Demonstration That Thiazole-Orange–Positive Platelets in the Dog Are Less Than 24 Hours Old

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Approximately 6% of dog platelets are positive for staining with thiazole orange, a dye frequently used to stain ribonucleic acid. In this report, thiazole-orange positivity is shown to mark platelets that are less than 24 hours old. Dog platelets were derivatized in vivo with N-hydroxysuccinimido biotin such that greater than 95% of all platelets were biotinylated. Newly synthesized, nonbiotinylated platelets were then monitored by flow cytometry for their ability to bind thiazole orange. After biotinylation, the percentage of biotin-negative, thiazole-orange–positive platelets increased gradually from 0.72% at 30 minutes to 5.44% at 24 hours. These data indicate that thiazole-orange staining does label newly synthesized platelets.

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In this report we use in vivo biotinylation of dog platelets to show that newly synthesized platelets are thiazole-orange positive. Specifically, greater than 95% of existing platelets are biotinylated by infusion of N-hydroxysuccinimido-biotin (NHS-biotin). At subsequent times, the appearance of new, nonbiotinylated platelets allows evaluation of thiazole-orange binding by these newest platelets. The data show that newly synthesized platelets are thiazole-orange positive and that platelets remain thiazole-orange positive for less than 24 hours.

MATERIALS AND METHODS

Materials. Thiazole orange was obtained as Retic-COUNT from Becton-Dickinson (Mountain View, CA). N-Hydroxysuccinimido-biotin (NHS-biotin) was from Calbiochem (La Jolla, CA). Streptavidin TRI-COLOR was from Caltag Laboratories (South San Francisco, CA). Dimethylsulfoxide (DMSO) was supplied by Research Industries, (Midvale, UT). Ribonuclease from bovine pancreas (#1119-915) was obtained from Boehringer-Mannheim (Indianapolis, IN). Saponin was from Sigma Chemical Co (St Louis, MO).

Buffers. We used the following buffers: buffered saline-glucose-citrate (BSCG), pH 7.3; 129 mM/L NaCl; 13.6 mM/L Na, citrate; 11.1 mM/L glucose; 1.6 mM/L KH,POI; 8.6 mM/L NaH,PO, pH 7.3; acid citrate dextrose (ACD); 38.1 mM/L citric acid; 74.8 mM/L Na, citrate; 136 mM/L glucose; phosphate buffered saline (PBS); 150 mM/L NaCl; and 10 mM/L NaH,PO, pH 7.4.

In vivo biotinylations. Adult beagle dogs were obtained from Marshall Laboratories (North Rose, NY) and housed according to regulations of the Institutional Animal Use and Care Committee of the University of Oklahoma Health Sciences Center (accredited by the American Association for Accreditation of Laboratory Animal Care). Dogs were biotinylated in vivo as described previously. Briefly, N-hydroxysuccinimido-biotin (0.3 mg/kg body weight) was dissolved in DMSO at 20 mg/mL with mild warming at 37°C. One half of the NHS-biotin was diluted into 20 mL of 0.9% saline and immediately infused into a front leg vein of the dog. Infusions were performed with a 21-g butterfly infusion set and a syringe pump at a flow rate of 6 mL/min. Thirty minutes after the first infusion, the second half of the NHS-biotin was diluted into saline and infused as before. This staggered infusion of NHS-biotin results in greater than 95% of all platelets being labeled with biotin (compare A and B in Fig 1).

A previous report used a single infusion of NHS-biotin with a resulting 80% of platelets biotinylated. The proposed explanation for the incomplete biotinylation was that platelets pooled in the spleen were not exposed to NHS-biotin because of the very short in vivo life span of NHS-biotin and the sluggish circulation of the spleen. Subsequently, we have adopted the double infusion technique described here with a 30-minute time period between biotinylations with the expectation that platelets present in the spleen during the first biotinylation would reequilibrate to the general circulation before the second labeling. This hypothesis has been recently strength-
NEW PLATELETS ARE THIAZOLE-ORANGE POSITIVE

