DFP Labeling of Platelets During Recovery from Thrombocytopenia

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A CUTE DEPLETION OF CIRCULATING PLATELETS IN ANIMALS is followed by a period of reactive thrombocytosis before the platelet counts finally return to normal. This overcompensation suggests that the rate of production of platelets is increased in response to thrombocytopenia, and this notion has been substantiated by the demonstration of changes in megakaryocyte size, maturation rate and number. However, the magnitude of post-thrombocytopenic compensatory thrombocytosis may be determined in part by changes in the rate of platelet destruction as well as platelet production. To investigate this possibility, platelet survival was measured with tritiated diisopropylfluorophosphate ($^{3}$H-DFP) in rats during the period of recovery from acute platelet depletion. Our previous evaluation had indicated that this was a reliable technique for determining survival of circulating rat platelets.

In normal rats, platelet survival curves suggested that about 40 per cent of circulating platelets were destroyed daily in a predominantly random manner. There was no evidence of either labeling of megakaryocytes by $^{3}$H-DFP or delivery of labeled platelets into the circulation.

MATERIALS AND METHODS

Measurements were made in male Sprague–Dawley rats which weighed 200–400 Gm. Larger animals of the same strain were used as blood donors. Each test animal was used for only one determination; to accumulate a series of determinations, a number of rats were treated and sequentially sacrificed. Platelet counts were done by phase microscopy on cardiac or arterial blood anticoagulated with dry K$_{3}$EDTA.

Platelet Survival with $^{3}$H-DFP

Platelet survival studies were done as previously described. $^{3}$H-DFP, dissolved in propylene glycol (New England Nuclear Corp.) was injected intramuscularly in a dose of 12 mGm./100 Gm. body weight. The specific activity was 880 mCi./mM., and the concentration was 210 mGm./ml. Circulating platelet-bound radioactivity was determined from

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cardiac blood samples taken two hours after injection of $^3$H-DFP, and daily thereafter. The average two-hour value was considered to be 100 per cent for each experiment, and subsequent values were expressed as a percentage of it. Platelet specific activity was calculated as counts per minute per $10^9$ platelets. Total circulating radioactivity was calculated from the specific activity and the whole blood platelet count as counts per minute for the platelets in one milliliter of blood. In spite of differences in platelet counts at the time of injection of $^3$H-DFP, control and thrombocytopenic rats had comparable values for the two-hour specific activity in each experiment ($\sim 250-500$ cpm./$10^9$ platelets). Therefore, platelet-bound radioactivity per milliliter of blood was lower in thrombocytopenic than control animals in the two-hour samples.

**Transfusion of Labeled Platelets**

Platelets were labeled in vivo by intramuscular injection of $^3$H-DFP, 17-26 $\mu$Gm./100 Gm., in 400-600-Gm. donor rats. Two hours later, cardiac blood was collected from etherized donors into ACD-A anticoagulant. Platelets were separated and concentrated by differential centrifugation and transfused into 150-200 Gm. normal female recipients. Survival of transfused platelets was determined as previously described.

**Exchange Transfusion with Platelet-Poor Blood**

Rat blood was collected by cardiac puncture into ACD-A anticoagulant. Platelets were removed by differential centrifugation. Exchange transfusion was performed under chloral hydrate anesthesia. A femoral artery and vein were cannulated, and exchange transfusion was accomplished in 2-ml. increments as previously described.

**Antiplatelet Serum**

Rabbit antirat platelet serum was prepared as previously described.

**RESULTS**

Average platelet counts for the groups of rats studied with $^3$H-DFP are shown in Fig. 1. The results were similar to those which have been published previously and showed that reactive thrombocytosis developed during the period of recovery from acute platelet depletion. For the first two days after lowering the platelet count to $< 10$ per cent of normal, platelet counts increased at the rate expected if both production and destruction remained normal. During the third postthrombocytopenic day, the increase in platelet count could be explained only by some measure of increased production, since it was greater than would be seen with normal production even if destruction ceased completely. Platelets were restored in splenectomized rats at the same rate as in intact animals, but the reactive thrombocytosis was somewhat greater in magnitude. The numbers on Fig. 1 are for reference and indicate the populations of platelets which were labeled with $^3$H-DFP in the experiments to be presented below.

