Red Blood Cell Life Span Using DFP$^{32}$ as a Cohort Label

By Martin J. Cline and Nathaniel I. Berlin

Diisopropylfluorophosphate (DFP) belongs to the class of chemical compounds known as phosphorofluoridates. The phosphorus-fluorine bond is strongly polarized with the result that DFP is a highly reactive compound. DFP interacts with biological materials to form esters; probably with the hydroxyl groups of serine.$^{1,2}$ Its most prominent biological interaction is with cholinesterase which it irreversibly inhibits.$^{3}$ During the labeling process the phosphorus-fluorine bond of DFP is broken and the resultant diisopropylphosphate is no longer capable of active protein labeling.$^{4}$

In 1947, Grob, Lilenthal, Harvey and Jones$^{5}$ suggested that DFP might be used to measure the life span of the circulating erythrocyte. These investigators found that after administration of DFP, red blood cell cholinesterase returned at a rate consistent with new cell synthesis. This rate can be used to calculate the red cell life span, assuming steady state conditions prevail.

In 1954, Cohen and Warringa used DFP labeled with phosphorus-32 to measure red cell life span in man.$^{6}$ Since that time several investigators have used DFP-32 as an erythrocyte marker in animals$^{7,8}$ and in man.$^{9,10}$ In these studies, a population of cells heterogeneous with respect to age was labeled either in vitro or in vivo.

The red blood cell has a limited capacity to bind DFP; consequently, it is theoretically possible to label a population of cells of similar age. A large blocking dose of unlabeled DFP is given to an experimental animal and after a wait of several days a smaller dose of DFP labeled with P$^{32}$ is then given. If saturation of the available red cell sites has been achieved by the blocking dose, then DFP-32 will label only those red cells synthesized in the period between administration of the blocking and isotopically-labeled doses.

DFP-32 as a cohort label would be superior to isotopically labeled metabolites such as glycine-2-C$^{14}$ and radioiron in that it is not reutilized after destruction of the labeled red cell.

Methods

Adult mongrel dogs of either sex, weighing between 14 and 25 Kg., were dewormed with butyl chloride and kept for at least four months before being used for study. Microhematocrits were performed on heparinized blood, using the method of Strumia et al.$^{13}$

$^{13}$-glycine-labeled erythrocyte survival: Following the intravenous administration of 40 $\mu$C of glycine-2-C$^{14}$, heparinized venous blood samples were obtained at approximately weekly intervals for 98 to 120 days. After removal of the plasma, the red cells were washed three times with saline and hemolyzed with an equal volume of distilled water. The stromal lipids were extracted with toluene and the partially purified hemoglobin solution was

From the Metabolism Service, National Cancer Institute, National Institutes of Health, Bethesda, Md.


Blood, Vol. 19, No. 6 (June), 1962
GLINE AND BERLIN

combusted to carbon dioxide and counted in a liquid scintillation counter after suspension in a thixotropic gel as barium carbonate.\textsuperscript{14} The erythrocyte life span was taken as the interval between the times at which the ascending limb and the descending limb of the curve of specific activity versus time reached one-half the maximal value.

DFP\textsuperscript{32} of specific activity 80–220 μCi/mg. was administered intravenously in sterile propylene glycol. Blood samples were obtained at three to ten day intervals after DFP\textsuperscript{32} administration, the plasma was separated, the red cells washed three times in cold saline and then diluted in an approximately equal volume of saline. The hematocrit of the red cell suspension was determined in quintuplicate and one ml. aliquots were pipetted in duplicate into disposable boats of aluminum foil and dried at 70°C. The boats were then sealed in cellophane, wrapped around a Geiger-Müller tube and counted. Correction was made for physical decay. Maximum counting error was three per cent.

For a population of DFP\textsuperscript{32}-labeled red cells heterogeneous with respect to age, the counts/min./ml. of cells plotted against time were fitted to a straight line by the method of least squares. The red cell life span was taken at the intersection of this line with the abscissa.

The life span of a cohort of red cells labeled with DFP\textsuperscript{32} was taken as the interval between the time of DFP\textsuperscript{32} administration and the time required to reach one-half the initial plateau isotope concentration.