1823

Fig 1. Flow-cytometric analysis of thiazole-orange positivity after in vivo biotinylation. Dog platelets were analyzed for thiazole-orange fluorescence (FL,) and surface biotinylation (streptavidin TRI-COLOR; FL,) before and after in vivo biotinylation. Gate R1 indicates platelets that are thiazole-orange positive and biotin negative. Gate R2 depicts platelets that are thiazole-orange positive and biotin positive. A through I represent time points -0.5, 0.17, 2, 4, 5, 6, 8, 10, 12, and 24 hours after biotinylation of dog no. 503. Note the time-dependent increase in thiazole-orange-positive, biotin-negative events (gate R1). The total level of thiazole-orange-positive events for these nine samples was 5.55±0.28% (mean ± 1 SD; n = 9) with a range of 5.11% to 5.97%. Ribonuclease treatment of fixed and permeabilized platelet samples eliminates the thiazole-orange staining indicated in gates R1 and R2 (see Table 1).

Streptavidin–TRI-COLOR labeling. Blood samples were drawn into EDTA tubes and mixed continuously at room temperature. Analysis was initiated within 60 minutes of drawing the blood. Ten microliters of whole blood was diluted into 4 ml of BSGC, pH 7.3, and 400 µL of 3.7% formalin in BSGC, pH 7.3, was added. (All solutions used in this analysis were filtered through 0.45-µm filters.) After 60 minutes of fixation, the sample was centrifuged at 1,500g for 10 minutes, and the pellet was resuspended in 1 ml BSGC, pH 7.3. These fixed samples can be stored at 4°C for at least 24 hours before analysis. Two hundred microliters of the fixed, washed blood sample was incubated with 4 µg/ml of streptavidin TRI-COLOR for 30 minutes at room temperature. TRI-COLOR is a Cy-5–derivatized phycoerythrin that is activated at 488 nm and has an emission peak at 667 nm. The 667-nm emission is observed in the FL, channel of the FACScan flow cytometer (Becton Dickinson, Mountain View,
The log settings used were FSC temperature. Five micrograms/milliliter of ribonuclease were subsequently added and the incubation continued for 15 minutes at 37°C. Staining for streptavidin TRI-COLOR and thiazole orange were then performed as outlined above.

RESULTS

In vivo biotinylation results in covalent labeling of greater than 95% of platelets in dogs. The biotin label is stable in vivo and allows determination of platelet life spans. As a result of these characteristics, biotinylation can also be used to tag existing cells and thereby identify newly synthesized cells by their lack of surface-bound biotin (see Discussion).

Figure 1A shows the streptavidin TRI-COLOR and thiazole-orange labeling of a prebiotinylation sample. With this particular dog, 5.27% of all platelets are thiazole-orange positive (gate R1), and this positivity is lost after incubation of fixed, permeabilized platelets with ribonuclease (see below). Ten minutes after in vivo biotinylation, greater than 95% of all platelets are biotin positive as indicated by the binding of streptavidin TRI-COLOR (Fig 1B). The thiazole-orange-positive cells are also biotinylated and are now present in gate R2; furthermore, the total level of thiazole-orange-positive events did not change significantly as a result of biotinylation (5.55% thiazole-orange-positive events at 10 minutes postderivatization).

After biotinylation, samples were analyzed for the number of platelets that were thiazole-orange positive and biotin negative (gate R1); biotin-negative platelets are newly synthesized (see Discussion). The number of new platelets that are thiazole-orange positive and biotin negative increased with time (for example, compare Fig 1, C and H). The progressive nature of the change in biotin-negative, thiazole-orange-positive platelets is illustrated in Fig 2, which summarizes data from three dogs. At 30 minutes, the number of biotin-negative, thiazole-orange-positive cells dropped from the prederivatization level of 5.21% ± 0.07% (mean ± 1 SD; n = 3) to 0.72% ± 0.20% of the total population. Over the next 24 hours, the number of platelets in this R1 gate gradually increased to 5.44% ± 0.53%.

An analysis of biotin-positive and thiazole-orange-positive platelets (gate R2) produces a similar but reciprocal plot (data not shown). These cells represent those already thiazole-orange positive at the time of biotinylation, which are subsequently lost from gate R2, presumably into the large population of biotin-positive, thiazole-orange-negative platelets.