Immediately after exchange transfusion with platelet-poor blood, the average platelet count for 66 normal rats was 78,000/mm$^3$, or $\sim 7$ per cent of normal. Two days later, platelet counts had increased to $\sim 700,000$/mm$^3$, and this population, of which $> 88$ per cent had been produced within 48 hours, was labeled with $^3$H-DFP (platelet population no. 1, Fig. 1). Their apparent survival in the circulation, as judged from the rate of fall of circulating platelet-bound radioactivity, was not the same as the normal for a population of platelets of mixed age (Fig. 2).
Fig. 1.—Platelet counts of intact and splenectomized rats after platelet depletion by exchange transfusion. Each point for intact rats depleted to about seven per cent of normal (open circles) is average of two to 10 rats. Each point for intact rats depleted to ~40 per cent (closed circles) is average of two rats. Each point for splenectomized rats is average of four to six rats. Numbers refer to platelet populations labeled with $^3$H-DFP.

Fall in platelet specific activity in these rats was identical to that of normal controls. However, total platelet-bound radioactivity fell more slowly than normal for the first three days, after which it decreased at a normal rate. Three days after labeling, only about 30 per cent of the newly formed platelets had apparently been destroyed; at the same time, ~80 per cent of the control platelet-bound radioactivity had been cleared from the circulation. The "plateau" of platelet-bound radioactivity occurred coincidentally with the rapid rise in platelet count and persisted during the period of maximum reactive thrombocytosis until the platelet count began to fall.

To evaluate the possibility that this period of apparent retention of platelets in the circulation may have been dependent upon the labeled cells reaching senescence before destruction, an even younger population was labeled. One day after plateletapheresis, platelet counts had returned to ~400,000/mm$^3$, and at least 80 per cent of the circulating platelets had been produced during the preceding 24 hours (platelet population no. 2, Fig. 1). The results of labeling of this population are shown in Fig. 3. The rate of fall of platelets specific activity again was about the same as for the controls. The overall fall
in total platelet-bound radioactivity occurred less rapidly than normal for the week after labeling. During the first day, however, the "cohort" of newly formed platelets appeared to be destroyed at a normal rate. The partial plateau of platelet-bound radioactivity occurred from the second to sixth post-thrombocytopenic days and coincided with the period of increased platelet production and maximum thrombocytosis.

Less-severe thrombocytopenia (platelet counts of \(~40\) per cent of normal), when induced by exchanged transfusion, has been shown to be followed by macromegakaryocytosis of a degree comparable to that which occurred after lowering the platelet counts to <10 per cent of normal.\(^3\) Platelet counts did not increase as rapidly, but moderate thrombocytosis was present on the fourth to sixth postthrombocytopenic days (Fig. 1). One day after depletion of platelets to \(~40\) per cent of normal, the counts had risen very little, and, presumably, the proportion of circulating platelets which were less than a day old was less than in the animals in which more severe thrombocytopenia had been produced. However, labeling the circulating platelets one day after moderate thrombocytopenia (platelet population no. 3, Fig. 1) produced a pattern of circulating platelet-bound radioactivity almost identical to that seen when \(>80\) per cent of the labeled platelets were new (Fig. 4). Again, specific activity fell at a normal rate. Total circulating platelet-bound radioactivity decreased the same as normal for a day, then, coincident with the rising
Fig. 3.—Circulating platelet radioactivity after injection of \(^{3}\)H-DFP into intact thrombocytopenic rats and normal controls. Thrombocytopenic rats injected one day after acute platelet depletion (platelet population no. 2, Fig. 1). Each point represents one rat, and lines connect average values.

platelet count, remained constant until the fifth postthrombocytopenic day. Thereafter, platelet radioactivity gradually decreased even though platelet counts showed an additional rise.

To consider the possibility that splenic sequestration of newly formed platelets may have influenced the shape of platelet survival curves, a cohort of one day-old platelets was labeled with \(^{3}\)H-DFP in rats which had been splenectomized four to six weeks earlier (platelet population no. 4, Fig. 1). The average pretreatment platelet count in these splenectomized rats was \(1.3 \times 10^{9}/\text{mm}^3\) (about 18 per cent higher than normal). This was reduced to \(\sim 100,000/\text{mm}^3\) (about eight per cent of control) by exchange transfusion with platelet-poor blood. A day later, platelet counts were \(\sim 400,000/\text{mm}^3\), and these platelets were labeled with \(^{3}\)H-DFP. Platelet specific activity for the next six days was the same as in splenectomized, but not thrombocytopenic, controls (Fig. 5). Average total platelet-bound radioactivity dropped less than controls during the first day after labeling then stayed nearly constant until the fourth post-labeling day. Thereafter, it gradually decreased. All of these observations in which measurements of platelet survival were made in situ during recovery from acute platelet depletion suggested the possibility that a cohort of newly formed platelets was destroyed at a slower rate than a normal population of
mixed ages. The role of obligatory senescence as a determinant of platelet destruction was evaluated by transfusing young platelets to normal recipients. The donors were pretreated with rabbit antirat platelet serum to produce thrombocytopenia four days and again two days before their platelets were labeled with \(^3\text{H}\)-DFP. Two hours after injection of \(^3\text{H}\)-DFP, donor platelets, which were two days or less old, were collected, concentrated and transfused. Survival of these platelets in the recipients is shown in Fig. 6. These young platelets were destroyed at the same rate as were transfused platelets of mixed ages, and there was no evidence that they were retained in the circulation of recipients.

