LEUKOKINETIC STUDIES. II. A METHOD FOR LABELING GRANULOCYTES *IN VITRO* WITH RADIOACTIVE DIISOPROPYLFLUOROPHOSPHATE (DFP[32])*

By A. M. MAUER,[†] J. W. ATHENS, HELEN ASHENBRUCKER, G. E. CARTWRIGHT AND M. M. WINTROBE

(*From the Department of Medicine, University of Utah College of Medicine, Salt Lake City, Utah*)

In a previous publication from this laboratory (1), radioactive diisopropylfluorophosphate (DFP[32]), a potent and irreversible esterase inhibitor, was shown to be a suitable label for granulocytes. Evidence was presented that the label is firmly attached to granulocytes; it does not modify the viability of the cells, and the label is not reutilized when the cells are degraded. Experiments designed to determine whether or not elution of the label occurs failed to provide such evidence. More recent studies (2), carried out with tritiated DFP and autoradiography, have confirmed the observation that the label is attached to granulocytes and little, or not at all, to lymphocytes or to monocytes.

A method for labeling granulocytes *in vivo* with DFP[32] was described in the previous publication (1). The purpose of this paper is to describe a method for labeling granulocytes *in vitro,* and to describe the manner in which granulocytes so labeled leave the circulation of normal subjects. Several preliminary reports of this work have been published (3, 4).

MATERIAL AND METHODS

Subjects. A total of 82 volunteers from the Utah State Prison was used in these studies. All of the subjects were normal, healthy males, 20 to 50 years of age, with normal leukocyte values. Each subject was used only once in the study. All of the studies were started between 8 and 10 a.m.

Method of labeling granulocytes in vitro. Five hundred ml of blood was withdrawn from the subject into a sterile, plastic bag [1] containing 67.5 ml of ACD-A solu-

tion.[2] DFP[32] was added to the blood in the bag in a concentration of 0.3 μg per ml of whole blood and the contents were mixed gently. The blood was allowed to remain at room temperature for 1 hour with occasional gentle mixing. At the end of this time, a 20 ml sample of blood was obtained from the bag and the remainder of the blood was infused into the subject over a 10 to 15 minute period.

Samples of blood, 20 ml each, were withdrawn at 0, 3, 6, 10, 24 and 30 hours after completion of the infusion. The methods for the isolation of the leukocytes from the blood samples and for determination of leukocyte radioactivity have been described previously (1).

Calculations. The percentage of cells infused which could be demonstrated in the circulation immediately after the infusion was calculated in the following manner. The specific activity (counts per minute per milligram leukocyte nitrogen) of the leukocytes in the bag was determined at the time of the infusion. From this determination, the volume of blood injected, and the calculated blood volume (5, 6), the expected counts per minute of the leukocytes in the circulation immediately after completion of the 15 minute infusion (t_0) were calculated:

$$\text{expected specific activity cells in blood at } t_0 = \frac{\text{specific activity of cells in bag} \times \text{volume of blood labeled}}{\text{blood volume}}.$$

The specific activity of the cells in the blood immediately after the infusion (t_0) was determined by extrapolation of the exponential granulocyte disappearance curve to the ordinate. The percentage of infused cells which was present in the circulation at t_0 was then calculated as follows:

$$\frac{\text{determined specific activity of cells at } t_0}{\text{expected specific activity of cells at } t_0} \times 100$$

$$= \text{percentage of infused cells present in the circulation at } t_0.$$

The time for half of the labeled granulocytes to leave the blood ($T_{\frac{1}{2}}$) was determined by plotting the leukocyte radioactivities against time in hours on semilogarithmic paper.

Radiation dosage to the subjects. When DFP[32] with a specific activity of 200 μc per mg was used, a total of 30 μc of radioactivity was administered to each subject. The

2 Each 100 ml contains 2.45 g of dextrose, 2.20 g of sodium citrate, and 0.73 g of citric acid.
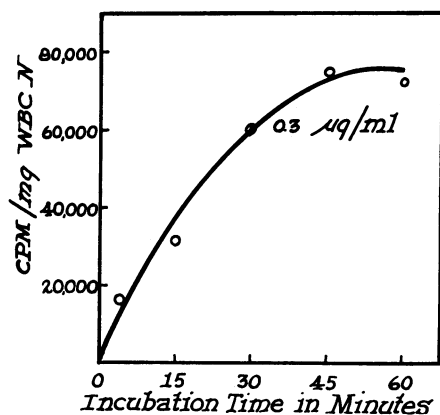
FIG. 1. UPTAKE OF DFP[32] BY GRANULOCYTES
IN RELATION TO TIME.

