LABELING OF ERYTHROCYTES WITH ^{103}Ru-RUTHENIUM RED

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The preparation of ^{103}Ru-ruthenium red and its use in the labeling of erythrocytes is described. Since in vivo and electrophoretic studies indicate that the tag is firmly bound to the cell, this technique appears to be very convenient for spleen scintigraphy and for the study of the in vivo fate of tagged cells.

The reaction of ruthenium trichloride with ammonia produces a cationic complex $[Ru_3O_2(NH_3)_{14}]^{6+}$, called ruthenium red. This complex exhibits interesting biologic properties. Among them, its selective binding to acid mucopolysaccharides (*1,2*) suggests its possible use in the radioactive tagging of cells.

The present communication deals with the preparation of the complex and with the labeling of erythrocytes with ^{103}Ru-ruthenium red.

MATERIALS AND METHODS

In order to reduce higher valence impurities, $^{103}RuCl_3$ (0.8 mg Ru and 1 mCi/mg Ru) in 0.25 *N* HCl (10 ml) was refluxed 30 min with ethanol (5 ml). Then it was evaporated to one-fourth its volume and concentrated ammonia (10 ml) was added. The solution was exposed to air for about 2–3 hr while heated in a water bath at 90°C and finally evaporated to dryness. The unreacted $^{103}RuCl_3$ and the NH_4Cl formed by HCl-ammonia interaction were extracted (four times) with hot ethanol (20 ml). The residue was dissolved in normal saline and filtered, first through filter paper and then through Millipore membrane filter (0.22-micron of pore size). The yield of this preparation was 65–70% of the original radioactivity.

Erythrocytes were separated by centrifugation from the plasma corresponding to 5 ml of oxalated rat blood. After being washed three times with cold saline, they were incubated with 0.4 mCi of ^{103}Ru-ruthenium red (1 mCi/mg Ru) in a volume of 5 ml. At the end of the 30-min incubation at 37°C, the erythrocytes were washed three times with saline and resuspended in their own plasma. Cells, supernate, and the three washings were counted in a scintillation well counter. The corresponding values are shown in Table 1. Immediately thereafter the erythrocytes were heat-altered in a water bath at 50°C for 20 min.

TABLE 1. COMPARATIVE DISTRIBUTION OF ^{103}Ru-RUTHENIUM RED IN TAGGED RAT RED CELLS*

Number of batches	3
Erythrocytes	8
Supernate	85
First washing	5
Second washing	2
Third washing	—

* Mean value of the percent of total ^{103}Ru.

The ^{103}Ru-tagged rat erythrocytes were injected into groups of rats through the tail vein. The animals were then sacrificed by bleeding at 1, 4, 24, and 72 hr. Radioactivity was counted in samples of blood, liver, spleen, kidneys, heart, and lungs (Table 2).

Aliquots of the labeled rat red blood cells were evaluated by electrophoresis. With isotonic $NaHCO_3$ as a buffer, free ruthenium red migrated toward the cathode (8 cm after a 2-hr run with a voltage gradient of 10 V/cm) while the erythrocytes remained at the origin (Fig. 1).

RESULTS AND DISCUSSION

The electrophoretic studies have shown that the ^{103}Ru-ruthenium red tag is firmly bound to the washed labeled cells. On the other hand, the tagging yield has been 6–9% of the original ^{103}Ru-ruthenium

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TABLE 2. RADIOACTIVITY DISTRIBUTION AT VARIOUS TIMES AFTER INTRAVENOUS INJECTION OF ^{103}Ru-RUTHENIUM RED-LABELED ERYTHROCYTES*

	Weight (gm)	Percent of injected dose per gram of tissue			
		1 hr	4 hr	24 hr	72 hr
Blood	5.0 ± 1.0	0.83 ± 0.09	0.46 ± 0.08	0.14 ± 0.01 (0.13 ± 0.02)†	0.05 ± 0.008
Liver	4.5 ± 0.4	1.90 ± 0.27	1.95 ± 0.15	1.73 ± 0.12 (0.42 ± 0.04)	1.80 ± 0.29
Spleen	0.8 ± 0.4	20.7 ± 3.66	23.8 ± 3.57	23.4 ± 3.08 (0.28 ± 0.02)	20.9 ± 3.89
Kidneys	1.8 ± 0.8	1.56 ± 0.22	1.50 ± 0.22	1.57 ± 0.14 (1.42 ± 0.28)	1.64 ± 0.20
Heart	0.8 ± 0.3	0.24 ± 0.03	0.18 ± 0.03	0.10 ± 0.01 (0.11 ± 0.03)	0.07 ± 0.01
Lungs	1.5 ± 0.5	2.58 ± 0.53	1.99 ± 0.29	1.44 ± 0.96 (0.23 ± 0.04)	0.77 ± 0.12

