THE ^{99m}Tc LABELING OF ERYTHROCYTES

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The availability and the favorable radiation characteristics of ^{99m}Tc have suggested the possibility of using it to label erythrocytes for scintigraphy of the spleen. Unfortunately, no reliable method of accomplishing this has been described. In the one case in which labeling has been reported, details of the analytic procedure used to determine the stability of the binding were not given (*1*). Furthermore, having developed a suitable analytical method of assaying the stability of the ^{99m}Tc tag, our laboratory was unsuccessful in obtaining good labeling with the reported technique.

In order to develop a simple and efficient technique for the analytical assay of this label and to improve the tagging yield by using a technique similar to the one widely used for spleen scanning with ^{51}Cr-labeled red blood cells (*2*), this experimental work was undertaken.

MATERIALS AND METHODS

Ten milliliters of human blood were added to 2.5 ml of ACD solution. The erythrocytes were separated from the plasma and suspended in physiological saline solution. Then 100 μg of Na_2CrO_4 and 1 mCi of ^{99m}Tc-pertechnetate were added. After incubating at 37°C for 15 min, 5 mg of ascorbic acid were added. After 1–2 min at room temperature, the pH was neutralized by addition of 0.1 ml of isotonic $NaHCO_3$ (1.39%). The erythrocytes were then centrifuged and washed twice with normal saline. Finally, they were resuspended in their own plasma and heat-altered by incubation in a water bath at 50°C for 20 min.

Samples were assayed by electrophoresis. With isotonic $NaHCO_3$ as a buffer, free pertechnetate migrates toward the cathode (10–12 cm after a 30-min run with a voltage gradient of 16 volts/cm) while the erythrocytes remain at the origin. The electrophoresis has to be carried out at 4°C because of heat release due to the high ionic strength of the buffer. Also, aliquots were centrifuged and the cells washed four times with normal saline. Cells, supernatant and the four washings were counted in a scintillation well counter. The corresponding values are shown in Table 1.

Rat red blood cells were similarly tagged with ^{99m}Tc, and after their intravenous injection, scanning was carried out at 1 hr, 4 hr and 24 hr (Fig. 1) using a Picker 3-in. Magnascanner. At the end of the experiment the two animals were sacrificed and the radioactivity was counted in blood, liver, spleen, kidneys, heart and lungs (Table 2).

A comparative assay between our proposed method and Fischer's (*1*) was carried out using mouse red blood cells. Groups of three animals were injected intravenously with one of the two different types of tagged red cells and sacrificed 5 or 24 hr later. The radioactivity was counted in blood, liver, spleen, kidneys, stomach, small intestine and thyroid in a well scintillation counter with total counts of 2,500–5,000. The radioactivity in each sample was compared with suspension 0.1 ml of injected ^{99m}Tc-tagged RBC standards. Table 3 shows the results of these determinations.

In order to determine the nature of the ^{99m}Tc labeling, the radioactivity distribution pattern was studied electrophoretically (with isotonic $NaHCO_3$ as a buffer) in tagged mouse red cells prepared according to Fischer's and to our technique. Electrophoretic runs of intact and hemolyzed erythrocytes are shown in Fig. 2 and Table 4.

The blood clearance of ^{99m}Tc-tagged erythrocytes labeled by our technique was studied in a dog. Fig. 3 shows the values of the radioactivity in cpm/5 ml whole blood withdrawn at 0 min, 15 min, 30 min, 1 hr, 2 hr, 3 hr, 4 hr and 24 hr after the intravenous administration of the tagged red cells.

RESULTS AND DISCUSSION

By combining the radioactivity yield after tagging (percentage of total ^{99m}Tc incorporated into the RBC

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and remaining after the washings) and the electrophoretic determination of the firmly bound ^{99m}Tc in the washed tagged RBC, the actual yields are: for the Fischer *et al* method 0.2% and for the chromate plus ascorbic acid method 10.7%. The electrophoretic assay of intact and hemolyzed ^{99m}Tc-labeled erythrocytes suggests that the radioactivity is bound mainly to the hemoglobin fraction. After the electrophoresis of hemolyzed erythrocytes the radioactivity remaining at the origin is probably associated with some hemoglobin retained by the cellular stroma (Fig. 2). The labeling process could be interpreted as follows: The Na_2CrO_4, acting as a penetration carrier, allows the $^{99m}TcO_4^-$ to reach the interior of the erythrocyte. Also, the use of chromate will minimize the ion-adsorption phenomenon on the cell membrane—a situation which could be critical when carrier-free radioisotopes are used. Subsequent treatment with ascorbic acid reduces the chromate to Cr^{3+}, and the ascorbic acid also could chelate partially the Fe^{II} present in the iron-porphyrin prosthetic group, thereby enabling it to complex the pertechnetate in a fashion similar to that used in the preparation of the ^{99m}Tc-iron complex (*3*). Also the reduced Cr^{3+} probably does not facilitate exit of TcO_4^- from the cell. In this method of red cell tagging the pH has to be lowered by the addition of an excess of ascorbic acid and then neutralized after a very short period (1–2 min); otherwise, it will induce a high rate of hemolysis clearly observable by electrophoresis. Figure 2 shows the results of a sample deliberately hemolyzed by letting it stand 15 min in acidic conditions.