compound was presumably bound firmly to erythrocytes, leukocytes, platelets and plasma proteins and, therefore, 30 μc of isotope was distributed predominantly in about 5 kg of blood with very little radioactivity reaching the bone marrow or other tissues. In three subjects, in whom the urinary excretion of radioactivity was studied, 40 per cent of the administered radioactivity appeared in the urine within the first 8 hours after infusion of the labeled blood. Thereafter, the biological half-life of the radiophosphorus in the body was approximately 6 days. The effective half-life was calculated to be 4.4 days (7). With the nomogram of Bertinchamps and Cotzias (7), the infinite dose of radioactivity administered to blood was calculated to be about 860 millirads. This figure represents the maximum dose, since the above calculation of the biological half-life does not take into consideration the excretion of DFP[32] through routes other than the kidneys.

Analysis of the DFP[32] preparations. DFP[32] was purchased from a commercial source [3] as a dilute solution in sterile, anhydrous propylene glycol. The amount of DFP[32] present in the solution was determined by the colorimetric method of Marsh and Neale (8) as modified by Wilcox and Neurath.[4]

Nine different batches of DFP[32] were used in these studies. The determined DFP concentrations were found to range from 15 to 100 per cent of the concentration stated by the manufacturer. The specific activities of the batches, at the time the material was used, ranged from 103 to 336 μc per mg.

RESULTS

Rate of uptake of DFP[32] into leukocytes. To determine the period of incubation required to obtain optimal labeling of granulocytes, blood was

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[8] Manning Research Laboratories, Waltham, Mass.
[4] We are indebted to Dr. Hans Neurath, University of Washington, Seattle, Wash., for supplying us with this method.

drawn into a plastic bag and incubated for 1 hour at room temperature with 0.3 μg of DFP[32] per ml of whole blood. The uptake of DFP[32] into the leukocytes was maximal in about 45 minutes (Figure 1).

The following experiment was performed to determine whether or not any DFP[32], which would be capable of labeling the leukocytes of the recipient, remained in the plasma after a 1 hour incubation period. From each of three subjects, 500 ml of blood was withdrawn. The blood was incubated at room temperature for 1 hour with 0.3 μg of DFP[32] per ml. At the end of this time, the blood was centrifuged to remove all cells and the cell-free plasma was infused into the donors. No radioactivity could be demonstrated subsequently in either the leukocytes or the erythrocytes of the subjects.

Disappearance of labeled granulocytes from the circulation. The disappearance of labeled granulocytes from the blood was measured in 45 normal subjects. The labeled leukocytes left the circulation in an exponential fashion in all subjects. The leukocyte radioactivity disappearance curves obtained in four representative subjects are shown in Figure 2.
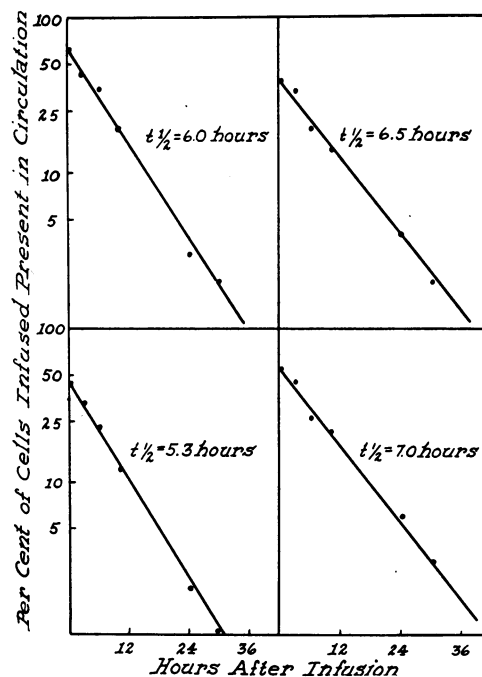

FIG. 2. GRANULOCYTE RADIOACTIVITY CURVES IN
FOUR REPRESENTATIVE SUBJECTS.

