Platelet Tagging with Tritium Labeled Diisopropylfluorophosphate

By Edward Adelson, Richard M. Kaufman, Ciro Berdeguez, Arnold A. Lear and Jack J. Rheingold

For the past 10 years, $^{32}$P labeled diisopropylfluorophosphate has been used to tag platelets. This compound attaches to various proteins, during which process it changes from diisopropylfluorophosphate (DFP) to diisopropylphosphate (DIP). The DIP is irreversibly bound to the protein molecule until that molecule breaks down and then the DIP is not reutilized. Although its major attachment has generally been considered to be to the cholinesterase molecule, DFP will attach to any protein that has a serine group. Its major pharmacologic action depends on its combining with cholinesterase and inhibiting the action of that compound, resulting in symptoms associated with overactivity of the parasympathetic nervous system. Usually doses of up to 3 milligrams of DFP in humans will produce little or no side effects. However, when the dose of DFP reaches 4 milligrams, side effects are often seen.

$^{32}$P labeled diisopropylfluorophosphate has the drawback of a relatively low specific activity since there is only one atom of phosphorus in the molecule. The short half-life of $^{32}$P results in a short shelf-life for the compound, further reducing the specific activity toward the end of the usual 10-day to 2-week survival studies. The specific activity of the tag cannot be increased by increasing the dose of DFP, because of the pharmacologic toxicity of the compound. Furthermore, increasing the dose of DFP gives diminishing results, as will be shown in the in vitro studies reported here.

This low specific activity has resulted in some difference of opinion as to whether the survival curves of platelets labeled with DFP$^{32}$ are linear or exponential. Leeksma and Cohen, Alfos et al., Zucker et al., and others

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have reported linear survival curves. Mizuno et al.9 and Hjort et al.10 have reported curves which appear to be exponential. If the specific activity of the DFP could be increased, it would be possible to obtain more reliable curves of platelet survival.

Recently, tritium labeled DFP has become commercially available. It is manufactured with a specific activity of 5 to 8.6 millicuries per milligram. This is nearly 40 times more radioactive than the best activity available with DFP.12 The long half-life of tritium results in a further twofold increase in the radioactive levels toward the end of the survival studies. Cline and Berlin12-14 have reported the use of tritium-tagged DFP to label red cells. We have applied a similar technic to platelets. We shall report the results of in vitro and in vivo tagging of platelets with tritium labeled DFP and present survival curves of dog platelets tagged in vivo with DFP-H3.

METHODS

For in vivo tagging of dog platelets, we injected 0.1 to 0.2 milligrams of tritiated DFP in propylene glycol intravenously into mongrel dogs weighing approximately 15 kilograms. The injection was given over a period of 2 minutes.

For our in vitro studies, whole blood was obtained from human volunteers, using the acid citrate anticoagulant of Aster and Jandl.15 Platelet-rich plasma was separated by differential centrifugation. To this plasma we added various amounts of tritiated DFP diluted in propylene glycol. The mixture of platelet-rich plasma and DFP-H3 was incubated at room temperature for 45 minutes.

For both the in vitro and in vivo work, platelets were separated by differential centrifugation. The whole blood was centrifuged at 350 g for 8 minutes at 4°C. The supernatant platelet-rich plasma was separated and centrifuged at 2000 g for 30 minutes. The platelet button was washed three times in saline. The platelets were counted by phase microscopy and transferred to bags made of cellophane casing and scotch tape and marked for identification with india ink.

The cellophane bags containing the wet platelets were hung on nails and dried by exposure to an infrared lamp which was set to warm the bags to a temperature of approximately 37°C. The cellophane bags with dry platelets were then burned by the Schöniger combustion technic, using the Oliverio et al.16 modification of the method of Kelly et al.17 Following this technic, the cellophane bag was placed on a platinum wire basket inside a 2-liter, heavy-walled filter flask that had been previously flushed with pure oxygen. The flask was placed in a Thomas Ogg apparatus and a beam of infrared light was aimed at the india ink marking of the bag. The black pigment absorbed the heat, the platelets burned, and the tritium was converted to tritiated water. The flask was then taken out of the chamber and placed in a vat containing a mixture of dry ice and alcohol. This condensed the water vapors onto the bottom of the flask. The flask was then moved to an ice water bath in order to raise the temperature to 0°C. The side arm was unclamped and exactly 20 cc. of scintillation fluid were poured into the flask. The fluid we used had the following composition: 5.0 Gm. PPO (2,5-diphenyloxazole), 0.15 Gm. POPOP [1,4-bis-2-(5-phenyloxazolyl)-benzene], 100 Gm. naphthalene, 133 ml. absolute ethyl alcohol, and 867 ml. dioxane. Exactly 15 cc. of the fluid were withdrawn, placed in a polyethylene vial, and counted in a liquid scintillation counter at 0°C. A mathematical correction was made for the 5 cc. of fluid left behind in the flask. The counting efficiency averaged 23 per cent and there was approximately an 8 per cent quench. Each vial was corrected for quench by internal standardization with tritiated water.

Prior to burning, an aliquot was removed from each sample and the hemoglobin con-

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tent was determined by the method of Crosby and Furth. Simultaneously, a red cell sample was prepared and burned. The radioactivity of the red cells was expressed in counts per minute per milligram of hemoglobin and an appropriate correction was made for hemoglobin contamination in each platelet sample.

RESULTS

Figure 1 shows the results of adding DFP-H3 to platelet-rich plasma. An increasing amount of DFP was attached to the platelets until a plateau was reached at about 300 counts per minute per 10⁸ platelets. That portion of the curve leading up to this plateau did not appear to form a straight line on semilog plot, suggesting that the absorption of DFP onto the platelet was not a simple exponential relationship. There may be several different mechanisms whereby DFP absorbs onto the platelet.

