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# Physicochemical Properties of Circulating Red Blood Cells of Lethally X-irradiated Mice Treated with Rat Bone Marrow

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SUCCESSFUL TRANSPLANTATION of rat bone marrow in lethally x-irradiated mice has been demonstrated in this laboratory,[1-3] and similar findings have been reported independently by others.[4-6] In these mice (called here, for convenience, 950 r-RBM), circulating rat granulocytes were demonstrated by the alkaline phosphatase test,[1, 3, 4] circulating rat platelets by serologic methods,[3] and circulating rat red blood cells (RBC) by a qualitative[5] and a quantitative immunohematologic test.[1] Twenty-five days after treatment, there were approximately 50 per cent rat RBC in the circulating blood of the mouse, and 100 per cent by the 65th day. At this writing, this level has been maintained for more than a year in several mice. However, by the double serum agar diffusion method, the serum proteins were found to be of the mouse type. No apparent ill effect has been observed among the 950 r-RBM mice after recovery from the secondary immunologic effect.

Since serologically detectable normal *rat* RBC existed in the presence of *mouse* serum proteins in the circulation, we felt that a study of some of the physicochemical properties of rat RBC produced in the irradiated mouse would give some insight into the role of environment in cell growth and structure. Such a study is reported here.

## Materials and Methods

Normal $C_3H \times 101F_1$ and $101 \times C_3HF_1$ mice, normal Sprague-Dawley rats, and 950 r-RBM mice of these strains were used. The irradiation conditions, injection of bone marrow, and the quantitative immunohematologic method have been described elsewhere.[1] Versenated blood was collected from 950 r-RBM animals after 100 per cent rat RBC were serologically detectable in the blood. The RBC obtained from versenated blood were washed three times with 50 volumes of 0.15 M NaCl. For each of the following experiments, 4–12 samples were analyzed in duplicate.

The mechanical fragility test was carried out at 2 C. according to the method suggested by Anderson[7] with a device consisting of two 50-ml. syringes connected by a stainless steel 18-gage needle. A 30-ml. volume of 1% RBC suspension was pressed from one 50-ml. syringe into the other through the needle four times, a brass weight of 1500 g being used as the driving force. The RBC suspensions were centrifuged at 1500 r.p.m. for 30 minutes, and the clear supernatant fractions used for hemoglobin determination. The following formula was employed to express the relative mechanical fragility:

$$\text{RMF} = \frac{\text{Abs}_{\text{expt}} - \text{Abs}_0}{\text{Abs}_{\text{max}} - \text{Abs}_0} \times 100$$

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where $Abs_{exptl}$ represents absorption after shearing RBC suspension; $Abs_0$, absorption before shearing; and $Abs_{max}$, absorption after complete hemolysis; i.e., 1 per cent RBC in distilled water.

Two volumes of thrice-distilled water was added to one volume of packed cells for determination of osmotic fragility. The suspension was mixed thoroughly, incubated for 10 minutes, centrifuged at 1500 r.p.m. for 30 minutes, and the supernatant diluted 250-fold with distilled water. This solution was recentrifuged before the 540-mμ absorption in the Beckman spectrophotometer Model DU was read. Experiments were carried out at 4, 27, and 37 C.

Attempts to crystallize hemoglobin were made according to Drabkin's method,[8] in which either saturated $(NH_4)_2SO_4$ or 2.8 M phosphate buffer at pH 8.6 was used, and according to the method described by Reichert and Brown.[9]

In determination of the sedimentation property of the hemoglobin in water, the Spinco analytic ultracentrifuge Model E was used. The Spinco paper electrophoresis apparatus Model R was used to determine the electrophoretic property. Veronal buffer of 0.05 ionic strength and pH 8.6 was used. The solutions were subjected to electrophoresis at 22 ma per cell at constant current for 4 hours at 27 C. The paper strips were dried in an oven and then analyzed for unstained hemoglobin with the Spinco Analytrol Model RA.

The denaturation properties of hemoglobin were determined at 27 and 11 C. according to the method described by Haurowitz et al.[10] Hemoglobin was determined by the absorption at 575 mμ with the Coleman Junior Spectrophotometer Model 6A. The 30- to 60-minute readings were taken as the time for maximum denaturation at 27 C. and overnight readings for maximum denaturation at 11 C.

## Results

Immunohematologic tests, in which specific mouse antirat RBC and rat antimouse RBC were used, showed that circulating RBC of these 950 r-RBM mice were 100 per cent of the rat type from the 65th to the 200th day after treatment. The results are summarized in table 1. These cells were collected and used for the following experiments.