TABLE I

Influence of DFP³² concentration *

Concentration	No. of subjects	Percentage of infused cells present at t₀		T½	
		Mean	Range	Mean	Range
μg/ml		*%*	*%*	*hrs*	*hrs*
0.1	4	33	27–39	7.0	6.0–8.0
0.2	3	42	26–68	7.5	6.0–9.0
0.3	8	49	27–62	6.9	5.5–8.0
0.7	3	23	14–33	7.2	5.8–8.5
1.0	3	42	29–57	8.3	7.0–9.0

* Five hundred ml of blood was incubated in a plastic bag with ACD-A for 60 minutes at room temperature with the concentrations of DFP³² given above.

The mean $T_{\frac{1}{2}}$ ($\pm$ one standard deviation) in the circulation was 6.6 $\pm$ 1.4 hours in the group of 45 normal male subjects, with a range of from 4.0 to 9.0 hours.

The percentage of infused cells which could be demonstrated in the circulation immediately after the infusion (t_0) averaged 49, with a range of from 27 to 62.

Influence of various factors on the disappearance rate of labeled granulocytes. The procedure was varied with respect to concentration of DFP³², temperature of incubation, volume of blood labeled, type of anticoagulant and container used, and the time of incubation. The influence of these procedural changes on the $T_{\frac{1}{2}}$ and on the percentage of infused cells which could be demonstrated in the circulation immediately after the infusion (t_0) was studied.

Varying the concentration of the DFP³² between 0.1 and 1.0 μg per ml had no influence on either the percentage of infused cells present in the circulation at t_0 or the $T_{\frac{1}{2}}$ (Table I). Incubating the blood at 1°, 20° and 37° C had no significant influence on these measurements (Table II). Likewise, labeling 100 or 250 ml of blood rather than 500 ml had no influence on the percentage of infused cells present at t_0 or on the $T_{\frac{1}{2}}$ (Table III). In single trials the use of heparin or sequestrene as an anticoagulant in place of ACD-A or the substitution of a siliconized glass container for the plastic container had no influence on these measurements.

Modifying the time of incubation from 15 minutes to 24 hours influenced significantly the percentage of infused cells present in the circulation at t_0 (Table IV). The proportion of the infused

TABLE II

Influence of incubation temperature *

Temperature	No. of subjects	Percentage of infused cells present at t₀		T½	
		Mean	Range	Mean	Range
		%	*%*	*hrs*	*hrs*
37° C	4	57	38–68	7.6	6.0–8.7
20° C	8	49	27–62	6.9	5.5–8.0
1° C	3	44	31–54	5.5	4.4–6.1

* Five hundred ml of blood was incubated in a plastic bag with ACD-A containing 0.3 μg of DFP³² per ml for 60 minutes at the temperatures given above.

cells present in the circulation at t_0 decreased exponentially as the incubation period was prolonged (Figure 3). When blood was stored for more than 8 hours, less than 12 per cent of the infused cells was present in the circulation at t_0 and a disappearance curve could not be obtained.

No correlation was observed (correlation coefficient, − 0.03) between the percentage of infused cells present in the circulation at t_0 and the $T_{\frac{1}{2}}$.

Transfusion of blood from normal subjects into normal recipients. Five hundred ml of blood was withdrawn from each of ten normal subjects. The blood was incubated for 60 minutes at room temperature with 0.3 μg of DFP³² per ml and then transfused into a normal recipient other than the donor. All subjects were cross-matched for the major blood group and Rh type. None of the subjects had ever received a blood transfusion prior to the experiment.

In seven of the subjects the granulocytes disappeared in an exponential fashion. The $T_{\frac{1}{2}}$ values in this group (5.4 to 9 hours) were within normal limits for autogenous infusions. In one subject the experimental values were widely scattered, presumably due to technical errors, and a line

TABLE III

Influence of the volume of blood labeled *

Volume of blood labeled	No. of subjects	Percentage of infused cells present at t₀		T½	
		Mean	Range	Mean	Range
ml		*%*	*%*	*hrs*	*hrs*
100	1	49		7.3	
250	3	43	37–46	6.4	5.0–7.0
500	3	42	26–68	7.5	6.0–9.0

* The volumes of blood given above were incubated in a plastic bag with ACD-A for 60 minutes at room temperature with 0.2 μg of DFP³² per ml of blood.

TABLE IV

Influence of incubation time *

Time of incuba-tion	No. of subjects	Percentage of infused cells present at t₀		T½	
		Mean	Range	Mean	Range
hrs		*%*	*%*	*hrs*	*hrs*
0.25	3	47	26–81	5.3	5.0– 5.7
1	21†	41	14–72	7.0	5.2– 9.0
2	5	39	19–52	9.4	7.5–12.0
8‡	5	24	12–48	9.0	
16‡	5	12	5–21		
24‡	4	7	5– 9		

* Five hundred ml of blood was incubated at room temperature in a plastic bag with ACD-A containing 0.3 μg of DFP³² per ml of blood, with the exceptions noted.

