Effects of Chelates and Incubation Media on Platelet Labeling with Indium-111

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We studied the effects of various [^{111}In]chelates and incubation media on labeling efficiency (LE) and in vivo survival of platelets. High LE of human and rabbit platelets in plasma were obtained with [^{111}In]tropolone and [^{111}In]mercaptopyridine-N-oxide. Indium-111 oxine in plasma resulted in a moderate LE and required a longer incubation time, while [^{111}In]oxine sulfate had low LE and inconsistent labeling. High LE for all forms of [^{111}In]chelates were achieved in labeling media free of plasma. However, in vivo platelet survival in rabbits was markedly reduced when platelets were labeled in the absence of plasma.

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Indium-111 (^{111}In) platelet labeling has become widely accepted during the last several years for both scintigraphic and in vivo kinetic studies. The labeling procedure using ^{111}In chelated with oxine (8-hydroxyquinoline), which was first introduced by Thakur et al. (*1*), has been modified by a number of investigators (*2–10,20–23,25,35*) in an effort to achieve the highest labeling efficiency without loss of platelet viability. However, there is no agreement as to which labeling conditions are ideal for platelet labeling. This has lead to a large variation in the reported experimental conditions, making comparison of the data from different laboratories difficult.

In this study, we compared the labeling efficiency for human and rabbit platelets using various [^{111}In]chelates in media containing plasma or free of plasma while other labeling conditions were held constant. The in vivo kinetics and distribution of rabbit platelets labeled under the different conditions were also studied as tests of in vivo platelet viability. The [^{111}In]chelates studied were oxine, oxine sulfate, tropolone and mercaptopyridine-N-oxide, while the incubation media studied were plasma, saline, modified Tyrode's solution, Hepes, and Tris buffers. All these [^{111}In]chelates and incubation media have been previously utilized by various investigators (*3–10,14,20–23*).

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MATERIALS AND METHODS

Isolation of Platelets for In Vitro Studies

Platelets were isolated from 42.5 ml blood obtained using a 19-gauge needle and 50-ml plastic syringe containing 7.5 ml acid citrate dextrose (ACD NIH-A). For rabbits, the central ear artery was used, while the blood sample from human volunteers used the antecubital vein. All procedures were done at room temperature with sterile plastic equipment. Platelet-rich plasma (PRP) was obtained by centrifugation at 220 *g* for 15 min. The PRP was the acidified to pH 6.5–6.7 with ACD NIH-A. After centrifugation at 1,000 *g* for 10 min, the platelets were counted and 5×10^9 platelets were suspended in 2 ml of the designated labeling media.

Preparation of [^{111}In]Chelates

Indium-111-oxine was prepared as previously described (*24*). Briefly, [^{111}In]chloride,* supplied in 0.01 N HCl, was chelated to oxine,† extracted into methylene chloride, evaporated to dryness, and dissolved in a mixture of ethanol and normal saline (1:4 by volume). This routinely resulted in 10 μl of ethanol added per ml of platelet suspension (or 1.0% [v/v]). For human platelets, the optimum oxine concentration of ~6.5 μg/ml platelet suspension was used (*25*). The optimum oxine concentration of 12 μg/ml platelet suspension was used for rabbit platelets (*24*). The specific activity ranged from 1.3 to 6.8 μCi/μg oxine.

The method of McAfee et al. (*14*) for the preparation of [^{111}In]oxine sulfate for leukocyte labeling was modified for platelet labeling as follows. The stock solution of oxine sulfate† contained 1 mg/ml in distilled, sterile, pyrogen-free water. Fifty microliters of the stock solution was mixed with 300 μl of [^{111}In]chloride, and 300 μl of Tris buffer (pH 7.5) was then added. The final concentration of oxine sulfate used for both human and rabbit platelets was 4.2 μg/ml platelet suspension.

The specific activity of this [^{111}In]chelate ranged from 6.4 to 35.7 μCi/μg oxine sulfate.

A stock solution of tropolone† of 1 mg/ml in Hepes-saline buffer (pH 7.6), containing 20 m*M* Hepes in normal saline was prepared by a modification of the method of Burke et al. (*26*). A working solution of [^{111}In]tropolone was made by adding 500 μl of the stock tropolone solution dropwise into 300 μl [^{111}In]chloride. A concentration of 31.3 μg tropolone per ml platelet suspension (2.4×10^{-4} *M*) was used for both human and rabbit platelet labeling. The range of specific activity used was 0.7 to 3.9 μCi/μg tropolone.

