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Further Studies on the Erythrocytic Host Response in Moloney Murine Leukemia

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SUMMARY

The red blood cells of 2-day-old normal and 8-week-old Moloney leukemia-infected BALB/c mice were examined using fragiligraphy, electron microscopy, and hemoglobin electrophoresis. Results of fragiligraphy revealed similar bimodal curves representing two RBC populations for the young normal and adult leukemic animals. Electron microscopy confirmed these two populations and revealed similarities in morphology between RBC's from the 2-day-old normals and 8-week-old leukemic animals. Electrophoresis showed no evidence of fetal hemoglobin in any of the samples. Thus, it is assumed the cells from the osmotically more resistant second populations in the leukemic animals are not fetal type cells in the sense of hemoglobin-types, but similar to the fetal erythrocytes which start to develop in the fetal liver (12th to at least the 17th day of gestation) and subsequently in the fetal bone marrow (after Day 16).

INTRODUCTION

Perk *et al.* (9), using a fragiligraph (Elron Electronics Industries, Haifa, Israel) which records gradual osmotic hemolysis levels, showed that the blood of the majority of adult BALB/c mice, regardless of sex, exhibited during the interval from four to six-weeks after Moloney leukemia virus injection until the time of death a bimodal fragility curve indicating two erythrocyte populations: (*a*) the normal and (*b*) a more osmotically resistant population. Such bimodal curves have never been reported for the normal BALB/c mouse after four weeks of age. However, bimodal fragiligrams have been described for the blood of healthy newborn calves, lambs, pigs, rabbits, mice, rats, hamsters, and guinea pigs (7). The second more resistant red blood cell population disappeared after one RBC life span of the respective animal species and were therefore designated as "fetal"-type cells.

Further, the use of light microscopy showed that a close resemblance existed between the mouse fetal type of erythrocyte and those from the second more resistant population in the blood of mice with Moloney leukemia. It was suggested, therefore, that the Moloney leukemia virus stimulated the hematopoietic tissue to release a fetal type of cell which under normal conditions is not recognizable in a healthy adult mouse.

In the present study, the red blood cells of 2-day-old normal mice and 8-week-old Moloney leukemia-infected mice were examined using fragiligraphy, electron microscopy, and hemoglobin electrophoresis.

MATERIALS AND METHODS

Fragiligraphy

Blood (0.1 ml) was removed with a heparinized capillary pipet from the retroorbital venous plexus of 8-week-old BALB/c mice which were injected at four weeks of age with Moloney leukemia virus (MLV). Blood from 2-day-old normals was removed by etherizing the animals, opening the axillary region, and drawing the blood with the pipet. The samples in each case were deposited in 1 ml of 0.86% phosphate-buffered saline (PBS), pH 7.2, and fragility curves (Fig. 1) were obtained using the fragiligraph. The time was recorded to the end of the first peak (end of hemolysis of first erythrocyte population) and up to the time necessary for the second more resistant population to hemolyze.

Electron Microscopy

Other samples of blood (0.2 ml) were removed from 8-week-old leukemic mice and from 2-day-old normals (pooled blood was used in this case because of the small amount of blood obtainable from 2-day-old mice).

Having determined from the fragiligraph recordings the time to the end of the first peak and to complete hemolysis, two blood samples, one from the normal mice and one from leukemic mice, were placed in 5 ml of PBS and washed 3 times at 1000 rpm for 3 minutes, resuspending the pellets in 5 ml of PBS each time. The final suspension in each case was transferred to a dialysis bag and completely dialized at 4°C against 4000 ml of distilled water to which approximately 0.1 gm of NaCl had been added to slightly retard the rate of hemolysis.