It is known that, in general, mechanically injured red blood cells release hemoglobin. In spite of the large shearing force of the syringe technic, only a negligible amount of hemoglobin was released by rat and 950 r-RBM mouse RBC. In contrast, a hemoglobin concentration approximately ten times as

Table 1.—Serologic Property of Red Blood Cells

| RBC | Serum | |
|---|---|---|
| | Mouse Antirat RBC | Rat Antimouse RBC |
| Mouse | − | + |
| Rat | + | − |
| 950 r-RBM mouse | + | − |

Table 2.—Mechanical Fragility at 2 C.

| 1 % RBC | Percentage Hemoglobin in Supernatant × $10^{-1}$ |
|---|---|
| Mouse | 6.0 |
| Rat | 0.6 |
| 950 r-RBM mouse | 0.7 |

great was found after shearing of mouse RBC. The results indicating that the RBC of the 950 r-RBM mice behaved like rat RBC are summarized in table 2.

As shown in table 3, the osmotic property is dependent on the temperatu[illegible]
A maximum release of hemoglobin was found in all three types of RBC at [illegible]
and a minimum at 4 C. Mouse RBC released the highest amount of heme[illegible] n
and was the least susceptible to temperature change. Rat RBC, on the other extreme, released the least amount of hemoglobin and was the most susceptible to temperature change. RBC of the 950 r-RBM mouse showed properties of both mouse and rat RBC. As summarized in column 3 of table 3, exposure to water at 37 and 27 C. caused release of hemoglobin in concentration only slightly greater than rat RBC but far below that amount released by mouse RBC. On the other hand, the temperature dependence of RBC of 950 r-RBM mice (column 4, table 3) is more mouse type than rat. A little more hemoglobin was released when the temperature was elevated from 4 to 27 to 37 C. Comparable results were obtained with mouse RBC. With rat RBC, however, there was approximately a two- and threefold increase in release of hemoglobin at comparable temperature change.

The results of our attempts to crystallize hemoglobin are presented in table 4. Mouse hemoglobin failed to crystallize out with any of the methods employed. Only amorphous materials were detected microscopically. On the other hand, the hemoglobin of rat and 950 r-RBM mice was crystallized by all three methods. It was noted that, for both types, only about 90 per cent of the total hemoglobin crystallized out. Photomicrographs of the hemoglobins crystallized in water at 2 C. are shown in figure 1.

The results of the denaturation property of hemoglobin are depicted in figure

TABLE 3.—Osmotic Property of RBC

| RBC | Temperature (C) | Mean Hemoglobin Concentration of Supernatant 1/250 dil. 540 mμ Abs. × $10^{-3}$ | Temperature Dependency | | |
|---|---|---|---|---|---|
| | | | $\frac{Abs._{37}}{Abs._{4}}$ | $\frac{Abs._{27}}{Abs._{4}}$ | $\frac{Abs._{4}}{Abs._{4}}$ |
| Mouse | 37 | 354 | 1.18 | 1.05 | 1.00 |
| | 27 | 315 | | | |
| | 4 | 289 | | | |
| Rat | 37 | 209 | 3.03 | 2.02 | 1.00 |
| | 27 | 139 | | | |
| | 4 | 69 | | | |
| 950 r-RBM mouse | 37 | 246 | 1.37 | 1.08 | 1.00 |
| | 27 | 194 | | | |
| | 4 | 180 | | | |

TABLE 4.—Ease of Crystallization of Hemoglobin at 2 C.

| RBC | Water | $(NH_4)_2SO_4$ | Phosphate Buffer |
|---|---|---|---|
| Mouse | − | − | − |
| Rat | + | + | + |
| 950 r-RBM mouse | + | + | + |

2. It can be seen that at 27 C. the rate of denaturation of hemoglobin by dilute alkali took place in an apparent first-order reaction. The velocity constants were found to be: $K^{27}_{mouse} = 10.0 \times 10^{-3}$ seconds$^{-1}$; $K^{27}_{rat} = 8.9 \times 10^{-3}$ seconds$^{-1}$; and $K^{27}_{950\text{-r-RBM mouse}} = 9.0 \times 10^{-3}$ seconds$^{-1}$. At 11 C., it became obvious that more than one type of hemoglobin, in terms of the alkali denaturation property, was present in all three types studied. Inspection of the curves showed at least two "breaks" for the rat and 950 r-RBM mouse curves and three "breaks" for the mouse curve. Regardless of the nature of rate-reaction curves, it is clear that the 950 r-RBM mouse hemoglobin was almost, if not completely, identical to the rat hemoglobin.