Indium-111-mercaptopyridine-N-oxide was prepared according to the method of Thakur et al. (*22*). Briefly, 1 mg of mercaptopyridine-*N*-oxide, as the sodium salt‡ (MPO), was mixed with 400 μl of [^{111}In]chloride and 4.5 ml of sterile normal saline was then added. A final concentration of 10 μg MPO per ml platelet suspension was used for both human and rabbit platelets. Specific activity ranged from 0.5 to 2.6 μCi/μg MPO.

All chelates were reagent grade and the purity of these agents was >98%. Each was used as supplied commercially.

In Vitro Platelet Labeling

For the in vitro evaluation of labeling efficiency, 100 μl of the [^{111}In]chelate solution was added to 2 ml of platelet suspended in various labeling media. For all experiments, labeling factors such as platelet concentration, centrifugal forces, handling techniques, anticoagulant type, and temperature were held constant. Chelate solutions were prepared on the day of the experiments. Labeling media which were studied included plasma, saline, modified Tyrode's (*35*), Hepes, and Tris. To determine labeling efficiency (LE) at various time points, a 100-μl sample of the radiolabeled platelets was removed at 1 min and selected intervals over 60 min. The platelets were separated from the labeling media by immediate centrifugation in a microcentrifuge and removal of the supernatant to another container. Percent LE was determined by measuring the fraction of ^{111}In radioactivity that remained associated with the platelets following the timed incubation with the various [^{111}In]chelates. Indium-111 radioactivity was determined by counting platelet pellets and supernatants in an automated sodium iodide well counting system.§

In Vivo Platelet Survival Studies

These were performed as described previously (*24*). Briefly, rabbit platelets were isolated as stated above for in vitro studies. Platelets were separately labeled with [^{111}In]oxine (12 μg/ml platelets), [^{111}In]tropolone (31.3 μg/ml platelets), and [^{111}In]MPO (10 μg/ml platelets) in plasma, modified Tyrode's, and saline incubation media. Following the i.v. injection of 30 to 120 μCi ^{111}In-labeled platelets (5×10^9 to 1.5×10^{10} platelets) into the marginal ear vein of 3.5–4.5 kg female New Zealand white rabbits, heparinized blood samples (3 ml) were obtained from the central ear artery at 30 min, 90 min, and daily thereafter for 6 days using 3-ml plastic syringes and 25-gauge needles. Separate aliquots of 1 ml whole blood and 0.5 ml platelet-free plasma from each sample were prepared for counting. Aliquots of 50 μl of the ^{111}In-labeled platelet injectate in 2 ml of water were used as counting standards. All samples were counted to <3% counting error in an automated gamma well counting system. The results were expressed as percentages of the injected dose in circulating platelets, assuming a blood volume of 4.25% of total-body weight (*3*).

To estimate the mean platelet survival time, the data were subjected to computer analysis using linear, exponential, and multiple-hit survival curve models, as described previously (*3, 7,9,24*). The percent initial recovery was determined by extrapolation of the multiple-hit survival curves to time zero.

Biodistribution Studies

The rabbits were killed on the sixth day of the study by an overdose of i.v. pentobarbital. Multiple samples of liver, spleen, lung, kidney, muscle, tibia, and bone marrow were obtained at necropsy. The radioactivity of each sample was determined by counting all samples with standards in the same manner as described for the blood samples. The radioactivity was expressed as percentages of the injected dose per organ using the following assumptions: skeletal muscle mass, 43% total-body weight (TBW); skeletal mass, 10% TBW; and bone marrow mass, 2.2% TBW (*27–29*).

Statistical Methods

Results are expressed as mean and standard error of the mean (s.e.m.) unless otherwise indicated. Differences between means were tested for statistical significance by the Student's t-test, with p-values of the differences <0.05 being considered statistically significant (*30*).

RESULTS

In Vitro Labeling Studies

We first assessed various [^{111}In]chelates as platelet labeling agents using a variety of incubation media. The concentrations of chelates used were those suggested by previous investigators as optimal for labeling platelets (*14,22,24–26*). The labeling of human platelets with [^{111}In]oxine in plasma (Fig. 1A) resulted in a mean labeling efficiency of 27% at 3 min, increasing to 37% at 10 min, and 57% by 60 min. The mean LE of the chelating agents, tropolone and MPO, were 82% and 67%, respectively, at 3 min, reaching a maximum LE of 89% and 81%, respectively, by 10 min. Oxine sulfate was found to be a poor platelet label at the concentration used with a maximum LE of only 22% and to have considerable variation in LE among experiments, e.g., a large standard error (data not shown).