**EXPERIMENTAL DESIGN AND RESULTS**

**Erythrocyte Uptake of DFP\textsuperscript{32}**

DFP\textsuperscript{32} from a single preparation was given to each of four dogs in doses varying between 0.03 and 0.26 mg./Kg. The erythrocyte uptake of DFP\textsuperscript{32} expressed as counts/min./ml. of red cells at time 0 was found to be dose dependent with a rapid increment in erythrocyte uptake in the dose range between 0.01 and 0.03 mg./Kg., and with a slower increment thereafter (fig. 1).

The fraction of the administered dose of DFP\textsuperscript{32} which was bound to erythrocytes was calculated from the uptake per ml. of red cells and an estimated total red cell volume of 38 cc./Kg. An inverse relationship between the dose of administered DFP\textsuperscript{32} and the fractional erythrocyte uptake was found (fig. 1).

**Erythrocyte Life Span by Random Labeling**

Four dogs were each given a single injection of DFP\textsuperscript{32} from one of several preparations in doses ranging between 0.02 and 0.12 mg./Kg. Blood radioactivity was followed for 70 to 84 days. A linear decrease of radioactivity with time was found (fig. 2). Erythrocyte life spans of 114 to 139 days with a mean of 125 days were obtained. This range of erythrocyte survival time is comparable to that found in the normal dog using C\textsuperscript{14}-glycine.

**The Effect of a Blocking Dose of Unlabeled DFP**

Four animals were each given a single injection of unlabeled DFP in doses of 0.02 to 1.0 mg./Kg. Twenty-four hours later, a dose of 0.04 mg./Kg. of either of two preparations of DFP\textsuperscript{32} was given. A similar dose of DFP\textsuperscript{32} was injected into two control animals who had received no prior unlabeled

\*Obtained from New England Nuclear Corp.
Fig. 1.—Erythrocyte uptake of DFP³² as a function of dose of administered DFP³². The per cent labeling is that percentage of the administered DFP³² that is bound to circulating red cells.

DFP. Each control dog received DFP³² from one of the two preparations used in the experimental group.

When compared to the two control dogs, the animals who had received unlabeled DFP prior to receiving DFP³² showed a reduced erythrocyte uptake of label which was proportional to the blocking dose (fig. 3). In the "blocked" animals there was a rapid decrease in blood radioactivity lasting four to six days, and then no further significant fall in the red cell P³² content to day 35, indicating that there was no removal of labeled cells.

Labeling a Cohort of Red Cells with DFP³²

Five dogs were each given a blocking dose of a 0.5 mg./Kg. of unlabeled DFP. Six or nine days later, DFP³² from either of two preparations was given to each dog in a dose of 0.04 to 0.05 mg./Kg. The red blood cell radioactivity was followed for 29 to 70 days. After an initial period of rapidly decreasing radioactivity lasting between four and 12 days, the red cell P³² content then remained unchanged or fell less than 0.25 per cent per day during the remainder of the observation (fig. 4). The low initial specific activity and physical decay of the isotope precluded further counting after 40 to 70 days in four animals. One animal died from unrelated causes 29 days after administration of isotope.

The data were interpreted as demonstrating that the tracer dose of DFP³² had labeled a cohort of cells of similar age, in this case ranging either be-
between one and six days or one and nine days of age, and that there had been no significant loss of labeled cells during the period of observation.

Application of Cohort Labeling to the Erythrocytes Produced in Response to Acute Blood Loss

Four dogs were each given a red cell blocking dose of unlabeled DFP (0.5 mg./Kg.). Twenty-four hours later they were each phlebotomized of 12 cc./Kg. of whole blood, approximately 15 per cent of their blood volume. Each of the four dogs was given a single injection of 40 μc of C14-glycine on a different day following bleeding. The intervals selected for glycine administration were one day, two days, six days and nine days after bleeding. All dogs were given DFP32, 0.04 mg./Kg. on the sixth day following bleeding. In all instances the venous hematocrit had returned to prephlebotomy.

Fig. 2.—Red cell life spans obtained by “random” labelling with DFP32.
levels by the seventh day. Higher initial DFP$^{32}$ specific activity and the availability of a larger number of cells for labeling as a result of an increased rate of synthesis in the bled dogs permitted a longer period of observation of erythrocyte isotope content than in the non-bled control animals of the previous experiment.

