Splenic Imaging with ^{99m}Tc-Labeled Erythrocytes: A Comparative Study of Cell-Damaging Methods

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Several methods of damaging red blood cells (RBCs) for splenic imaging were compared to determine the optimum approach. The RBCs from donor animals were labeled with $^{99m}TcO_4^-$ and damaged by heat, excess acid citrate dextrose (ACD), excess Sn(II) ion, or the sulfhydryl inhibitors N-ethylmaleimide (NEM) or p-hydroxymercuribenzoate (PMB). The organ distributions of undamaged and damaged RBCs were determined in rats, and splenic imaging studies were performed in rabbits. Splenic deposition and spleen-to-liver ratios with heat- or sulfhydryl-damaged ^{99m}Tc-RBCs were significantly greater ($p < 0.001$) than the values obtained using ACD or Sn(II) ion. Heat-damaging produces good splenic localization of ^{99m}Tc-RBCs but requires rigidly controlled incubation conditions. NEM-damaging provides an excellent and predictable alternative approach.

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Specific imaging of the spleen (i.e., with only minimal activity in the liver) is desirable in a number of clinical situations. These include the detection of accessory spleens, the evaluation of patients with suspected congenital asplenia or polysplenia, and the evaluation of patients with suspected splenic injury. The ideal agent for splenic imaging would have the high photon yield and low radiation dose of ^{99m}Tc-sulfur colloid and the high splenic specificity of damaged ^{51}Cr-tagged red blood cells (RBCs) (*1,2*) or ^{197}Hg-mercurihydroxypropane-labeled RBCs (*3–6*). Theoretically, ^{99m}Tc-RBCs fulfill these requirements (*7,8*). Recently, reproducible efficient methods of labeling RBCs with $^{99m}TcO_4^-$ have become available (*9–12*), and the ^{99m}Tc-RBCs may then be damaged to augment their uptake by the spleen (*13*). Some workers have used heating as the means of damaging (*11,13–15*), whereas others have used such chemicals as acid citrate dextrose (ACD) or Sn(II) ion (*16*). The sulfhydryl inhibitors N-ethylmaleimide (NEM) and p-hydroxymercuribenzoate (PMB) have also been employed (*13,17–19*), but these agents have not been used with ^{99m}Tc-RBCs. Because of the potential utility of splenic imaging with ^{99m}Tc-RBCs, we compared these various damaging methods to determine which was optimal for use with this radiopharmaceutical.

METHODS

The splenic deposition and organ distributions of ^{99m}Tc-RBCs (with and without damaging) were investigated in male Sprague–Dawley rats weighing 200–400 gm.

Labeling methods. *Method 1.* Utilizing the method of Harwig et al. (*9*), 6 ml of whole blood from a donor rat was infused into a sterile tube containing 0.5 ml of ACD. Two milliliters of packed cells was then incubated with 1 μg of electrolytically produced stannous ion and then washed with 3 ml of saline. Five millicuries of $^{99m}TcO_4^-$ was then added to the packed red cells, and after a 5-min incubation the cells were washed with 3 ml of saline.

Method 2. Following the method of Smith and Richards (*10*), 6 ml of whole blood was directly injected into their sterile kit, which contains sodium heparin, sodium citrate, dextrose, and 2.6 μg of stannous citrate. Two milliliters of packed red cells was then added to a sterile vial containing 5 mCi of $^{99m}TcO_4^-$.

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Both methods provided high labeling efficiency (90–95% by the Harwig method, above 97% by Smith and Richards). These efficiencies were determined by washing the red cells twice after the incubation and then counting the ^{99m}Tc activity in the two supernatants. Cells labeled by the two methods revealed no significant difference in splenic deposition or spleen-to-liver ratios. Accordingly, the results are combined in the data presentation.

Damaging techniques. In preliminary studies, we determined which damaging techniques maximize uptake and spleen-to-liver ratios (S/L = activity per gm spleen/activity per gm liver). Higher concentrations and longer incubation times than those described below resulted in greater RBC deposition in the liver and thus lower S/L ratios. The damaging techniques studied were as follows:

