

Red Blood Cell Metabolism in AKR Mice in the Prelymphomatous Phase¹

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SUMMARY

The purpose of this study was to evaluate erythrokinetics and *in vitro* red blood cell (RBC) glucose utilization, 2,3-diphosphoglycerate production, and adenosine triphosphate levels following incubation in AKR mice from early in life until the onset of AKR lymphoma. Normal BALB/c mice served as controls. While hemoglobin concentration and RBC survival remained constant and similar in both groups of mice, the half disappearance time of injected radioactive ⁵⁹Fe was longer and the 48-hr reappearance of ⁵⁹Fe was less in AKR mice, compared with those of BALB/c mice. *In vitro* RBC metabolic studies indicated increased glucose utilization and 2,3-diphosphoglycerate production and decreased adenosine triphosphate levels following incubation in AKR RBC, in contrast to those in BALB/c RBC. RBC metabolic studies were also done in a small group of low leukemic C3H mice, and were similar to BALB/c mice. These differences became most marked in RBC from mice aged 15 to 30 weeks. Overt lymphoma began to occur after age 40 weeks. Hence, these erythropoietic changes occurred prior to the onset of lymphoma.

The data imply a direct effect of virus infection on RBC or their precursors. The results are similar to changes in RBC metabolism noted in Rauscher-infected BALB/c mice. The broader implication of these findings in reference to viral host interactions and human leukemogenesis is discussed.

INTRODUCTION

Murine leukemias are induced by viruses and, during their course, a number of nonneoplastic hematopoietic phenomena may occur. For example, Rauscher leukemia virus infection is characterized by a biphasic illness (7). Initially, dose-dependent thrombocytopenia, splenomegaly, granulocytosis, monocytosis, and hemolytic anemia are seen (7). Mice given high titers of virus frequently do not survive this phase, and death is due to splenic rupture and hemorrhage. Platelet survival studies performed during this period reveal a sharp decrease in platelet survival (8). Platelet metabolic studies reveal alterations that suggest that the initial thrombocytopenia is due to a direct effect of the virus on the platelet (4). Erythrokinetic studies reveal relative bone marrow failure, ineffective erythropoiesis, and shortened red

cell survival (6). RBC metabolic studies demonstrate increased osmotic fragility, increased autohemolysis, increased glucose utilization, and decreased RBC ATP levels during incubation *in vitro*. Similar changes in thrombopoiesis and erythropoiesis have been noted in Friend virus leukemia (14, 25). These results also suggest a direct effect of the virus on the RBC membrane.

The above hematopoietic responses have been designated the preleukemic phase of Rauscher leukemia, since the hematological response seems to be a reaction to destruction of circulating RBC and platelets by the virus. Others define these responses as malignant proliferation of cells in response to viral infection (12, 15).

The latter phase of Rauscher infection is characterized by increased lymphopoiesis. Rauscher (23) considered this phase a lymphatic leukemia, and investigations in other laboratories have supported this concept (10). Other investigators who have studied Friend disease consider this illness a form of erythroleukemia, in that the lymphatic proliferation is much less prominent, while the erythroblastic proliferation persists for a longer period of time (22). However, the Friend virus complex is a mixture of viruses (11). The response to infection is variable depending upon virus strain, virus titer, and genetic background of the host. For example, 1 virus strain leads to a polycythemia (22). Furmanski *et al.* (16) have also isolated another virus strain that spontaneously regresses following infection. These workers also point out that the conventional Friend virus complex consists of 2 components, 1 a spleen focus forming virus, the 2nd a lymphocytic leukemia virus. Each component is necessary for full expression of Friend disease (16).

The panmyelosis of the preleukemic phase of the murine leukemias has been compared with human myeloproliferative disorders, such as polycythemia vera, myeloid metaplasia, and chronic myeloid leukemia (3). There seems to be developing a consensus that the myeloproliferative disorders may be preleukemic manifestations of an ultimate leukemic disease. The final phase of these disorders, if the patient survives, may be acute granulocytic leukemia (1).