Having determined by this method the time to the end of hemolysis, two new samples were prepared in like manner and dialyzed for a time proportional to that necessary for the first population to hemolyze in each case. For example, in the case of the normal blood, the fragiligraph tracing indicated complete hemolysis in an average time of 6.5 minutes, and the time to completion of the first peak averaged 3.2 minutes. In the dialysis bag, hemolysis was accomplished in 40 minutes; thus,

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the time required for hemolysis of the first population was estimated to be approximately 20 minutes.

After removal of the samples from dialysis, they were placed immediately into PBS for 3 minutes to stop hemolysis. Following this, the samples were removed from the dialysis bags and centrifuged at 1000 rpm for 3 minutes. This sedimented the unhemolyzed, more resistant cells from the second population, leaving the ghosts of the first population in the supernatant. The pellet was again placed in a dialysis bag, allowed to completely hemolyze, and the ghosts were spun down at 10,000 rpm for 10 minutes.

The supernatant containing the ghosts of the more fragile first population was also centrifuged at 10,000 rpm for 10 minutes to sediment them. Finally, all pellets were fixed in Dalton's chrome osmium (3) for 30 minutes at 4°C, followed by four washings in 5 ml of distilled water, each followed by centrifugation at 10,000 rpm for 10 minutes, with final resuspension of the pellets in 1 ml of distilled water.

A drop of each sample was placed on carboned formvar-coated grids, air dried, and shadow cast with 6 mg of chromium at a height of 3 cm from a distance of 12 cm. The specimens were examined in an RCA EMU-3G electron microscope.

Electrophoresis

Blood samples were taken from leukemic, 2-day-old normal, and 8-week-old normal control mice and analyzed for fetal hemoglobin using cellulose acetate electrophoresis (1) with Beckman B-2 buffer at pH 8.6 and ionic strength 0.075 M.

RESULTS AND DISCUSSION

Fig. 1*A* represents a typical unimodal cumulative fragiligram and its derivative as obtained from the blood of normal adult BALB/c mice. The cumulative curve describes the degree of hemolysis on the ordinate as a function of time (abscissa) which is a result of the gradually decreasing saline concentration surrounding the erythrocytes.

Blood samples obtained from newborn 2-day-old normal BALB/c mice or from the examined Moloney leukemic adult BALB/c yielded cumulative fragiligrams which were of a distinct bimodal type, and their derivatives presented two peaks, indicating two erythrocyte populations (Figs. 1 *B, C*). One corresponded to the normal adult type of red blood cell and the other to a second population of erythrocytes which hemolyze at a much lower saline concentration. These findings are in accordance with previous investigations reported for the normal newborn (7) and Moloney leukemic mice (9). Further, phase contrast microscopy of blood samples withdrawn from the dialysis membrane at different time intervals during the hemolysis process revealed that the second RBC population consisted mainly of anucleated erythrocytes of various sizes (9). It is also noteworthy that reticulocyte counts, performed randomly, revealed an increase in number following MLV inoculation; however, there was no clear correlation between the reticulocyte number or percentage and the relative percentage of the more osmotically resistant RBC population.

From four weeks after MLV inoculation to the time of death, in animals where two populations were found, the proportion of cells which constitute the two erythrocyte populations was fairly uniform, regardless of the clinical progression of the leukemia (9). Therefore, in the present comparative study, the blood samples of 8-week-old BALB/c mice which were injected at four weeks of age with MLV and blood samples from normal 2-day-old and from healthy adult mice were examined.

Electron microscopic observation revealed an almost complete separation of populations in the samples from the normal and leukemic mice. Using the criteria of Danon and Perk (4) for distinguishing old and young red blood cells in the electron microscope and applying it to mice, it was evident that the second population from each sample was composed almost entirely of young cells and the first population consisted mainly of old cells (Figs. 2, 3). Also, cells from the second population of the 2-day-old normal and the 8-week-old leukemic animals were morphologically similar (Figs. 4, 5). These findings support the assumption that the Moloney virus elicits production of a fetal-type erythrocyte (9).