† This group includes studies done with concentrations of DFP³² from 0.1 to 1.0 μg per ml.

‡ Incubated for 1 hour at room temperature and at 5° C for the remainder of the incubation period.

could not be drawn through the points. In two of the subjects the granulocytes disappeared from the circulation in an exponential fashion but at an accelerated rate (T½ = 2.5 and 1.2 hours).

A transfusion of blood from a normal subject was given to a patient with hemophilia. This patient had received approximately 100 previous transfusions of plasma or whole blood. The T½ of the labeled cells in the circulation of the patient was 0.8 hour, in contrast to the normal mean of 6.6 hours. Twenty-two per cent of the transfused cells was present in the circulation of the patient at t₀.

DISCUSSION

This study was undertaken to determine the manner and rate of removal of normal granulocytes from the circulation of normal subjects. The technic described for labeling granulocytes *in vitro* has the advantage that only granulocytes circulating in the blood are labeled and, therefore, interpretation of the disappearance curves is more simple than interpretation of curves obtained following the intravenous administration of DFP³² (1).

Interpretation. With the method outlined, only about 50 per cent of the infused cells was present in the circulation at the completion of the infusion. Those cells which were not removed immediately disappeared from the circulation in an exponential fashion with a mean T½ of 6.6 hours. Thus, from a kinetic point of view, the labeled granulocytes appeared to behave as two distinct cell populations. Several explanations for this phenomenon may be offered.

The immediate disappearance from the circulation of about 50 per cent of the labeled cells could be due to rapid elution of the label, rapid removal of leukocytes damaged by the labeling procedure, or to distribution of the infused granulocytes in a larger pool of cells than that calculated from the blood volume and the granulocyte count.

As the result of studies previously reported (1) the possibility of elution seems remote but cannot be entirely excluded. However, if the label eluted *in vivo*, it might be expected that the percentage of labeled cells present at t₀ would decrease as the concentration of DFP³² was increased. Such was not found to be the case (Table I).

The possibility of damage to the cells during the labeling procedure deserves serious consideration since granulocytes removed from the body are highly susceptible to damage and it has been shown that damaged granulocytes are removed by the lungs, possibly the first time they pass through the circulation (9–13). In the studies reported here, a greater proportion of the infused granulocytes was missing from the circulation when blood was stored for 8 to 24 hours and then infused than when blood was incubated for an hour or less (Table IV and Figure 3) and infused. It appears, however, that rapid removal of labeled cells which have been damaged as a function of the time of incubation is not the sole reason for the immediate
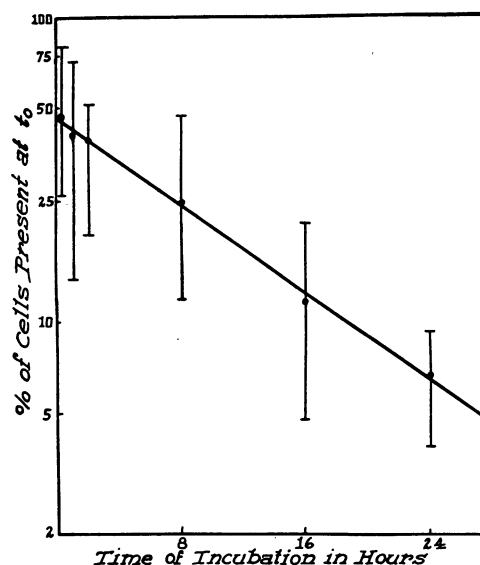

FIG. 3. THE EFFECT OF TIME OF INCUBATION ON THE PERCENTAGE OF INFUSED CELLS PRESENT IN THE CIRCULATION AT TIME ZERO (t₀).

disappearance of about half of the labeled granulo-
cytes. Extrapolation of the exponential curve
(Figure 3) to the ordinate, i.e., zero incubation
time and therefore presumably zero damage, sug-
gests that 54 per cent (100–46) of the infused cells
was removed rapidly for a reason unrelated to the
time of incubation.