* Mean value ± s.d. of a four-animal group.
† Animals injected intravenously with ^{103}Ru-ruthenium red (*4*).

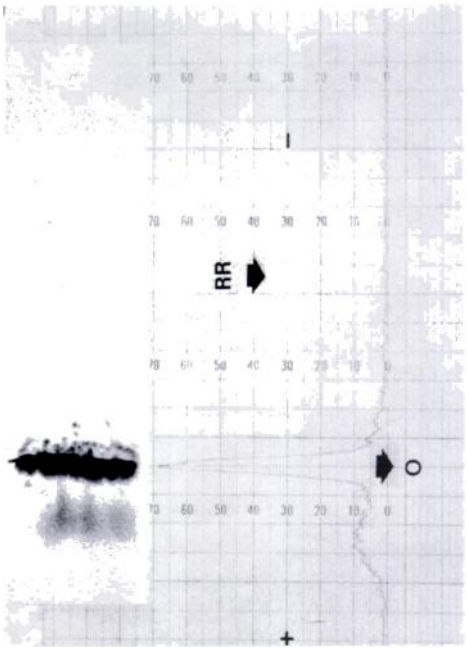

FIG. 1. Electrophoresis of ^{103}Ru-ruthenium red-tagged erythrocytes. O is origin and RR is ruthenium red's migration. Radioactivity found in RR was 0.8% of total.

red. Since the tagging of cells with ^{103}Ru-ruthenium red is based on its specific interaction with the acid mucopolysaccharides of the cell (*1,2*), there is a limitation in the tagging yield for stoichiometric reasons. For a given volume of red blood cells, therefore, a higher ^{103}Ru specific activity (a lower mass of the complex) will produce a better relative tagging yield (as percent of total ^{103}Ru used). It is interesting to note that in any case this tagging yield is within the range of the one obtained with ^{99m}Tc by the author using the method of Fischer, et al, 9–10% (*3*).

In comparison with the ^{99m}Tc-labeled erythrocytes (*3*), the radioruthenium-labeled red blood cells show no electrophoresis-released tag, which is an indication of a stronger chemical bond.

The experimental finding that ^{99m}Tc from labeled erythrocytes decreases in spleen from 86.7% at 5 hr to 44.2% after 24 hr while the ^{103}Ru radioactivity from ruthenium red-labeled erythrocytes remains practically constant (23.8% at 4 hr and 23.4% after 24 hr) is an indication of better in vivo stability of the radioruthenium tag. In addition to this, the in vivo distribution of ^{103}Ru-ruthenium red (*4*) has shown a blood-to-spleen ratio (radioactivity per gram of tissue) of 2.2 at 24 hr and 6.6 at 72 hr whereas, in animals injected with ^{103}Ru-labeled erythrocytes, these ratios are 16.7 and 41.8, respectively. These observations indicate that the ^{103}Ru accumulated in spleen is bound to erythrocytes and not as free complex.

The in vivo distribution studies and the electrophoretic analysis show that the stability of the ^{103}Ru tag is considerably higher than the corresponding ^{99m}Tc-tag. The difference seems to be due to the fact that two distinct processes are involved in the radioactivity binding. Technetium-99m as well as ^{51}Cr are attached to hemoglobin and they are lost when hemoglobin degradation occurs (*5*). In the case of ^{103}Ru-ruthenium red the tag is bound to acid mucopolysaccharides which are considerably more resistant to in vivo degradation.

The ratio of radioactivity distribution (Table 3) between spleen and other tissues shows a high spleen-to-liver uptake ratio that indicates the potential value of this method of erythrocyte labeling for scintigraphy of the spleen. In this respect, the availability of a ruthenium radioisotope (^{97}Ru) with a shorter half-life and with excellent physical properties (Table 4) (*6*) suggests the feasibility and convenience of this technique.