The possibility of a Cr^{3+} pertechnetate complex formation was assayed by electrophoresis of the incubation mixture without the red cells. After reduction with ascorbic acid, all of the radioactivity was

TABLE 1. COMPARATIVE STUDY OF THE RADIOACTIVE DISTRIBUTION OF ^{99m}Tc IN TAGGED HUMAN BLOOD RED CELLS (AS PERCENT OF THE TOTAL ^{99m}Tc)

Method	Fischer *et al* (1)	Chromate + ascorbic acid
Number of batches	4	12
Erythrocytes	10.1 ± 0.3	32.31 ± 4.0
Supernatant	49.5 ± 0.9	51.00 ± 3.6
1st washing	18.7 ± 0.8	10.45 ± 1.0
2nd washing	10.0 ± 0.4	4.28 ± 0.3
3rd washing	7.0 ± 0.4	1.26 ± 0.4
4th washing	5.2 ± 0.4	< 0.1%
ELECTROPHORESIS		
Erythrocytes	1.9 ± 0.5	22.8 ± 4.6
Free pertechnetate	99.1 ± 1.2	76.5 ± 8.2

TABLE 2. RADIOACTIVITY DISTRIBUTION IN RAT 24 HR AFTER INTRAVENOUS INJECTION OF ^{99m}Tc-LABELED BLOOD RED CELLS (MEAN VALUE OF TWO ANIMALS)

	% injected ^{99m}Tc/gm	Ratio spleen/tissue
Blood	0.2	34.0
Liver	0.1	68.0
Spleen	6.8	1.0
Kidneys	0.8	8.5
Lungs	0.1	68.0
Stomach	0.1	68.0

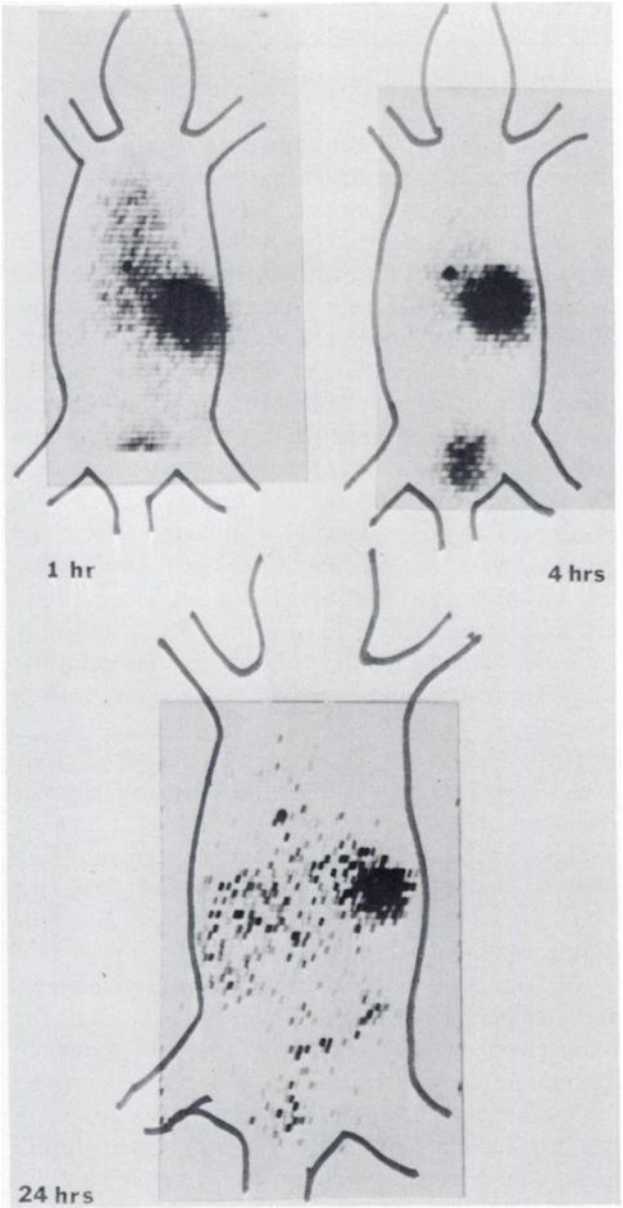

FIG. 1. Serial scanning a rat injected with ^{99m}Tc-tagged erythrocytes.