For the in vivo work, a dose of DFP-H3 that would yield approximately 30 counts per minute per 10⁸ platelets was selected to keep the amount of DFP on the platelet at a level equivalent to the lower part of the in vitro curve. Each determination was corrected for hemoglobin contamination as previously described. A correction could not be made for white cell contamination, but there was less than one white cell per thousand platelets. The highest counts per minute per platelet was arbitrarily considered 100 per cent in each dog, and all other counts were expressed in terms of the 100 per cent figure for the corresponding dog.

From all the individual observations of the per cent survival, we derived the ideal curve for platelet survival by least squares fitting to an exponential curve using a digital computer. Figure 2 shows this regression curve plotted on semilog scale together with the mean per cent survivals. The standard error for each mean is shown. The half-life of the survival curve is 2.4 days.

Since this graph assumes an exponential relationship, we carried out a test for lack of fit to determine the validity of this assumption. The F ratio of this test was 0.955. The 95 per cent level of F for our data was 2.28. This showed that lack of fit was not significant when the regression curve was assumed to be exponential.

We then attempted to fit our data to a linear regression by the method of least squares using the digital computer. This time the lack of fit had an F
PLATELET TAGGING WITH P³² LABELED DFP

Fig. 2.—A semilog plot of the regression curve obtained by a least squares fitting of all the observations of per cent platelet survival in six normal dogs with in vivo DFP-H³ platelet tag. The open circles are the mean per cent platelet survivals for each time interval. The number next to each open circle is the standard error for that mean.

With tritium labeled DFP, the limiting factor in dosage no longer is the pharmacologic effect of the compound, but rather permissible levels of tritium radioactivity. In preliminary studies on humans with malignant diseases, we have used 0.6 milligrams of DFP-H³ containing 5 millicuries of tritium. This dose of DFP is to be compared with doses of up to 4 milligrams used in experiments with DFP³².⁴,⁸
Figure 1 shows the manner in which increasing amounts of DFP label platelets in vitro. Platelet radioactivity rises at a gradually decreasing rate until a plateau is reached at a level of 300 counts per minute per 10⁷ platelets. Since the rising protein of the graph does not form a straight line of semi-log plot, the absorption of DFP onto the platelet is not a simple exponential function. Any portion with a serine group will be labeled, and therefore more molecules are involved than simply the platelet cholinesterase. Hjort et al. have pointed out that some of these proteins might have a more rapid turnover than the platelet itself, and this could result in some elution of the tag.

While elution does occur during the first few days of DFP red cell survival curves, it is lessened by lowering the concentration of DFP. Although elution of DFP tag has not been shown to occur in the case of platelets, this possibility should be borne in mind. To lessen the chances of elution, we chose for our in vivo tag a dose of DFP-H³ that gives only one-tenth the maximum label. In spite of this precaution, the exponential nature of the curve of platelet survival in figure 2 may still be due to elution. An alternate explanation is that platelet life span is not determined by aging, which would result in linear curves, but by random destruction.

One of the advantages of DFP-H³ over DFP² is that the tritium label permits smaller doses of DFP, and therefore less danger of elution and of pharmacologic toxicity. The long half-life of the tritium compound results in a longer shelf-life, which is another advantage. A third advantage is the increased levels of radioactivity, which improves counting statistics and gives more reliable curves.

These higher levels of radioactivity will also permit many experiments that could not be carried out with the lower specific activity of DFP². Survival studies can be carried out using platelets labeled in vitro with DFP; cross transfusion experiments can be carried out with DFP-tagged platelets; platelet cohorts tagged with DFP can be prepared and studied in a manner similar to that used by Cline and Berlin for red cells, and the effect of storage on the viability of DFP-tagged platelets can be studied.

**Summary**

A method is described for tagging platelets either in vitro or in vivo with tritium labeled diisopropylfluorophosphate, burning the tagged platelets by a modification of the Schöniger combustion technic and counting the resultant tritiated water by liquid scintillation counting.

The curve of in vitro uptake of DFP-H³ by the platelet suggests that more than one protein in or on the platelet takes up the DFP. However, a saturation point is reached, as indicated by a plateau in the uptake curve.

The survival curve of in vivo tagged platelets in five normal dogs is exponential with a half-life of 2.4 days. This can be explained by random destruction of platelets or by elution of the tag.

The DFP-H³ tag has several advantages over the DFP² tag. Either smaller doses of DFP or higher levels of radioactivity or both may be achieved with this technic.
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SUMMARIO IN INTERLINGUA

Es describite un methodo pro le marcage de plachettas in vitro o in vivo con diisopropylfluorophosphato a tritium, le arsura del marcate plachettas per un modification del technica de combustion de Schöniger, e le examine del resultante aqua a tritium per medio de contation scintillatori de liquido.

Le curva del acceptation in vitro de diisopropylfluorophosphato a tritium per le plachettas suggere que plus que un proteina in o super le plachetta participa in le processo. Tamen, le attingimento de un puncto de saturation es reflectite in le presentia de un plateau in le curva de acceptation.

Le curva del longevitate de plachettas marcate in vivo in cinque normal canes es exponential, con un tempore de medie valor de 2.4 dies. Isto pote esser explicate per le occurrentia de destruction aleatori de plachettas o de elution del marca.

Le uso de diisopropylfluorophosphato a tritium como marca ha plure avantages super le uso, pro le mesme objectivo, de diisopropylfluorophosphato a phosphoro 32. Isto es que plus micre doses de diisopropylfluorophosphato suffice pro le attingimento de un certe nivello de radioactivitate o que plus alte tal nivellos pote esser attingite per un certe dose de diisopropylfluorophosphato.

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