Representative results of paper-strip electrophoresis patterns are shown in figure 3. The relative hemoglobin concentrations, obtained when RBC were lysed in water, in terms of the 540-m$\mu$ absorption, were 3.8, 3.2, and 3.8 for mouse, rat, and 950 r-RBM mouse types, respectively. The difference observed was evidently in the degree of adherence of hemoglobin to the paper strip at the source of application. It can be seen that, relative to the mouse hemoglobin, rat and 950 r-RBM mouse hemoglobins adhered to the paper strip in significantly higher concentrations.

Rat and mouse hemoglobin samples were ultracentrifuged at the same time at 20 C. by use of a 1° prismatic window to deflect the Schlieren pattern from one cell. No significant differences were found in the sedimentation rates.

## Discussion

The relative importance of genetic and *intra*species bioenvironmental factors in the growth of somatic cells has long been a subject of great interest. The effect of *inter*species bioenvironmental factors is of equal interest but is difficult to assess because the normal immunologic defense mechanism of the host must be overcome. It has, however, now been demonstrated in this and other laboratories that, in lethally irradiated mice, rat bone marrow can be transplanted permanently.[1-6] As many as 95 per cent of these lethally irradiated mice can be protected from acute radiation death with injections of rat bone marrow, but on about the third week after treatment, they undergo a severe immune reaction involving the rat transplant and host antibody-producing cells. The maximum reaction period appears on about the sixth to seventh week after treatment. A certain percentage of these mice will eventually return to normalcy; the survivors then become truly tolerant to the interspecific rat bone marrow transplant.

The results presented here show that, 65 days or more after treatment, the circulating RBC of the 950 r-RBM mice are indistinguishable from rat RBC. These results confirm previous findings that such cells are identical to rat RBC in (1) their ability to react with specific antirat RBC serum, (2) their absorbing capacity of rat RBC antibodies, and (3) their ability to induce rat antibody formation in normal mice.[1, 2] It can therefore be concluded that the surface molecular configuration of these RBC is of the rat type.

Our findings on the osmotic properties of the RBC of 950 r-RBM mice were of interest. The amount of hemoglobin released by these cells when lysed in

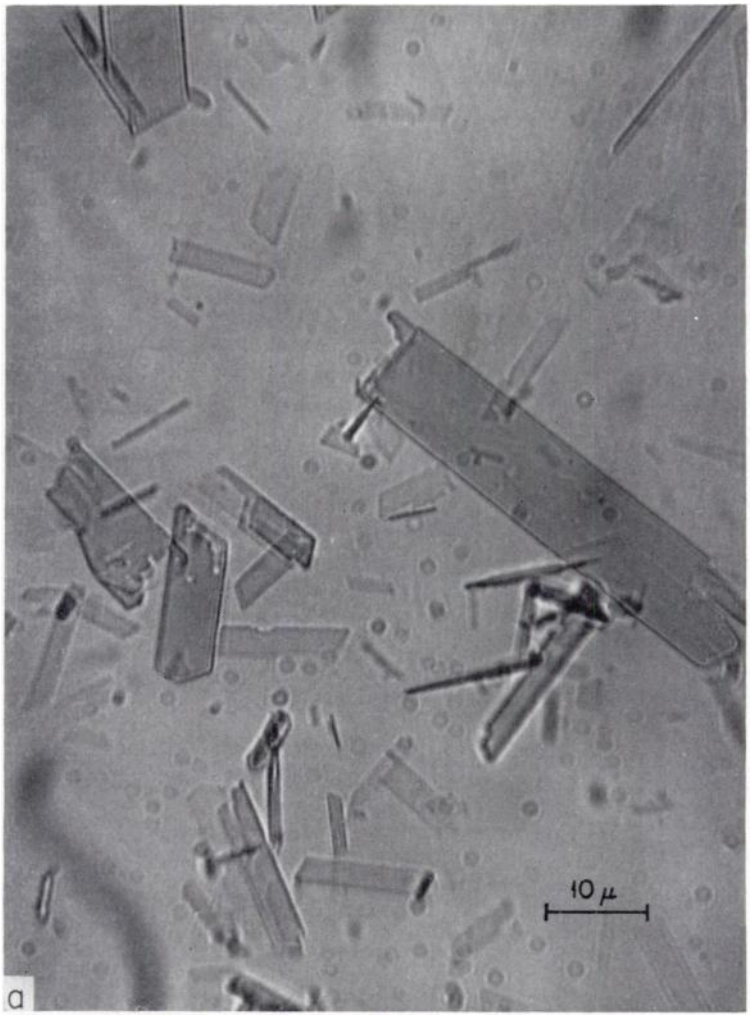


FIG. 1.—Photograph of hemoglobin crystals
a. Rat-in-mouse hemoglobin

water was comparable to that in the rat RBC, but their temperature-dependent nature is comparable to the mouse RBC. It has been shown that the serum proteins of these treated mice were of the mouse type[1]; therefore, these RBC were suspended in mouse serum proteins in vivo. Thus it would appear that the serum proteins may have had some degree of influence on the osmotic properties of the cells.