1. Heat-damaging was performed using a thermostat-controlled water bath preheated to 49–50°C. Two milliliters of ^{99m}Tc-RBCs in a sterile test tube was incubated in this bath for 15 min, with gentle agitation at 30-sec intervals. To assess the need for a rigidly controlled constant-temperature water bath, an experiment was performed in which the incubation temperature varied over 48–51°C.
2. NEM* was added to washed ^{99m}Tc-RBCs to give concentrations of 5, 10, 15, 20, or 25 micromoles NEM per milliliter of packed RBCs, and the mixture was incubated for varying times (1, 3, 5, 15, or 30 min) at room temperature. After incubation, the cells were washed once in saline. In these preliminary studies, the best results were obtained using 20 micromoles NEM and 5-min incubations.
3. The PMB† incubation procedures were similar to those for NEM. Concentrations of 5, 10, 15, and 20 micromoles PMB per ml were examined initially. The results reported below are for 10 micromoles PMB and 5-min incubation at room temperature.
4. A short procedure using excess Sn(II) ion (Atkins et al., *16*) was used. Two milliliters of washed ^{99m}Tc-RBCs was incubated with 1 mg of $SnCl_2 \cdot 2H_2O$ and 1 ml of ACD‡ for 5 min at room temperature.
5. Both the short (5 min) and long (15 min) excess-ACD procedures were performed as described by Atkins et al. (*16*). Two milliliters of washed ^{99m}Tc-RBCs was incubated with 2 ml of ACD at room temperature. Only the longer incubation led to increased splenic deposition. These results are reported for the ACD method.

Tissue distribution study. At varying times (0.5, 1, 2, 3, or 4 hr) after intravenous injection of 0.1 ml of either damaged or undamaged packed ^{99m}Tc-RBCs, the rats were euthanatized by rapid cervical fracture and immediately frozen in liquid nitrogen to prevent RBC redistribution. The spleen, kidneys, testes, tibia and femur, and samples of liver, cardiac blood, and muscle were removed, weighed, and counted in a gamma well counter. The activity in each tissue was calculated as percent injected dose per gram of tissue. All significance testing was performed using the Student's t-test. Percent injected dose in the entire spleen was also determined for the time of maximal uptake. Control animals, injected with undamaged RBCs, were studied along with each experimental group.

Imaging study. In addition to the studies of splenic deposition and organ distribution, abdominal images were obtained with a scintillation camera in six New Zealand albino rabbits after intravenous injection of autologous ^{99m}Tc-RBCs. Three millicuries of ^{99m}Tc-RBCs was injected, and 400,000-count images were obtained at 0.5, 1, 2, 3, and 4 hr after injection. Each rabbit was imaged initially with undamaged ^{99m}Tc-RBCs. On subsequent days (to allow suitable time for ^{99m}Tc decay), each rabbit was reimaged after injection of ^{99m}Tc-RBCs altered by each of the damaging methods being investigated. Finally, each rabbit was imaged after the injection of 3 mCi of ^{99m}Tc-sulfur colloid, and these images were compared to those obtained using the damaged ^{99m}Tc-RBCs. A high-resolution parallel-hole collimator was used for these studies.

RESULTS

Table 1 shows the distribution (% injected dose/gm) of the undamaged and damaged ^{99m}Tc-RBCs at 2 hr after injection. Splenic uptake was significantly greater ($p < 0.001$) in animals whose red cells had been damaged by heat, NEM, or PMB. These animals also showed lower ($p < 0.001$) blood-activity levels than controls or animals injected with cells treated with ACD or Sn(II) ion. Deposition in other tissues in both the experimental and control groups was low. Whole-bone activity (not shown in Table 1) was low, with less than $0.2 \pm 0.03\%$/gm deposition in animals ($n = 18$) injected with control, heat-, or NEM-damaged cells. When the total splenic deposition (% injected dose) was assessed (rather than % injected dose/gm), a similar superiority of splenic uptake was seen with cells damaged by heat, NEM, or PMB. However, cells damaged by heating in a water bath permitting minor temperature varia-

TABLE 1. ORGAN DISTRIBUTION OF ^{99m}Tc-RBCs*

RBCs	n	Blood	Liver	Spleen	Kidney	Muscle	Testes
Controls	18	7.7 ± 0.32	1.0 ± 0.03	2.1 ± 0.15	1.86 ± 0.04	0.06 ± 0.001	0.05 ± 0.001
Heat-damaged	12	2.7 ± 0.21	1.6 ± 0.11	46.3 ± 5.21	1.84 ± 0.09	0.05 ± 0.001	0.05 ± 0.001
NEM-damaged	12	4.0 ± 0.28	1.2 ± 0.07	30.3 ± 2.50	0.90 ± 0.04	0.06 ± 0.001	0.05 ± 0.001
PMB-damaged	12	2.8 ± 0.17	2.4 ± 0.18	40.0 ± 4.80	0.90 ± 0.03	0.06 ± 0.001	0.05 ± 0.001
Excess ACD	5	6.3 ± 0.25	1.4 ± 0.09	9.0 ± 0.27	1.10 ± 0.04	0.07 ± 0.001	0.05 ± 0.001
Excess Sn(II)	5	6.2 ± 0.25	1.1 ± 0.08	4.4 ± 0.22	1.31 ± 0.05	0.06 ± 0.001	0.05 ± 0.001

* Two hours after injection; results are given in mean percent injected dose/gm ± s.d.