The purpose of the present experiments was to study in detail RBC metabolism and erythrokinetics in AKR mice during the lifetime of the animals. Study of the evolution of AKR lymphoma is important in that it avoids the multiplicity of problems engendered by diseases induced by experimentally passaged virus. The pattern of thrombocytopenia in the AKR mouse has been shown to follow in a qualitative fashion the thrombocytopenia of laboratory-induced Rauscher and Friend disease (2). In AKR mice, at 3 weeks of age, platelet counts are lower than in age-matched BALB/c con-

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trols. By 4 months, platelet counts have risen and are comparable to those of age-matched BALB/c controls. The severity of the early thrombocytopenia is not as pronounced as is the early thrombocytopenia of Friend or Rauscher disease, and the time sequences are prolonged in the natural disease when compared to the laboratory counterparts (2, 7). Platelet metabolic studies in the AKR mouse also suggest that the virus infects the megakaryocyte early in the disease (5). Death in AKR mice is caused by a lymphoma with marrow invasion and secondary thrombocytopenia.

RBC metabolic studies in AKR animals have not been reported. The data obtained herein indicate that AKR mice, in contrast to BALB/c mice, demonstrate subtle changes in erythropoiesis and *in vitro* RBC metabolism prior to the onset of lymphoma. The RBC alterations are qualitatively similar but quantitatively less than those noted in Rauscher and Friend disease.

MATERIALS AND METHODS

Mice

AKR weanling females were obtained from The Jackson Memorial Laboratory, Bar Harbor, Maine, and were maintained in plastic cages, fed Purina laboratory chow and Micro-mix pellets, and given water *ad libitum*. Two groups of approximately 125 animals each were purchased at age 6 to 8 weeks and maintained throughout the duration of these experiments. One group was used for the erythrokinetic experiments and the other for the RBC metabolic experiments. BALB/c weanling females of similar age were maintained in similar fashion. An additional group of 30 C3H low leukemia incidence mice were also maintained as described above.

Hematological Studies

Blood for the determination of the hematological data was obtained by puncture of the retroorbital venous plexus with a heparinized capillary tube. Hemoglobin and PCV² were determined by methods previously published (6).

Ferrokinetics

In individual mice, the $t_{1/2}$ and the percentage ⁵⁹Fe reappearence in the RBC were calculated by methods previously described (6).

RBC Survival

This was determined by cohort labeling with ⁵⁹Fe (Ferrutope; Squibb and Sons, New York, N. Y.). Mice were given i.v. injections of 0.25 ml of ⁵⁹Fe containing 3 μ Ci. Three hr later and daily thereafter, they were given i.p. injections of 0.5 ml nonradioactive iron (iron dextran injection; Imferon; Lakeside Laboratories, Milwaukee, Wis.; 50 mg elemental iron per ml) to block reutilization of ⁵⁹Fe. Individual mice

² The abbreviations used are: PCV, packed cell volume; $t_{1/2}$, half disappearance time of injected radioactive ⁵⁹Fe; DPG, 2,3-diphosphoglycerate; Hgb, hemoglobin.

were bled serially from the retroorbital plexus, and the cpm in 16 μ l whole blood were determined. In each mouse, the ⁵⁹Fe reappearence on Day 2 was arbitrarily assigned a value of 100%. Percentage survival was calculated from the reappearence of ⁵⁹Fe and plotted on semilogarithmic paper.

Preparation of RBC Suspensions for Metabolic Studies

Blood from AKR, BALB/c, and C3H mice was obtained by retroorbital plexus bleeding into heparin by means of Pasteur pipet. Heparin, 500 μ m/ml, was added in a ratio of 0.5 ml of heparin for each 5 ml of blood collected. A pool of blood from 10 to 20 mice was used in each experiment. Blood was centrifuged at 1500 \times g for 10 min. The plasma and buffy coat were removed. The packed RBC's were washed once in Krebs-Ringer bicarbonate buffer, pH 7.4 (13), and resuspended to approximately 10 to 25% PCV. Penicillin solution in buffer, 1000 μ g/ml, was added to give a final concentration of 10 μ g/ml. Streptomycin solution in buffer (4000 μ g/ml) was added to give a final concentration of 40 μ g/ml. Five % glucose in water was added in amounts varying from 0.1 ml/2 ml RBC suspension for ATP experiments to 0.2 ml/2 ml RBC in the glucose utilization experiments. Nucleated cell counts in these red cell suspensions were checked before each experiment and in all instances were less than 500/ μ l. The pH was adjusted to 7.4 with a pH meter by gassing with 5% CO₂, 95% O₂.