Studies on the physicochemical properties of the hemoglobin showed that the RBC of 950 r-RBM mice possessed rat-like hemoglobin whose denaturation by alkali is comparable to that of rat hemoglobin. The ability of the hemoglobin of 950 r-RBM mice to undergo a paracrystalline state at low temperature, as expressed by its inability to release hemoglobin when sheared mechanically, is also indicative of rat hemoglobin. Only rat hemoglobin undergoes a paracrystal-

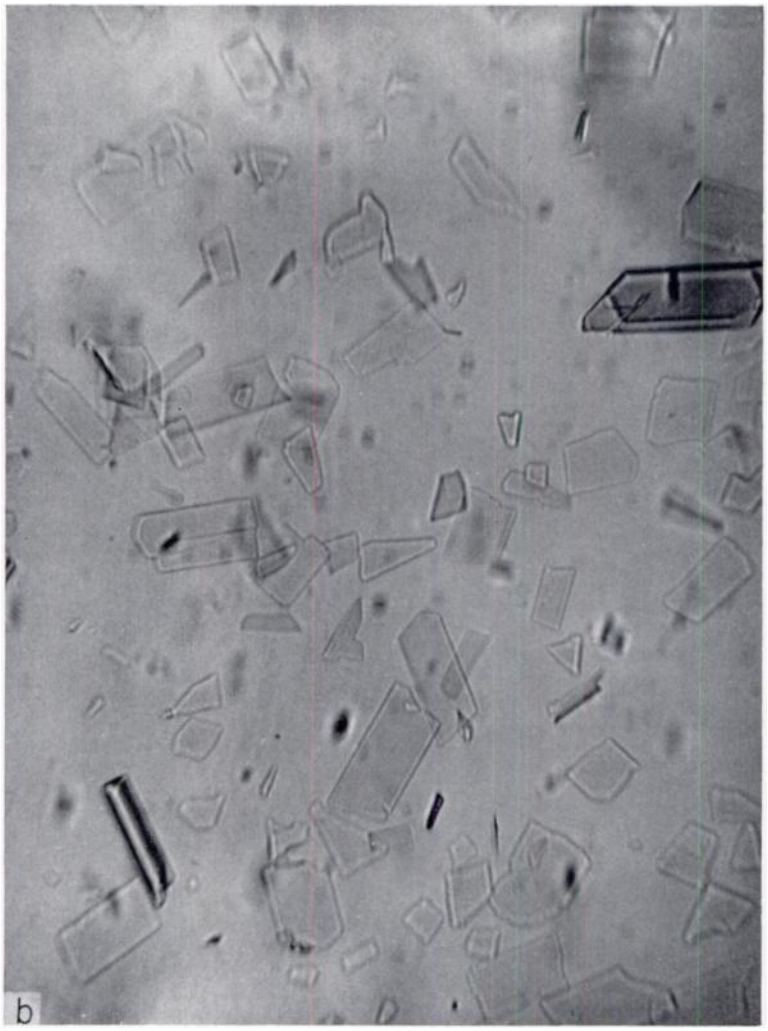


FIG. 1.—b. Rat hemoglobin

line state in the cell at low temperature.[11] Paper-strip electrophoresis also revealed rat hemoglobin characteristics. The amount of hemoglobin at the point of attachment was comparable to that of rat hemoglobin. The adherence to the paper strip has been suggested by Waldmann-Meyer and Schilling[12] as a characteristic physical property of a substance. Finally, the ease with which the 950 r-RBM hemoglobin crystallizes out under the experimental condition is comparable to that of rat hemoglobin, and microscopically the crystals of both types were alike. It is also of interest that in both cases a maximum of only 90 per cent of the hemoglobin crystallized.