Ruthenium red exhibits in mice a relatively low toxicity. Its LD_{50} is equal to 25 mg/kg and 22.3

TABLE 3. SPLEEN-TO-TISSUE RATIO OF ^{103}Ru RADIOACTIVITY AS FUNCTION OF TIME ELAPSED AFTER INTRAVENOUS INJECTION OF ^{103}Ru-RUTHENIUM RED-LABELED ERYTHROCYTES

	Spleen-to-tissue ratio (activity per gram of tissue)			
	1 hr	4 hr	24 hr	72 hr
Blood	24.9	51.7	167.1	418.0
Liver	10.9	12.2	13.5	11.6
Kidneys	13.3	15.9	14.9	12.7
Heart	86.3	132.2	234.0	298.6
Lungs	8.0	12.0	16.3	27.1

TABLE 4. PHYSICAL CHARACTERISTICS OF ^{103}Ru AND ^{97}Ru

Radio-isotope	Half-life (days)	Radiation	Energy	Percent
$^{103}_{44}$Ru	39.5	Beta	0.70 (maximum)	100
		Gamma	0.497	88
		Gamma	0.610	6
$^{97}_{44}$Ru	2.88	Orbital electron capture	—	100
		Gamma	0.215	91
		Gamma	0.324	8

mg/kg to 28.0 mg/kg is the 95% confidence interval (CI) for the LD_{50} as determined in mice by the method of Litchfield and Wilcoxon (*7,8*).

The comparative distribution of ^{103}Ru from labeled erythrocytes and ^{103}Ru-ruthenium red complex (Table 2) (*4*) shows the following patterns: for ^{103}Ru-ruthenium red-labeled erythrocytes, spleen > liver > kidney > lung > blood > heart, and for ^{103}Ru-ruthenium red complex, kidney > liver > spleen > lung > blood > heart. The ^{103}Ru-ruthenium red complex is excreted rapidly through the kidneys (about 40% of the injected dose 2 hr after intravenous injection) (*4*).

Finally, this way of tagging may be a useful tool for studying the in vivo fate of other cells such as tumor cells which in most cases exhibit a thick cell coat rich in mucopolysaccharides. This structural characteristic facilitates the labeling with radioactive ruthenium red. Under suitable experimental conditions (complex concentration, time of interaction, etc.), it is possible to minimize the cell viability impairment. The ruthenium red does not affect the morphologic integrity of the cell but by its binding with Ca^{2+}-binding sites (muco-sites) this dye inhibits the calcium distribution and alters cellular processes related to this cation (*8,9*).

REFERENCES

1. LUFT JH: The fine structure of hyaline cartilage matrix following ruthenium red fixative and staining. *J Cell Biol* 27: 61A, 1965

2. GUSTAFSON GT, PIHL E: Histochemical application of ruthenium red in the study of mast cell ultrastructure. *Acta Pathol Microbiol Scand* [A] 69: 393–403, 1967

3. ANGHILERI LJ, LEE JI, MILLER ES: The ^{99m}Tc labeling of erythrocytes. *J Nucl Med* 11: 530–533, 1970

4. ANGHILERI LJ: Radioactive ruthenium red accumulation by tumors: a potential scanning agent. *Strahlentherapie* [Sonderb] 149: 173–175, 1975

5. HOLLINGSWORTH JW: Disappearance of radioactive chromium tagged erythrocytes from tissues and body cavities of rabbits. *Proc Soc Exp Biol Med* 87: 493–498, 1954

6. LEBOWITZ E, KINSLEY M, KLOTZ P, et al: Development of ^{97}Ru and ^{67}Cu for medical use. *J Nucl Med* 15: 511, 1974

7. LITCHFIELD JT, WILCOXON F: A simplified method of evaluating dose-effect experiments. *J Pharmacol Exp Ther* 96: 99–103, 1949

8. ANGHILERI LJ: The in vivo inhibition of tumor growth by ruthenium red: its relationship with the metabolism of calcium in the tumor: to be published in *Z Krebsforsch*

9. MOORE CL: Specific inhibition of mitochondrial Ca^{2+} transport by ruthenium red. *Biochem Biophys Res Commun* 42: 298–305, 1971