-(4 Methyl-5 phenyloxazolyl)]-benzene (dimethyl POPOP) mixed five volumes to one with Triton X-100. A clear, stable emulsion was produced by 10 ml of this mixture and 0.5 ml of the 0.5 N HClO4 hydrolysate of DNA. No quenching of 32P was apparent and tritium counting efficiency in this system was about 35%. In doubly labeled samples, 1.2% of 32P counts spilled over into the tritium channel. No tritium counts appeared in the 32P channel. The third channel was used to monitor tritium quench with the external Ba standard. Samples were counted usually to the 1% level (cold...
samples were counted to at least the 3% level) and were corrected appropriately for background, spillover, quench, and decay from time zero. $^{32}$P activity has been expressed throughout as CPM/μg of DNA-P while $^3$H activity has been converted to DPM/μg of DNA-P by correcting for the degree of quench determined for each sample.

Plasma clearance of $^{32}$P was determined by measuring the radioactivity of the acid extractable phosphorus as previously described. $^5$ Tritium clearance from plasma was measured by determining $^3$H radioactivity /ml of plasma. An aliquot of each sample was dried by lyophilization, reconstituted with water and the $^3$H activity counted. This activity represented nonvolatile $^3$H while the difference between these counts and those of the whole plasma were taken to represent the counts in tritiated water.

**RESULTS**

The plasma clearance of $^{32}$P in the horse after an intravenous injection (Fig. 1) is comparable to that in man$^6$ and rabbit,$^5$ and the acid extractable radioactivity measured in plasma appears to be mainly in the orthophosphate form in which it is injected.$^8$ After an intravenous injection of $^3$HT in the horse, regression of plasma $^3$H activity (Fig. 2) also resembles that in man$^4$ but here it is obvious after a short time that most of this activity is in the volatile fraction, presumably as tritiated water. The nonvolatile $^3$H activity was not further identified, but in other species has proved not to be $^3$H-thymidine since that compound is rapidly metabolized.$^4, 9$

In one study, lymphocyte labeling was evaluated on daily samples for 9 days and the results are shown in Fig. 3. The lymphocyte labeling curve is low and flat for the 9 days of study while labeling in the mixed leukocytes shows the characteristic rise beginning on day 5. This pattern of lymphocyte labeling is the same as that found in previous studies.$^5$ During
the first 4 days the concentration of label in the DNA of the mixed leukocytes is nearly equivalent to that in the lymphocytes isolated from each sample. Since about 70% of the mixed leukocytes are granulocytes, the concentration of label in granulocyte DNA must be approximately the same as in the lymphocytes. In no instance were there enough monocytes to readily explain the amount of label in the granulocyte fraction. No undulations of granulocyte DNA label concentration are visible in these samples obtained at daily intervals. Convincing fluctuations in DNA label concentration were not noted in two other studies in which samples were obtained at daily intervals.

When sampling is done at 6 or 3-hr intervals, fluctuations in granulocyte DNA label concentration are apparent, as shown in the four studies in Fig. 4. The time scales of the four studies have been arranged in the graph to adjust for the duration of the low plateau in order to align the peaks. In section B there are two clear peaks that are 27 hr apart as are the three peaks seen in section D. The inception of frequent sampling was poorly timed in the study shown in C and the first cycle is not defined clearly enough to determine the interval between the first and second peaks. In the study in section A the first peak is not as clearly seen as in B and D although sampling was performed at 3-hr intervals. The animal in which this study was performed had changes in total and differential blood leukocyte counts during the course of study from a low of 3000 with 79% granulocytes to a high of 11,500 with 87% granulocytes. No other horse had significant variations in total or differential counts and no horse had a decrease in volume of packed red cells during study. The study in section D was done in a horse that had throughout the period persistent neutrophilic leukocytosis with a total count of 12–14,000 cu mm with about 85% segmented neutrophils. Even though the earlier rise in blood granulocyte DNA label content would be consonant with more rapid transit through storage, the interval between the peaks in DNA label content appears to be the same as that in section B. This horse received 3.5 rather than 3.0 $\mu$Ci of $^{32}$P/kg, accounting in part for the higher concentration of DNA label. Cell samples from this horse also had a greater per cent of granulocytes than those from the other animals contributing further to the height of the DNA labeling curve. No change in the cycles is produced by adjusting any of the DNA labeling curves for variations in the proportions of lymphocytes and granulocytes.
Three studies were done using $^{32}$P and $^{3}$HT simultaneously. The DNA labeling curves from one such study are plotted in Fig. 5. The curve for $^{3}$HT labeling and that for $^{32}$P labeling are strikingly similar and the cyclic fluctuation in label content is obvious. It should be noted that the regression of the curve for $^{3}$HT labeling parallels that for $^{32}$P after an initial slightly more rapid regression from the maximum.
DISCUSSION