TABLE 2. TOTAL SPLENIC DEPOSITION OF ^{99m}Tc-RBCs*

Cells	n	2 hr	4 hr
Controls	36	2.1 ± 0.3	2.2 ± 0.2
Heat-damaged†	24	46.3 ± 4.2	47.5 ± 5.0
Heat-damaged‡	24	19.1 ± 0.9	21.2 ± 1.5
NEM-damaged	24	30.1 ± 3.4	55.2 ± 2.0
PMB-damaged	24	38.5 ± 3.2	38.0 ± 2.6
Excess ACD	10	8.3 ± 0.6	26.7 ± 2.3
Excess Sn(II)	10	4.8 ± 0.3	8.0 ± 1.5

* Total splenic activity expressed as percent injected dose per total organ ± s.d.

† Heat-damaging performed in a constant-temperature water bath (49–50°C).

‡ Heat-damaging performed in a poorly controlled water bath (48–51°C).

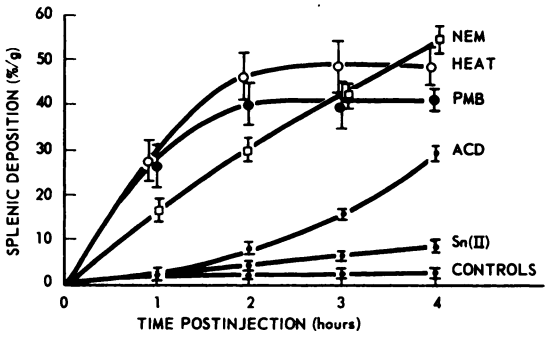

FIG. 1. Splenic deposition (%/gm ± s.d.) of ^{99m}Tc-RBCs obtained using several methods of damaging. Each ACD and Sn(II) data point represents five animal studies. All other data points represent at least 12 animal studies.

tions (48–51°C) were less likely to be deposited in the spleen than cells damaged in a constant-temperature (49–50°C) water bath (Table 2).

The 1- to 4-hr ^{99m}Tc-RBC splenic depositions obtained using the various damaging methods are compared in Fig. 1. Damaging by heat, PMB, and NEM consistently resulted in significantly greater splenic deposition than was obtained by excess ACD or excess stannous ion ($p < 0.001$). By 4 hr after injection the splenic uptake of heat- and NEM-damaged cells was similar, and both significantly exceeded ($p < 0.001$) splenic uptake of other types of damaged cells. However, heat-treated cells reached maximal levels of splenic deposition earlier than NEM-damaged cells.

Figure 2 shows spleen-to-liver (S/L) ratios obtained with the various damaging methods. Throughout the period of observation, the S/L ratios obtained with heat or the sulfhydryl inhibitors significantly exceeded ($p < 0.001$) those seen with ACD or stannous ion (maximum S/L ratios: ACD 15 ± 1.2; Sn(II) 7 ± 2.1). The peak S/L ratio (20 ± 2.3) for PMB-damaged cells was reached 2 hr after injection and did not increase significantly thereafter. With heat- and NEM-damaged cells, the maximum S/L ratios were observed 4 hr after injection. At this time the NEM S/L ratio (51 ± 4.1) was significantly higher than those for heat ($p < 0.01$) and PMB ($p < 0.001$) damage.

Comparative abdominal images performed in the same rabbit (2 hr after injection) with undamaged and damaged ^{99m}Tc-RBCs and with ^{99m}Tc-sulfur colloid are shown in Fig. 3. The specificity of splenic images obtained with the damaged ^{99m}Tc-RBCs is superior to that obtained with ^{99m}Tc-sulfur colloid. The effect of the postinjection interval on image quality is emphasized by a time-sequence study performed with NEM-damaged ^{99m}Tc-RBCs (Fig. 4). Imaging at 2–4 hr after injection results in increased splenic visualization, with little activity seen in the liver.