In all the animals, the red cells labeled with DFP$^{32}$, presumably the cells synthesized from 0–6 days after bleeding, had shortened survivals and the plot of DFP$^{32}$ content against time showed patterns of random erythrocyte destruction (fig. 5) in all animals except one (L697). This is to be contrasted with the absence of significant random erythrocyte destruction in the comparable time period in non-bled animals (fig. 4). The presence of random destruction in the bled animals permitted the calculation of only an approximate red cell life span with DFP$^{32}$. The estimated erythrocyte life spans are given in figure 5.

The red cells labeled with C$^{14}$-glycine administered one, two and six days after bleeding were also randomly destroyed and had shortened life spans. In the animal receiving C$^{14}$-glycine nine days following phlebotomy, the survival of the red cells labeled with glycine was normal and there was no random destruction (fig. 5). In this animal the initial sharp fall in erythrocyte DFP$^{32}$ content was only partially explicable on the basis of an increasing hemoglobin mass.
Discussion

Hjort and his colleagues demonstrated that the erythrocytes of man, the rabbit and the rat have a limited capacity to bind DFP. This phenomenon was confirmed by Athens and his co-workers by the finding that the red cell uptake of DFP in man was decreased by prior administration of unlabeled DFP. The ability to saturate the DFP-binding sites of the erythrocyte makes possible the labeling of a cohort of red blood cells of similar age with DFP. Such a cohort label has several advantages over labeled metabolites such as Fe and C-glycine. With DFP the age of the cells tagged is known. With C-glycine and to a lesser extent with radioiron there is a small component of the labeling "pulse" that is prolonged.

DFP is converted to DIP and cannot be reutilized, whereas iron and glycine from erythrocyte globin return to their metabolic pools and may be reutilized so that it is possible to have old red cells and newly synthesized red cells with the same label.

Cohort-labeling with DFP has the disadvantage of a short isotope half-life which precludes accurate measurement of radioactivity associated with red cells older than 70 to 90 days. This problem has been circumvented by use of DFP labeled with a longer-lived isotope, tritium. Cohorts of red cells tagged with tritium-labeled DFP in three normal dogs had survival times of 98, 103 and 104 days.

The early rapid loss of red cell DFP seen in some animals after labeling of a cohort of cells of similar age (figs. 4 and 5) may be the result of elution of label, of destruction of a group of heavily labeled and damaged cells, or—
Fig. 5.—Erythrocyte survival in acutely bled dogs.

less likely—the result of two erythrocyte populations with different life spans. Hjort and his co-workers have presented evidence from experiments using random red cell labeling with DFP that the initial loss of marker may be related to the dose of DFP, but that it occurs to some extent even with very small doses. Metz and his colleagues have presented evidence that large doses of a cholinesterase inhibitor do not affect red cell survival in man.

Using DFP as a cohort label, it has been possible to demonstrate that in the dog the red cells produced in response to acute blood loss have a shortened survival. Supporting evidence for this observation has been obtained using
C¹⁴-glycine as a red cell label. Similar evidence has been obtained using the rat and the rabbit as the experimental models.

From the erythrocyte C¹⁴-glycine data, one can conclude that in the dog the red cells produced by the ninth day following bleeding have a normal survival. Those red cells produced between days one and six following blood loss do not survive normally. The defect which predisposes this group of red cells to premature destruction has not been elucidated.

Summary

In the dog, the erythrocyte uptake of DFP was found to be dependent upon the dose of administered DFP. Effective blocking of red cell uptake of isotopically-labeled DFP was achieved by prior administration of unlabeled DFP. By allowing a time interval between the administration of DFP and DFP the cohort of red cells synthesized in that interval could be labeled with DFP. The cohort of erythrocytes synthesized in response to acute blood loss were so labeled and were found to have a shortened life span with a pattern of random destruction.

Acknowledgment

We are indebted to Mrs. J. G. Waggoner for her outstanding technical assistance.

References


Martin J. Cline, M.D., Clinical Associate, National Cancer Institute, Bethesda, Md.

Nathaniel I. Berlin, M.D., Ph.D., Clinical Director, National Cancer Institute, Bethesda, Md.
Red Blood Cell Life Span Using DFP$^{32}$ as a Cohort Label

MARTIN J. CLINE and NATHANIEL I. BERLIN