When modified Tyrodes solution was used as the platelet labeling medium (Fig. 1B), all chelating agents except oxine sulfate had LE of >90% by 3 min. Oxine sulfate had a mean LE of 69% at 3 min, reaching 90% LE by 45 min. With saline as the labeling medium (Fig. 1C), LE of 80–85% were obtained for all four chelating agents tested. Oxine, tropolone, and MPO showed no additional changes over the 60-min incubation period. Slightly higher LE (95%) was achieved by oxine sulfate. Labeling in Tris, or Hepes consistently resulted in mean LE of 90% or greater by 3 min for all ^{111}In chelating agents. Results are not shown for these labeling media

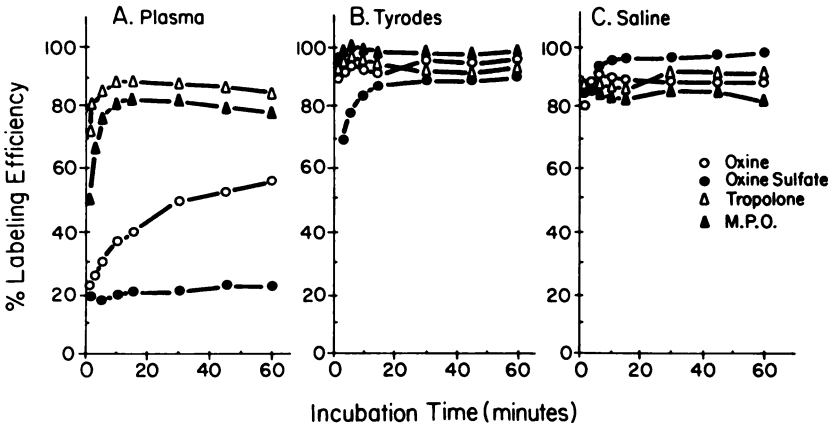

FIGURE 1
Labeling of human platelets with various [^{111}In]chelates in A: Plasma, B: Modified Tyrode's, and C: Saline. Samples of 5 × 10^9 platelets from normal volunteers were suspended in 2 ml of incubation media and labeled with the [^{111}In]chelates at room temperature. Aliquots of the platelet suspension were removed at different times and platelet-associated ^{111}In radioactivity determined. Each point represents the mean of three experiments.

since their labeling patterns are similar to those of saline.

Similar results were obtained using rabbit platelets (Fig. 2) with the following notable exceptions. Indium-111-oxine appeared to be a better labeling agent in plasma for rabbit platelets. The mean LE of 58% was achieved at 3 min, increasing to 90% by 30 min (Fig. 2A). Indium-111-oxine sulfate was a poorer labeling agent in saline for rabbit platelets (Fig. 2C).

Platelet Kinetic Studies in Rabbits

Based on the above in vitro results, oxine, tropolone, and MPO in plasma, modified Tyrode's, and saline were selected for in vivo kinetic and distribution studies in rabbits. All incubation times were 10 min, except for [^{111}In]oxine in plasma, which was 30 min.

The kinetics of ^{111}In-labeled platelets were analyzed using three different mathematic models, i.e., linear, exponential, and multiple hit models. As we have reported previously (*6,8,23*), the kinetic data fitted the multiple-hit model best as judged by the residual sum of squares, which was used as a measure of the precision of the curve fitting (data not shown). Therefore, only estimates derived from the multiple-hit model are reported here. There was no statistically significant difference in platelet survival among the three chelating agents in plasma (Table 1). However, all labeling done in media free of plasma resulted in significantly reduced mean platelet survival times (Table 1). The initial recoveries of the ^{111}In-labeled platelets are shown in Table 2. In contrast to the differences seen in mean survival times between plasma and plasma-free media, no statistical differences were seen for the initial recovery, except when platelets were labeled with [^{111}In]oxine in modified Tyrode's. The reason for this is unclear.