**DISCUSSION**

DFP, tagged with radioactive phosphorus (\(^{32}\text{P}\))\(^{10}\) or tritium (\(^3\text{H}\))\(^{11}\) has been widely used as a label for determination of rates of platelet destruction. It is attractive for this purpose because platelets can be labeled in situ, thereby avoiding the possibilities of injury during processing ex vivo and antigenic incompatibilities between a donor and a recipient. However, a number of observations have suggested that radioactive DFP may not give an accurate picture of platelet turnover.
Fig. 5.—Circulating platelet radioactivity after injection of $^3$H-DFP into splenectomized thrombocytopenic rats and splenectomized controls. Thrombocytopenic rats injected one day after acute platelet depletion (platelet population no. 4, Fig. 1). Each point represents one rat, and lines connect average values.

Bithell et al.\textsuperscript{12} evaluated platelet labeling in vivo and in vitro with $^{32}$P-DFP, compared the results to platelet survival curves with $^{51}$Cr, and concluded that $^{32}$P-DFP injured platelets. In contrast to the notion that DFP may injure platelets is the report that the apparent halflife of circulating platelet-bound radioactivity increased as the dose of $^{32}$P-DFP was increased in rabbits.\textsuperscript{13} In rats given different doses of $^3$H-DFP, however, this effect was not seen.\textsuperscript{6} Comparative studies of platelet survival determined in situ with $^3$H- or $^{32}$P-DFP and by transfusion of platelets labeled with $^{51}$Cr, $^3$H- or $^{32}$P-DFP have shown longer apparent survival times for platelets labeled in situ.\textsuperscript{8,12} Persistent radioactivity in circulating platelets has been shown to be due, in part, to reincorporation of the phosphorus portion of $^{32}$P-DFP into platelet lipids.\textsuperscript{14,15}

Labeling of megakaryocytes with subsequent production of labeled platelets has been the subject of a controversy which is unresolved. In the early description of the use of $^{32}$P-DFP for determining human platelet survival, there was no suggestion of an important delivery of labeled cells from the bone marrow.\textsuperscript{10} In the rat, however, the curves leveled off, and this was attributed to a gradual release of labeled platelets, probably from megakaryocytes.\textsuperscript{16} Other
studies in human beings indicated that there are depots in which platelets are labeled by \(^{32}\text{P}\)-DFP; one site which was postulated was the megakaryocytes.\(^{17}\) If platelets react with DFP, then the mature megakaryocytes should also react with it. Darzynkiewicz et al.\(^{18}\) reported that megakaryocytes became labeled with \(^{3}\text{H}\)-DFP when incubated on slides in vitro, but we were unable to demonstrate definite labeling when particles of rat marrow were incubated with \(^{3}\text{H}\)-DFP.\(^{6}\) Conflicting results have also been reported when megakaryocytes were examined for labeling after injection of radioactive DFP in vivo.\(^{11,12,15,19,20}\)

In the rat, platelet survival curves determined with \(^{32}\text{P}\)-DFP\(^{16}\) or \(^{3}\text{H}\)-DFP\(^{6}\) as a label, favor the concept of random removal of platelets from the circulation without regard for their age. However, data obtained with \(^{35}\text{S}\)-sodium sulfate,\(^{21}\) \(^{51}\text{Cr}\)-sodium chromate\(^{22}\) and \(^{14}\text{C}\)-serotonin\(^{23}\) indicate that the age of platelets may be a significant factor in determining when they would be destroyed. Comparative studies have shown a linear clearance (senescent) when rat platelets were labeled with \(^{51}\text{Cr}\) and an exponential clearance (random) with \(^{3}\text{H}\)-DFP.\(^{24}\) At this point it seems clear that much of the controversy about shapes of platelet survival curves and their significance originate with the methods which have been used to detect platelet destruction.