DISCUSSION

Radionuclide images of the spleen are obtained routinely with ^{99m}Tc-sulfur colloid. This agent localizes in the reticuloendothelial cells of the liver, spleen, and bone marrow and provides simultaneous images of these regions. In certain clinical circumstances, however, simultaneous imaging of the liver and spleen is undesirable. In a patient who has had a splenectomy as treatment for hemolytic anemia or idiopathic thrombocytopenic purpura, the recurrence

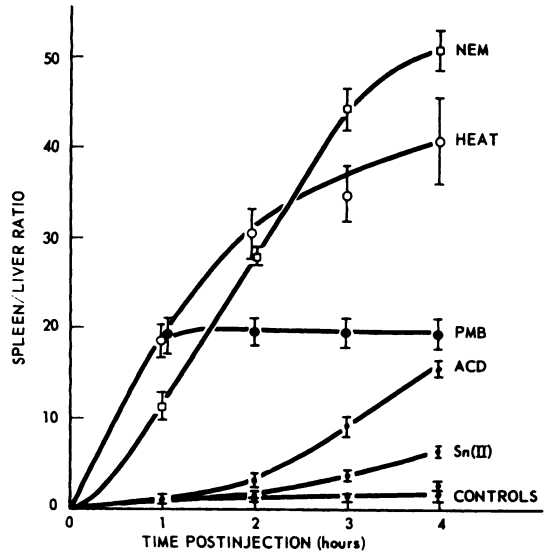

FIG. 2. Spleen-to-liver ratios (S/L ± s.d.) obtained using several methods of damaging. Data were obtained from same animal population as in Fig. 1.

of anemia or thrombocytopenia prompts a search for accessory spleens. In these patients the left lobe of the liver may "migrate" into the left upper quadrant and cause confusion when ^{99m}Tc-sulfur colloid images are obtained. Similarly, in pediatric patients with severe congenital heart anomalies, the question of asplenia versus polysplenia may arise. Unfortunately, these children often have large transverse livers that span the abdomen and thus make it difficult to determine the location of splenic tissue by ^{99m}Tc-sulfur colloid imaging (*20*). Specific splenic imaging could also benefit patients with a suspected splenic laceration or hematoma. The left lobe of the liver may extend into the left upper quadrant and appear to be an abnormal portion of the spleen. Caudal-angled and other oblique views may resolve this problem (*21*). Specific imaging would alleviate this cause of false-positive images in trauma patients.

Heat-damaging techniques have been reported to result in high splenic deposition of ^{99m}Tc-RBCs (*11,14,15*), and our results confirm the utility of these methods for ^{99m}Tc-RBC splenic imaging. Deposition of nearly 50% of the injected dose in the spleen can be obtained routinely. However, carefully controlled heating conditions are vital to the success of this method. This was emphasized by the poor splenic uptakes obtained when the temperature of the water bath was allowed to fluctuate over 48–51°C. This phenomenon might be even more important in the clinical laboratory, where larger volumes of red cells are used, these being more difficult to heat uniformly.

The current study also shows that high splenic deposition and S/L ratios can be achieved using sulfhydryl inhibitors to damage ^{99m}Tc-RBCs. Although NEM provided the highest S/L ratios among all the agents tested in this study, it did not result in early high splenic accumulation; maximum S/L ratios and splenic depositions (>50% of the injected dose) were found approximately 4 hr after injection. However, splenic imaging studies showed that the low liver accumulation found with this agent allowed excellent images to be obtained after 2 hr (Fig. 4). Increased splenic accumulation and S/L ratios at 3

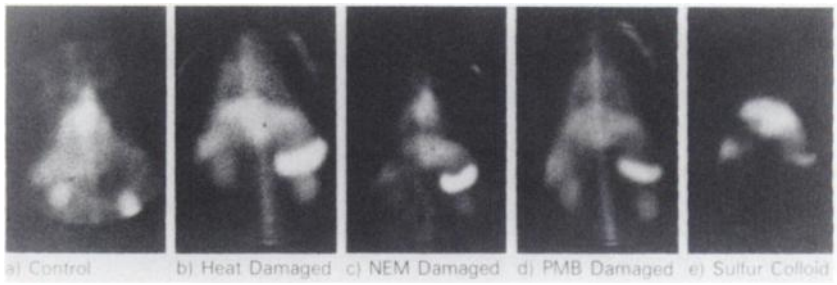

FIG. 3. Abdominal images obtained on successive days in one rabbit using several different damaging methods. All images were obtained 2 hr after injection. Area of diminished activity in right hepatic lobe on ^{99m}Tc-sulfur colloid image represents anatomic variant.