Biodistribution Studies

Six days postinjection of ^{111}In-labeled platelets, the rabbits were killed and major organs removed. Rabbit liver, spleen, and bone marrow are known to be the major organs of platelet sequestration (*24*). The mean percent dose per organ was <4% in bone, muscle, kidneys, and lungs and did not vary with different labeling conditions. As shown in Fig. 3, there was no statistical difference in platelet sequestration in liver, spleen, and bone marrow among the different chelating agents in each of the three different labeling media, except for hepatic sequestration of platelets labeled with [^{111}In]oxine in Tyrode's solution. The hepatic sequestration of rabbit platelets labeled under these conditions was 28.8%, which was significantly decreased from that seen with the same [^{111}In]chelate in plasma media

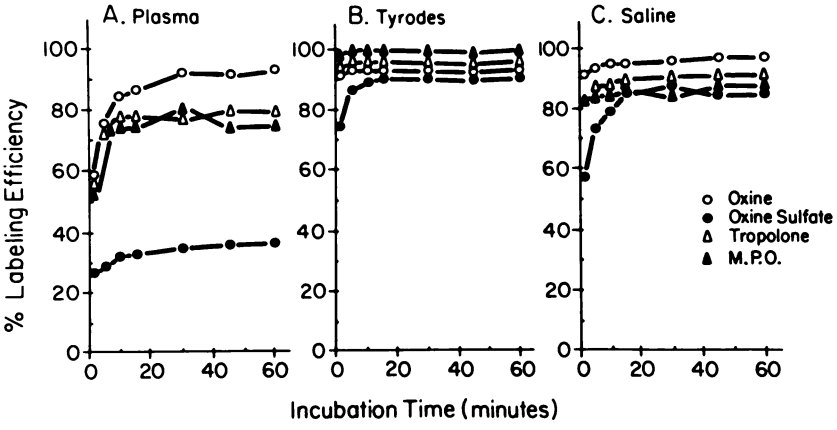

FIGURE 2
Labeling of rabbit platelets with various [^{111}In]chelates in A: Plasma, B: Modified Tyrode's, and C: Saline. Conditions for labeling were the same as those described in Figure 1. Each point represents the mean of three experiments.

TABLE 1
Effect of Various Chelates and Incubation Media on the Survival of Indium-111-Labeled Rabbit Platelets

	Mean platelet survival time (days)*		
	Plasma	Tyrode's	Saline
Oxine	2.62 ± 0.16 (8)†	1.10 ± 0.09 (4)	1.25 ± 0.34 (3)
	—	$p < 0.001$	$p < 0.005$
Tropolone	2.37 ± 0.38 (4)	1.35 ± 0.06 (4)	1.54 ± 0.20 (4)
	N.S.	$p < 0.001$	$p < 0.005$
MPO	2.86 ± 0.24 (4)	1.30 ± 0.05 (4)	1.61 ± 0.20 (4)
	N.S.	$p < 0.001$	$p < 0.005$

* Mean platelet survival time as estimated from the multiple-hit survival curve model. p Values given for comparison of means with the mean of oxine in plasma.
† Number of experiments is given in parenthesis.

TABLE 2
Effect of Various Chelates and Incubation Media on the Initial Recovery of Indium-111-Labeled Rabbit Platelets

	Initial recovery (%)*		
	Plasma	Tyrode's	Saline
Oxine	63.2 ± 3.5 (8)†	49.9 ± 1.8 (4)	72.9 ± 5.5 (3)
	—	$p < 0.05$	N.S.
Tropolone	67.8 ± 7.4 (4)	73.0 ± 5.3 (4)	75.5 ± 5.3 (5)
	N.S.	N.S.	N.S.
MPO	71.8 ± 2.9 (4)	75.3 ± 6.2 (4)	78.0 ± 10.3 (4)
	N.S.	N.S.	N.S.

* Percent initial recovery as estimated from the multiple-hit survival curve model. p Values given for comparison of means with mean of oxine in plasma.
† Number of experiments is given in parenthesis.

(40.0%, $p < 0.05$). In all cases, a total of 80 to 90% of the injected [^{111}In]platelets were found in these three organs.

DISCUSSION

As a platelet labeling agent, [^{111}In]oxine suffers from several disadvantages. First, the platelet labeling efficiency of [^{111}In]oxine in plasma is low as compared with that seen in media without plasma. This has led many investigators to omit plasma from the incubation medium (*1,5,6,8,10*). There is evidence, however, that incubation of platelets in the absence of plasma during the labeling procedure may damage the platelet and alter its in vivo survival (*2,4,13*). Second, [^{111}In]oxine is insoluble in aqueous solution. A small quantity of ethanol is routinely added to dissolve it for labeling platelets (*1–10*). However, ethanol is potentially toxic to the platelets (*11,12*).