Recent observations by Karpatkin\(^{25}\) substantiate other reports\(^{26,28}\) that newly formed platelets are larger and denser than platelets which have aged in the circulation. He also found that the large-heavy platelets incorporated more radioactivity when \(^{32}\text{P}\)-DFP was injected into rabbits than did the light-small platelets. These findings suggest that DFP may not uniformly label all
circulating platelets as had previously been supposed. He expressed his results as radioactivity per volume of platelets rather than per number of platelets. Since a given volume of large platelets would contain fewer platelets than would the same volume of small platelets, the radioactivity per platelet in his studies must have been much higher in newly formed platelets than in older ones. We did not measure platelet volume in the studies reported here, but, in all cases, comparable specific activities (cpm./10⁹ platelets) were found for populations of newly formed platelets and of mixed ages. Thus, labeling intensity did not appear to be greater for young cells.

From studies of platelet turnover in normal rats injected with ³²P-DFP, we concluded that rat platelets were mostly destroyed by randomly occurring processes and that labeling of megakaryocytes was insignificant. If both of these conclusions were correct, then platelet specific activity, which is dependent on replacement or dilution of labeled cells by unlabeled cells, should have fallen somewhat more rapidly than controls in the animals with increased platelet production. Total platelet-bound radioactivity should have fallen at a normal rate, since newly formed platelets would be subject to the same randomly occurring processes as would a population of platelets of mixed ages. However, these conditions were not found.

The data appear to be most compatible with significant random loss of platelets, together with megakaryocyte labeling. Thus, in animals receiving ³²P-DFP 24 hours after production of thrombocytopenia, there was a decrease of 40–50 per cent in circulating platelet-bound radioactivity within 24 hours after labeling. Senescence could not have accounted for such a loss, and random loss is clearly implicated. Thereafter followed a plateau in circulating radioactivity. This appears to be best explained by production of labeled platelets which implies megakaryocytic labeling. In animals labeled 48 hours after induction of thrombocytopenia, the initial drop was less marked, ~30 per cent, presumably because the megakaryocytic response to thrombocytopenia was more fully developed and there was an earlier release of larger numbers of labeled platelets. Similarly, as would be anticipated from these considerations, the duration of the plateau relative to the induction of thrombocytopenia was similar in all experiments. Splenic pooling, as a major contributing factor was eliminated by the studies in splenectomized animals. If megakaryocytes were more heavily labeled in animals stimulated by thrombocytopenia than in the normals, platelet specific activity might have been expected to fall at a rate slower than normal. The fact that it did not suggested that the newly formed platelets were labeled in the normal animals also.

If labeled platelets had been retained in the circulation until completion of a life span and, at the same time, only unlabeled cells had been produced, then platelet-bound radioactivity should have dropped precipitously when the labeled cells became senescent. No such precipitous drop was seen. If platelet destruction were predominantly senescent and labeled platelets had been produced, then a rising total circulating radioactivity would have been anticipated in the first few days after injection of ³²P-DFP. The striking fall in the first 24 hours clearly excludes this possibility. Because of these considerations it was concluded that an obligatory life span for rat platelets did not fully ac-
count for the unusual results when cohorts of platelets were labeled in situ with \(^3\)H-DFP during recovery from acute thrombocytopenia. This conclusion was further substantiated by the finding that newly formed labeled platelets were destroyed at a normal rate when transfused into normal recipients.

For radioactive DFP to be an accurate determinant of platelet survival, it should label only cells within the circulation and not the precursor cells. If megakaryocytes are significantly labeled, then platelet radioactivity curves are partially determined by rate of influx of new cells and relative intensity of labeling of platelets and megakaryocytes rather than by rate of platelet destruction alone. The intensity of labeling of the platelet units within megakaryocytic cytoplasm appears to be considerably less than that of platelets in the blood at the time of injection of the compound. However, the platelets subsequently produced carry enough of the radioactive label to significantly alter the decay curve for circulating platelet-bound radioactivity. The delivery of labeled platelets into the circulation of rats for several days after injection of \(^3\)H-DFP constitutes a serious drawback to the use of this compound for determination of peripheral platelet turnover in situ.

**SUMMARY**

Rat platelets were labeled with tritiated diisopropylfluorophosphate (\(^3\)H-DFP) during recovery from acute thrombocytopenia. The results indicated that there was significant labeling of megakaryocytes by \(^3\)H-DFP which, in the presence of an increased rate of platelet production, resulted in maintenance of relatively constant values for platelet-bound radioactivity during the period of maximum platelet production and reactive thrombocytosis. Significant random loss of platelets was apparent, and, when a cohort of young platelets was transfused to normal recipients, they were destroyed at a normal rate.

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