TABLE 3. RADIOACTIVITY DISTRIBUTION 5 HR AND 24 HR AFTER THE INTRAVENOUS INJECTION OF ^{99m}Tc-LABELED ERYTHROCYTES (GROUPS OF 3 ANIMALS)

(Percentage of injected dose/gm)

	5 hr			24 hr		
	Method I	Method II	Method I and hemolyzed	Method I	Method II	Method I and hemolyzed
Blood	1.1*(1.2– 0.9)**	2.5(2.9– 2.0)	0.3(0.4– 0.3)	0.2(0.3– 0.2)	0.2(0.3– 0.1)	0.4(0.6– 0.4)
Liver	20.8 (21.3–20.6)	3.3(4.1– 2.9)	4.7(5.5– 4.5)	4.8(5.6– 4.2)	0.9(1.1– 0.8)	1.1(1.3– 1.0)
Spleen	86.7 (90.0–83.0)	5.9(6.7– 5.0)	5.1(5.8– 5.0)	44.2(58.3–40.8)	1.7(1.8– 1.3)	2.5(3.3– 2.2)
Kidneys	3.2 (4.0– 2.7)	1.3(1.5– 1.2)	3.7(4.7– 3.0)	2.5(3.0– 2.0)	0.5(0.6– 0.3)	1.0(1.2– 0.8)
Stomach	5.6 (6.8– 5.2)	32.6(36.0–31.6)	4.2(4.8– 4.0)	0.6(0.7– 0.5)	3.4(3.5– 3.2)	0.6(0.7– 0.4)
Small intestine	1.3 (1.4– 1.0)	4.9(5.4– 4.1)	1.4(1.7– 1.1)	0.5(0.6– 0.4)	0.6(0.7– 0.6)	0.3(0.3– 0.2)
Thyroid	25.0 (30.0–15.0)	400(450–350)	35.0(45.0–15.0)	5.0(5.0– 5.0)	15.0(20.0–12.0)	15.0(18.0–14.0)

Method I = using chromate + ascorbic acid
Method II = according to Fischer *et al*
* = Mean value
** = Range

found to migrate as pertechnetate, ruling out the possibility of complexing by Cr^{3+}. It should be pointed out, however, that because of the well-known reactivity of chromium with proteins, this metal could play some role in red cell tagging, presumably changing physicochemical properties such as cellular permeability.

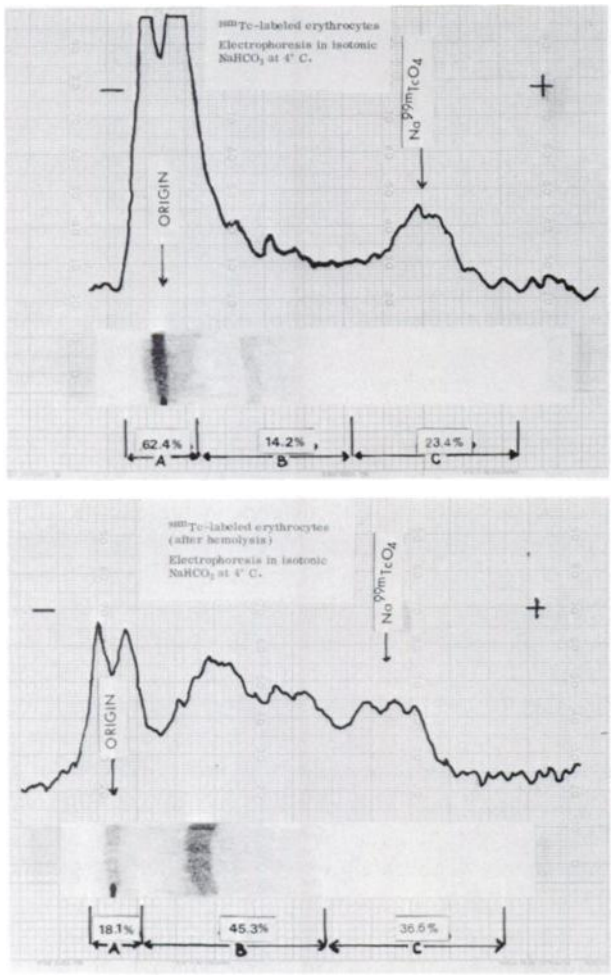

FIG. 2. Electrophoresis of intact and hemolyzed ^{99m}Tc-tagged mouse erythrocytes.