As an attempt to overcome the above shortcomings of [^{111}In]oxine, a number of lipid-soluble metal chelates, which complex with ^{111}In in both aqueous and non-aqueous media, have been described (*14–23*). Tropolone (*20*) and mercaptopyridine-N-oxide (*22*) label platelets with high efficiency even in the presence of plasma. The water soluble nature of these [^{111}In]chelates eliminates the need for the use of ethanol. Oxine sulfate, which is also soluble in the aqueous medium, has been used to label leukocytes (*14*), but as yet, has not been studied as a platelet label. Although several investigators have suggested that some [^{111}In]chelates are better platelet labeling agents than others' there has been no systematic comparison of the various [^{111}In]chelates. In addition, there has been no general agreement as to whether media without plasma can be used to label platelets with [^{111}In]chelates without compromising their integrity and in vivo survival.

In this study, we have compared several [^{111}In]chelates complexed with platelets under identical labeling conditions. Factors such as platelet concentration, centrifugal forces, handling techniques, time ex vivo, anticoagulant, and temperature were held constant. The concentrations of several [^{111}In]chelates were those shown by previous investigators to be optimal for platelet labeling (*14,22,24–26*). In vivo survival of ^{111}In-

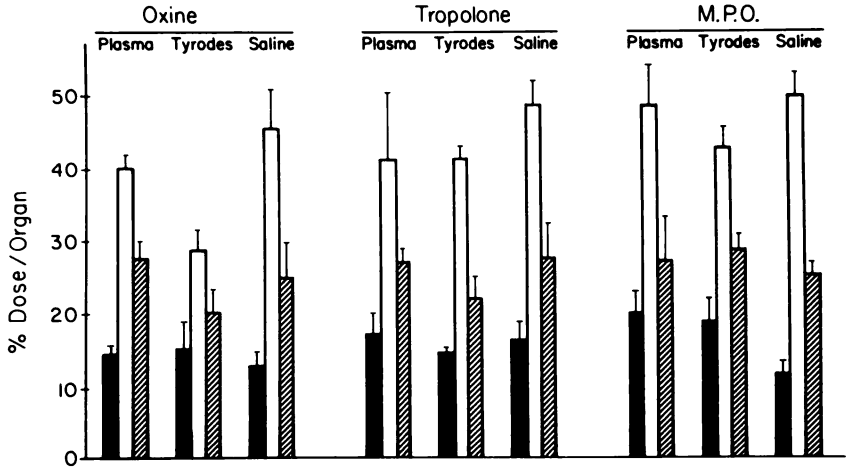

FIGURE 3
Organ distribution of [^{111}In]platelets in rabbits 6 days after infusion. Platelets were labeled using the various [^{111}In]chelates and incubation media. The results are the mean and s.e.m. of three experiments. (■) Spleen; (□) Liver; (▨) Bone marrow.

labeled rabbit platelets was used as an indicator of platelet integrity, instead of in vitro platelet function tests, since there is concern as to the relevance of in vitro testing to in vivo function (*36*).

Our results confirm those of others (*20,22,23*) that under identical labeling conditions, [^{111}In]tropolone and [^{111}In]MPO label platelets more efficiently than [^{111}In]oxine. In the rabbit model, platelets labeled in plasma, regardless of the [^{111}In]chelate used, survived longer in the circulation than those platelets labeled in incubation media that lacked plasma. Whether these latter results can be extrapolated to human conditions is not clear and requires further study.

It has been shown that [^{111}In]oxine labeled platelets have similar in vivo survival in rabbits as those labeled with chromium-51-sodium chromate (*24*). In this study, we have also shown that [^{111}In]oxine-labeled platelets exhibited similar survival as those labeled with [^{111}In]tropolone or [^{111}In]MPO. This suggests that the ethanol present in [^{111}In]oxine is not detrimental to platelets as judged by the in vivo survival of [^{111}In]oxine labeled platelets in rabbits.

NOTES

* Medi-Physics Inc., Richmond, CA.
† Aldrich Chemical Co., Milwaukee, WI.
‡ Sigma Chemical Co., St. Louis, MO.
§ Packard Instrument Co., Downers Grove, IL.

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