These studies in horses confirm the presence of cyclic fluctuations in blood granulocyte DNA label content and provide somewhat better definition of them by more frequent sampling. Cyclical fluctuations in granulocytopoiesis have been proposed on the basis of cyclical changes in blood granulocyte counts, but it is not likely that the fluctuations in blood granulocyte DNA label have any relation to this. Mathematical considerations dictate that the cyclical rise and fall of blood granulocyte DNA label is the consequence of essentially asynchronous replication in marrow with the dividing cells drawing construction materials from a pool in which labeled precursor rises abruptly from zero to a high concentration and then falls away rather rapidly. Mathematical considerations dictate that the cyclical rise and fall of blood granulocyte DNA label is the consequence of essentially asynchronous replication in marrow with the dividing cells drawing construction materials from a pool in which labeled precursor rises abruptly from zero to a high concentration and then falls away rather rapidly. The duration of high label concentration in the precursor is short in relation to the length of the generative cycle. More extensive studies of the consequences of altering the cell's generative cycle time or duration of the S period, the rate of DNA synthesis during different parts of the S period, label availability functions, degree of randomness of cell passage through the post mitotic pool, and the like have been published. As suggested earlier by Patt and Maloney the period of the cycles of blood granulocyte DNA label fluctuation is determined by the generative cycle of the replicating marrow elements. The generative cycle of the myelocytes dividing for the last time after label is introduced overpowers all other parameters including the cycle time of cells destined to undergo one or more subsequent divisions, while variations of parameters such as circulating half-time effect trivial changes in the cycle period. The data from these studies suggest that the last myelocyte generative cycle of the horse is around 27 hr. No explanation is obvious for the study (Fig. 4A) in which two peaks are apparent 54 hr apart unless that animal had myelocytes with a terminal generative cycle of 54 hr, or that change from the steady state occurred during the study, permitting expression of greater variability of labeling of cells or of release from storage into blood.

The visibility of cyclic undulations in blood granulocyte DNA label content necessitates an orderly passage through the storage pool as random exit from the postmitotic pool into blood completely dampens the oscillations. Additionally, a random exit from storage into blood would cause the blood granulocyte DNA activity to rise immediately and would preclude the occurrence of the low plateau that lasts for several days. Boggs et al. have interpreted their DF labeling studies in dogs as being compatible with first in first out kinetics in the postmitotic pool.

It is apparent that by day 1 after label injection there are in blood some granulocytes that are labeled. In radioautographic studies in man using 3HT labeled granulocytes have not been noted in blood for as long as 3 days after label injection. This difference may be due to lightly labeled cells that are missed by radioautographic techniques but are detected by counting in the aggregate. To account for labeled blood granulocytes soon after label injection, some cells must exit storage other than in a strictly orderly manner. In one model a wide variance about the time of exit from
storage has been utilized to explain experimental observations but analysis indicates that variance about time of exit must be rather small else the cyclic undulations in blood granulocyte DNA label will be damped and not visible.

The nearly identical labeling curves obtained simultaneously in the same animal with $^3$HT and $^{32}$P must be reconciled with the view that $^3$HT represents a "flash" or pulse label while $^{32}$P is known to be available for a much longer time. It can be shown that, other parameters held constant, a flash label will result in a halving of the DNA label peaks with each succeeding cycle while a non-flash label will produce a less rapid regression with succeeding cycles, the rate of regression being related to just how long label is available in high concentration in the precursor pool. The curves obtained in these studies are not compatible with a flash label. In view of the rapid degradation of $^3$HT there must be significant reutilization from DNA to provide label for incorporation into DNA formed after the first hour or so. $^3$HT reutilization has been clearly demonstrated in rodents. It has been said that such reutilization does not complicate the analysis of first generation (after label injection) labeled cells but it seems conceivable, especially if the generative cycle is very long, that label that has been incorporated into other cells immediately after label injection may well find its way to first generation cells still in or coming into their DNA synthesis period.

REFERENCES


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