Previous studies in which starch gel (2) or polyacrylamide gel disc electrophoresis (6) were used to separate hemoglobins have resolved three or four components in red cells from fetal mice, only one of which persists in the adult mouse. The fetal mouse erythropoiesis proceeds initially in yolk-sac blood islands (8 to 12 days) and, subsequently, in liver (12 to at least 16 days) and in bone marrow (after Day 16). Yolk-sac cells synthesize three hemoglobins, but fetal livers and bone marrow erythoid cells form only adult-type hemoglobin (5, 6). Thus, blood obtained from mouse fetuses at late stages of gestation show only the adult-type hemoglobin present in the fetal erythrocyte. Accordingly, the results of repeated electrophoresis examination in the present study revealed no differences in pattern of migration between 2-day-old normals, 8-week-old leukemic animals, and 8-week-old normals. Thus, by the electrophoresis method, there was no evidence of fetal hemoglobin (Fig. 6).

From these data it appears that the structural differences found between the membranes of the two cell types (first and second RBC populations) are responsible for the increased resistance to osmotic pressure. Similarly, even in cells where the fetal hemoglobin is present (new born calves' erythrocytes), it was found that the membrane structure and content is responsible for the increased osmotic resistance of the fetal cells (8).

Direct interaction of the Moloney leukemia virus with the matured RBC does not appear to be related causally to the presented changes in the osmotic and structural pattern of the erythrocytes. This view is based largely on the following findings: (*a*) *In Vitro* incubations of MLV with blood obtained from normal adult BALB/c mice revealed no differences in the fragility pattern when compared to the control incubated blood. (*b*) Electron microscopic examination of the RBC obtained from Moloney leukemic mice revealed no association of the virus to the cells. (*c*) The osmotic and structural similarity is evident between the blood of the 2-day-old normal and leukemic mice.

Further investigation is needed, however, to determine the significance of the second more resistant population of red blood cells in the 2-day-old normal and 8-week-old leukemic BALB/c mice.

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Fig. 1. *A*, Fragiligraph curves (cumulative, x; derivative, y) of blood from 8-week-old normal mouse showing only one peak. *B*, fragiligraph curves of 2-day-old normal mouse blood. Note bimodal peaks indicating two RBC populations. *C*, fragiligraph curves of blood from 8-week-old Moloney leukemic mouse. The degree of hemolysis is on the ordinate, which is a function of time (*abscissa*), which in turn is a function of decreasing salt concentrations. Note similarity to 2-day-old normal.

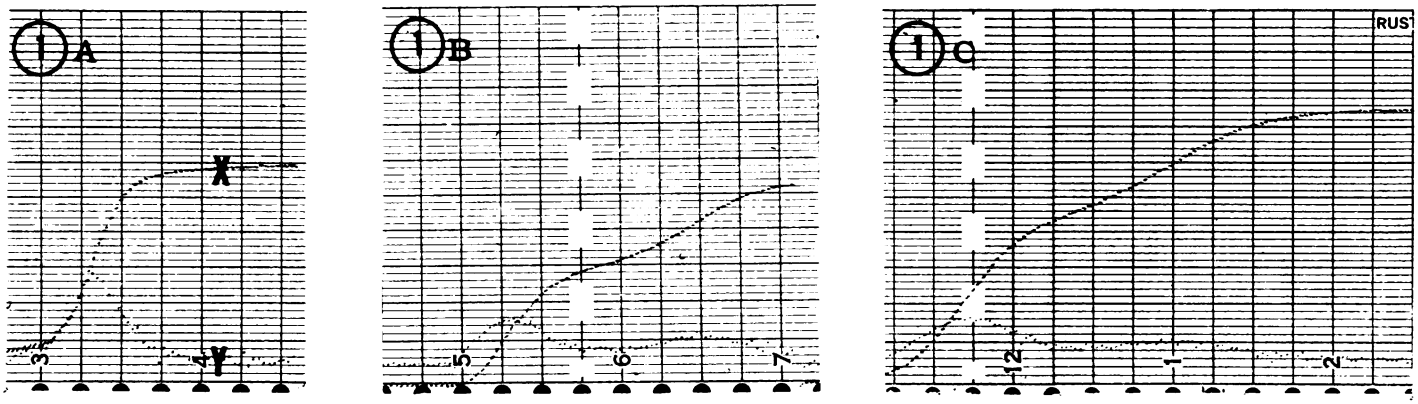
Fig. 2. Typical membrane ghost of old red blood cell from the first RBC population in a 2-day-old normal mouse. Approximately × 24,000.