These results of serologic and fragility tests show that the *interspecies* bioenvironmental factor has no influence in altering the surface molecular configuration of RBC. Similarly, hemoglobin formation appears to be unaltered in the

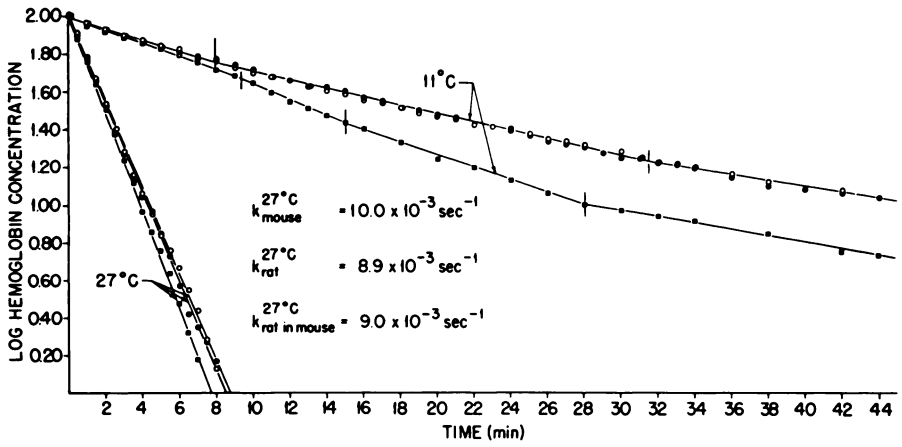


FIG. 2.—Denaturation of hemoglobin by alkali. ○, rat; ●, rat-in-mouse; ■, mouse. Vertical line denotes "break" in curve.

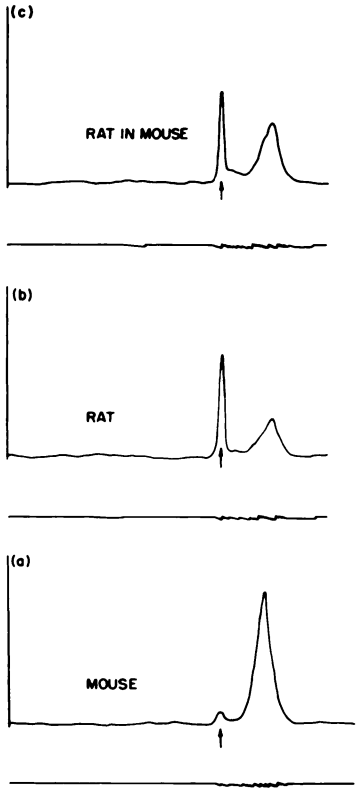


FIG. 3.—Electrophoretic pattern of hemoglobin. The arrow indicates point of application of hemoglobin; curve above arrow is pattern of light absorption of paper strip, and curve below arrow is pattern of integrator.

mouse environment. It is proposed, therefore, that cells of the 950 r-RBM mouse be called rat-in-mouse RBC.

It can be assumed that differences on the molecular synthetic level exist between rat and mouse; and since rat RBC precursors are firmly entrenched in the mouse, it is surprising that the bioenvironmental factor had relatively little influence in altering the formation of RBC. No information on the rate of formation of RBC or of life span has been obtained. These results emphasize very strongly the prominent role of the genetic factor.

## Summary

1. Two months after injection of rat bone marrow into lethally X-irradiated mice (950 r-RBM mice), 100% of the circulating RBC were serologically of the rat type, indicating that the surface molecular configuration of RBC from these experimental mice are of the rat type.

2. The hemoglobin was found to be also very much like the rat type in its ease in crystallization, its alkali denaturation property, its electrophoretic property, and its tendency to form a paracrystalline state at low temperature.

3. These cells possessed dual osmotic properties; the relative hemoglobin concentration released when cells were lysed in water was more comparable to the rat type, but its temperature dependency was more comparable to the mouse type.

## Summario in Interlingua

1. Duo menses post le injection de medulla ossee de rattos in muses subjicite a doses letal de irradiation X, 100 pro cento del erythrocytos circulante del muses esseva serologicamente del typo de ratto. Isto significa que le configuration molecular al superficie del erythrocytos de iste muses experimental esseva un configuration characteristic del erythrocytos de ratto.

2. Esseva etiam trovate que le hemoglobina esseva similissime al hemoglobina de rattos in su facile crystallisation, su proprietate de disnaturation per alcali, su proprietate electrophoretic, e su tendentia a formar un stato paracrystallin a basse temperaturas.

3. Le cellulas possedeva dual proprietates osmotic. Le relative concentration de hemoglobina liberate post lyse in aqua esseva comparabile al comportamento de erythrocytos de ratto, sed le dependentia ab le temperatura esseva plus tosto del typo trovate in muses.

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