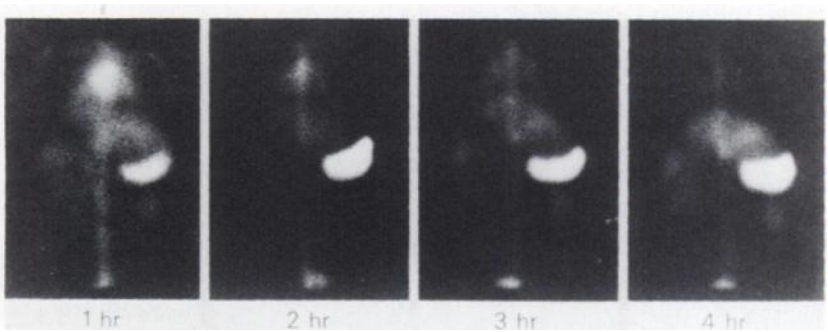

FIG. 4. Sequential abdominal images obtained after injection of NEM-damaged ^{99m}Tc-RBCs. Although absolute splenic deposition and S/L ratios increase through 4 hr, excellent splenic images can be obtained at 2 hr after injection. Due to increased splenic activity, the scintillation camera intensity was reduced slightly between 1- and 2-hr images; it was kept constant thereafter.

and 4 hr did not add significantly to image quality.

The use of sulfhydryl inhibitors in this study was suggested by earlier work of Jacob and Jandl (*17–19*) and Wagner et al. (*13*) who evaluated the effects of these agents on the splenic uptake of human ^{51}Cr-RBCs. Such PMB- and NEM-damaged RBCs accumulated in the spleen to a greater extent than in the liver. The in vitro studies of Jacob and Jandl (*18*) helped clarify the mechanism of action of these agents. Both chemicals bind to the sulfhydryl enzymes of the RBC membrane. This binding damages the membrane and results in K^+ loss and Na^+ entry into the cell. An associated influx of water leads to cellular swelling and spherocytosis, which accounts for the removal of the damaged cells in the splenic microcirculation. NEM, an uncharged molecule, enters the cell and binds to the intracellular glutathione as well as the membrane enzymes. Since PMB is a charged molecule and does not penetrate the red cell membrane, it attaches only to the membrane's sulfhydryl groups.

The chemical damaging methods evaluated in the current study (excess ACD and excess stannous ion) were significantly less effective in producing splenic localization than heat- or sulfhydryl-damaging methods. The results obtained with these chemicals were similar to those previously reported and indicate that these techniques should not be considered as damaging methods of primary importance. However, one must be aware of possible interspecies differences in the splenic sequestration of damaged cells. Rats were used for our bioassay experiments because their small size allowed rapid freezing, which prevented postmortem redistribution of RBCs. On the other hand, rabbits were used for the imaging studies because their larger size made for better-quality scintigrams. The excellent results obtained in these two species do not necessarily prove that results in humans would be as good. However, earlier human studies (*13,19,22*) using ^{51}Cr-RBCs damaged with NEM and PMB do support the contention that sulfhydryl inhibition will damage human RBCs and lead to efficient splenic sequestration.

Our results suggest that NEM rather than PMB is the sulfhydryl agent of choice and is the one that should be most closely compared with the heat-damaging methods. NEM is available at low cost in a readily usable form. Less time is required for NEM incubation (5 min) than for heat-damaging (15 min), but the NEM-damaged cells should be washed once to remove excess NEM before injection into the patient. This requires an additional 4–6 min. The micromolar quantities of NEM used for red cell damaging have shown no adverse effect in humans (*13,19,22*), but the potential toxicity of this compound should be more carefully studied. Heat-damaging provides good splenic localization of ^{99m}Tc-RBCs but requires rigidly controlled incubation conditions for optimal results, whereas NEM provides an excellent and predictable alternative procedure. If NEM were manufactured in an easily usable kit form, it could become the agent of choice to damage ^{99m}Tc-RBCs for splenic imaging studies.

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FOOTNOTES

* N-Ethylmaleimide, Sigma Chemical Co., St. Louis, Mo.
† p-Hydroxymercuribenzoate, Sigma Chemical Co.
‡ E. R. Squibb & Sons, Inc., Princeton, N.J.

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