The most plausible explanation for this rapid
removal is that the labeled granulocytes are dis-
tributed in a pool of granulocytes larger than that
calculated from the blood volume and the granulo-
cyte count. Evidence in support of this concept
will be presented in the next paper (14).

The exponential disappearance of labeled gran-
ulocytes from the circulation with a mean T_4 of
6.6 hours indicates that granulocytes leave the
blood in a random fashion. That is, unlike erythro-
cytes, the disappearance of granulocytes from blood
is unrelated to the time of entry of the cells into
the circulation, and, by chance, a particular gran-
ulocyte may remain in the circulation only a few
seconds or for an indefinitely long time. In this
regard it is important that a correlation was not
observed between the percentage of infused cells
in the circulation at t_0 and the granulocyte dis-
appearance rate (T_4). This suggests that the T_4
is representative of the disappearance rate of the
entire labeled population of cells, rather than that
of a selected population as, for example, the
youngest cells. The validity of the T_4 is further
substantiated by the observation (3) that granulo-
cytes labeled *in vivo*, without any handling, also
disappeared from the circulation in a random fash-
ion and at the same rate as do cells labeled *in vitro*
and then returned to the circulation. The mean
T_4 of the cells labeled *in vivo* in 30 normal subjects
was 6.4 hours; the mean T_4 for the cells labeled
in vitro in the 45 normal subjects in this study was
6.6 hours. The difference between these mean
values is not significant.

Application of the method. It is anticipated that
this method will be useful in studying the gran-
ulocyte disappearance rate in leukemia and in
other conditions. By the use of homologous trans-
fusions in normal subjects, as illustrated by the
ten preliminary studies reported herein, a reduc-
tion of the T_4 value may prove to be a highly
sensitive method for detecting leukocyte incom-
patibilities and granulocyte blood groups. By a
combination of autogenous infusion, transfusion of

the patient's blood into a normal subject, and the
transfusion of normal blood into the patient, it may
become possible to differentiate granulocytopenias
which are attributable to intracellular defects from
those which may be produced by extracellular
causes.

This method may also prove useful in the study
of the preservation of granulocytes. Our studies
clearly indicate that the longer granulocytes are
stored, the more susceptible they become to imme-
diate removal from the circulation. Ninety-one to
95 per cent of the granulocytes stored for 24 hours
was removed immediately. Whether this is due to
damage to the cells by the anticoagulant, by ex-
posure to a foreign surface, by the depletion in the
mixture of an essential nutrient, or by the accumu-
lation in the stored blood of a product of cellular
metabolism, is subject to experimental investi-
gation.

SUMMARY

1. A method has been described for labeling
granulocytes *in vitro* with radioactive diisopropyl-
fluorophosphate (DFP^{32}). Briefly, the method is
as follows: 500 ml of blood is withdrawn into a
plastic bag containing acid citrate dextrose solution
and incubated at room temperature for 1 hour with
0.3 μg of DFP^{32} per ml of blood. The blood con-
taining the labeled granulocytes, erythrocytes and
platelets is infused into the donor. At appropriate
time intervals, 20-ml samples of blood are with-
drawn. The leukocytes are separated free of ery-
throcytes and platelets and the leukocyte radio-
activity is determined.

2. Fifty-four per cent of the labeled granulo-
cytes disappeared from the circulation during the
infusion. The remainder of the granulocytes was
removed from the circulation in an exponential
fashion; the mean half-time in the circulation was
6.6 ± 1.4 hours in 45 normal male subjects.

3. The proportion of labeled cells infused which
was present in the circulation at the end of the
infusion was not influenced by the temperature of
incubation, the concentration of DFP^{32}, the volume
of blood labeled, or the type of anticoagulant or
container used, within the limits of the experi-
ments performed. The proportion was influenced,
however, by the time of incubation; the longer the
blood was incubated, the smaller was the propor-

tion of infused cells in the circulation immediately on completion of the infusion.

4. The time for the disappearance of one-half of the labeled granulocytes was not influenced by the proportion of infused cells which was demonstrable in the circulation at the end of the infusion, or by the time or temperature of incubation, the concentration of DFP^{32}, the volume of blood labeled, or the type of anticoagulant or container used.

5. Ten normal male subjects were transfused with labeled blood from other normal subjects. In seven of the subjects, the granulocytes were removed at the normal rate. In two of the subjects, the granulocytes disappeared at an accelerated rate. In one subject, the experimental values were widely scattered and a line could not be drawn through the points.

6. The validity, limitations, interpretations and applications of this technic are discussed.

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