Table 1 documents the effect of ribonuclease treatment on the thiazole-orange positivity of dog platelets. For these experiments, platelets were fixed with formalin as detailed above and permeabilized with 0.2% saponin. These data show that saponin exposure does not affect thiazole-orange staining; however, incubation of permeabilized platelets with ribonuclease does eliminate the thiazole-orange staining. Ribonuclease treatment of nonpermeabilized cells does not impact thiazole-orange staining (data not shown).

**DISCUSSION**

The data presented in this report document that thiazole-orange–positive platelets are newly synthesized platelets and...
NEW PLATELETS ARE THIAZOLE-ORANGE POSITIVE

that they remain thiazole-orange positive for less than 24 hours. The flow cytometric dot plots shown in Fig 1 clearly show the shift in the observed thiazole-orange–positive events from the biotin-positive gate (R2) to the biotin-negative gate (R1) with time after biotinylation. An approximation of the kinetics for the appearance of thiazole-orange positivity in the biotin-negative gate is shown in Fig 2. The overall conclusion is quite clear that thiazole-orange–positive platelets are the youngest cells.

The slope of the graph in Fig 2 should reflect the life span of thiazole-orange positivity; however, minor alterations in the gate set between biotinylated and nonbiotinylated platelets in Fig 1 dramatically affect the recovery slope in Fig 2. Modest adjustments in the gate will shift the apparent life span from the 24 hours shown in Fig 2 to values as low as 12 hours. As a result, the precise period of time a platelet remains thiazole-orange positive cannot be determined from these experiments.

Platelet biotinylation in vivo allows existing platelets to be tagged and thereby discriminated from newly synthesized, nonbiotinylated platelets. However, a careful analysis of the "nonbiotinylated" population of platelets in this study as well as a previous report indicated that after in vivo biotinylation, newly appearing platelets are not totally lacking in surface biotin as indicated by a modest binding of PE-streptavidin (compare FL3 levels of platelets in gate R1 in Fig 1, A and H). There are two possible explanations for this phenomenon. Either megakaryocytes are biotinylated and subsequently produce biotinylated platelets or newly synthesized platelets adsorb small quantities of plasma proteins, which, in this case, happen to be biotinylated because the in vivo biotinylation reaction is nonselective. We have directly examined megakaryocytes 2 hours after in vivo biotinylation and found very modest levels of biotinylation. Considering that megakaryocytes will fragment into many platelets, the biotin level per platelet will be insignificant, but may explain the modest levels seen here. The second explanation would require newly synthesized platelets to adsorb biotinylated plasma proteins (eg, fibrinogen or IgG) on their cell surface. There is no experimental support for this proposal, but it cannot be dismissed at this time. Whatever the explanation, the modest level of biotinylation exhibited by newly synthesized platelets does not interfere with their differentiation from fully biotinylated platelets.

These observations indicate that thiazole-orange analysis is potentially a reliable method to estimate platelet production. In addition, the percentage of thiazole-orange–positive platelets may serve as an estimate of mean platelet age under steady-state conditions. However, before thiazole-orange staining of platelets can be a useful tool clinically, several assay parameters need to be finalized. For example, whereas in this report and that of Kienast and Schmitz, whole blood was analyzed, others have used platelet-rich plasma. However, the primary problem with the use of thiazole orange is one of defining a threshold gate for positivity. Ault et al have set a gate that identifies 1% of platelets in a normal control as thiazole-orange positive. A simpler methodology for setting an analysis gate would enhance the assay, and recently Bonan et al have proposed use of thiazole-orange–stained reticulocytes as an internal marker for setting the fluorescence threshold for thiazole-orange–stained platelets. We and others have shown that ribonuclease treatment of permeabilized platelets eliminates thiazole-orange staining, but this approach may not be amenable to a routine assay.

Validation of thiazole orange as a marker of the very youngest platelets will also allow studies of anomalies associated with thrombocytopenias where bleeding may or may not occur despite low platelet counts. Because it is widely assumed and infrequently shown that young platelets are the most hemostatically active, a measure of mean platelet age, as determined by the percentage of very young platelets, may be useful in evaluation and management of thrombocytopenic patients.

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REFERENCES

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GL Dale, P Friese, LA Hynes and SA Burstein