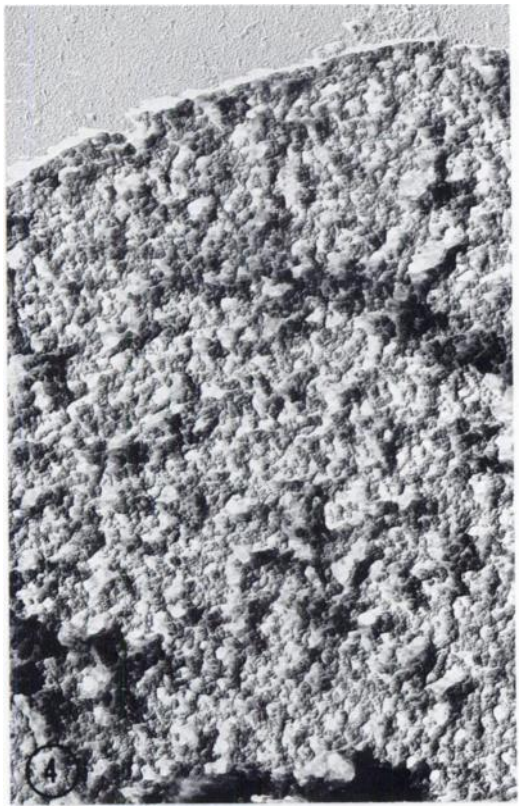
Fig. 3. Typical membrane ghost of old red blood cell from the first RBC population in an 8-week-old Moloney leukemic mouse. Approximately × 24,000.

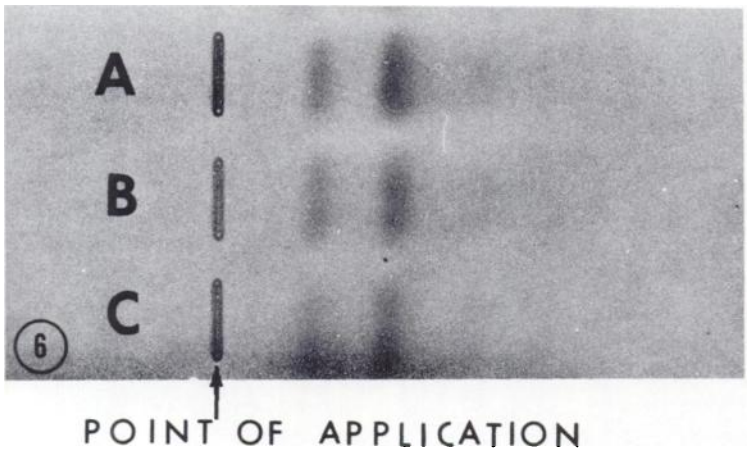
Fig. 4. Typical membrane ghost of young red blood cell from the second RBC population in a 2-day-old normal mouse. Approximately × 24,000.

Fig. 5. Typical membrane ghost of young red blood cell from the second RBC population in an 8-week-old Moloney leukemic mouse. Similarity of surface structures to Fig. 4 is readily seen. Approximately × 24,000.

Fig. 6. Acetate gel hemoglobin electrophoresis patterns. *A*, 8-week-old normal mouse; *B*, 2-day-old normal mouse; *C*, 8-week-old Moloney leukemic mouse.







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