The artificially induced spherocytes are retained by the spleen, while the intact erythrocytes, if any, will remain in the blood stream. When the erythrocytes are mechanically damaged or broken, the hemoglobin will leave the cells and will be taken up by the liver as well as by the kidneys. The presence of extracellular ^{99m}Tc-hemoglobin will result in a lower spleen-to-liver uptake ratio (Table 5). A comparison of the tissue distribution of ^{99m}Tc-hemolyzed erythrocytes and those prepared according to Fischer's method (with a large amount of adsorbed pertechnetate) shows a different pattern: the hemolyzed red cells produce a higher radioactivity in liver, spleen and kidneys, while the amount in stomach, small intestine and thyroid is smaller (Table 3). These values are in agreement with release of ^{99m}Tc-hemoglobin from the tagged erythrocytes and also indicate that the ^{99m}Tc-hemoglobin bonds are being slowly broken down.

This situation also is reflected in the blood-clearance study. Less than 10% of the initial radioactivity remains in blood at 2 hr, and the biological half-life is about 9¼ hr. We assume that the remaining activity is ^{99m}Tc-hemoglobin (or ^{99m}Tc slowly released from tagged hemoglobin) produced by the slow disintegration of tagged red cells.

The autopsy data corroborate the *in vitro* findings, showing that when Fischer's technique is used, the radioactivity which remains in the cell is mainly located intracellularly as free $^{99m}TcO_4^-$. Washing the tagged cells, however, does not rinse away all of this intracellular free ^{99m}Tc (Table 1). On the other hand, the presence of chromate ion facilitates the elimination—an experimental fact which substantiates the assumption that the chromate ion acts as a penetration carrier.

TABLE 4. ELECTROPHORETIC STUDY OF ^{99m}Tc-LABELED ERYTHROCYTES (MICE)

	Red cells (origin)		Hemoglobin (B)		Free TcO_4^- (C)	
	a	b	a	b	a	b
^{99m}Tc-labeled erythrocytes	3.1	62.4	3.5	14.2	93.4	23.4
Hemolyzed ^{99m}Tc-labeled erythrocytes	2.1	18.1	2.5	45.3	95.4	36.7

a = Fischer *et al* method (1)
b = Chromate + ascorbic acid method
Heparin was used instead of ACD solution

TABLE 5. RATIO OF RADIOACTIVITY DISTRIBUTION BETWEEN SPLEEN AND ADJACENT ORGANS (MOUSE EXPERIMENT)

Ratio spleen to	5 hr			24 hr		
	I	II	III	I	II	III
Liver	4.10	1.80	1.08	9.20	1.89	2.29
Stomach	15.4	0.18	1.21	73.6	0.50	4.16
Kidneys	27.0	4.49	1.37	17.6	3.40	2.50

I = Chromate method
II = Fischer's method
III = Chromate method + hemolysis

Mouse erythrocytes have shown higher fragility to manipulation than rat erythrocytes. This factor appears to account for the lower spleen-to-liver ratio in mice than in rats. As shown in Table 3, the larger amounts of radioactivity found in stomach and thyroid with Fischer's preparation corresponds to the high amount of free pertechnetate found electrophoretically.

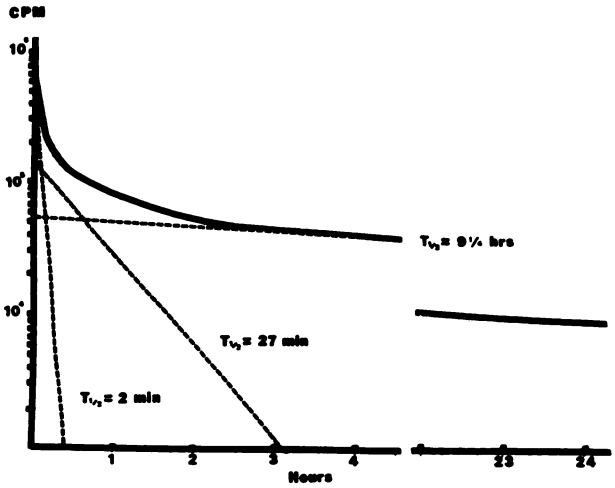

FIG. 3. Blood clearance of ^{99m}Tc-tagged erythrocytes in dog.

CONCLUSION

The labeling of erythrocytes by our method produces a yield of incorporated radioactivity (^{99m}Tc bound to the cells) 10–50 times higher than the previously proposed technique. A penetration into the cell of the ^{99m}Tc carried over by the chromate ion and the subsequent complexing by the reduced iron of the hemoglobin seems to be the mechanism involved in this tagging reaction.

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