ATP

ATP levels in RBC suspensions were measured with kits supplied by Boehringer Chemical Corporation-Boehringer Mannheim GmbH, Mannheim, Germany.

Osmotic Fragility

This study was performed on whole heparinized blood by the method of Dacie and Lewis (9).

Glucose Utilization

Glucose utilization was measured by the method of Hutton (18).

DPG Levels

The DPG levels were measured with kits supplied by the Sigma Chemical Company, St. Louis, Mo.

Statistical Analysis

Significant differences between means, by Student's *t* test, had a *p* value of <0.05.

Experimental Groups

Erythrokinetic Studies. One group of approximately 75 AKR mice, age 6 through 8 weeks, were given injections of ⁵⁹Fe, and the $t_{1/2}$, 48-hr reappearence of iron, and RBC survival studies were performed. Prior to injection of ⁵⁹Fe,

Hgb concentration and PCV were obtained. The animals were sacrificed by cervical dislocation at the end of the RBC survival experiment. Sixteen AKR mice, approximately 16 to 25 weeks old, were also evaluated in the same way as were a group of 34 animals, 33 to 40 weeks of age. At the conclusion of these erythrokinetic studies, these animals were sacrificed. The same studies were performed on groups of BALB/c mice (Table 1).

RBC Metabolic Studies. A group of approximately 125 AKR mice and 125 BALB/c mice were obtained when about 6 weeks of age. After a period of adjustment to laboratory living, serial studies were performed on these mice. Ten to 20 of each group of mice were bled serially at intervals of approximately 4 weeks. The animals were bled from 8 through 50 weeks of age, and each animal was bled approximately 3 times. A group of 30 C3H mice were studied at 9, 35, and 40 weeks of age.

ATP Stability. Approximately 4 ml of RBC suspension

obtained from 10 to 20 mice were placed in a 25-ml Erlenmeyer flask. Glucose was added to one flask and nonglucose Krebs-Ringer bicarbonate buffer was added to the other. Duplicate flasks were placed in a Dubnoff metabolic shaker at 37° and agitated at moderate speeds. RBC specimens were withdrawn from the flask at times zero, and at 1, 2, and 4 hr. The blood was deproteinized and the supernatants were frozen. Within 24 hr, ATP levels were measured.

DPG Levels. These were measured on the same RBC suspensions as were used in the ATP determinations.

Osmotic Fragility. Whole heparinized blood obtained from 10 to 20 mice was pooled, and osmotic fragility on these samples was measured on the day of collection.

Glucose Utilization. Ten to 30 mice of various ages were used (Table 2). RBC suspensions were prepared and placed in 25-ml Erlenmeyer flasks. After the addition of radioactive glucose, the flasks were incubated at 37° in a Dubnoff metabolic shaker.

Table 1
Erythrokinetic data in AKR and BALB/c mice

Strain	Age (wk)	No. of mice	⁵⁹ Fe t ₁ (min)	⁵⁹ Fe reappearance at 48 hr (%)	RBC survival (days)	Hgb concentration (g/100 ml)/PCV (%)
AKR	5-8 (Group 1)	67	70.6 ± 23.3 ^a	59.6 ± 23.4	43.0 ± 5.1	16.0 ± 0.98/47.5 ± 3.4
	16-25 (Group 2)	16	64.9 ± 15.3	66.1 ± 16.0	40.5 ± 5.2	ND ^b
	32-40 (Group 3)	34	74.0 ± 16.3	87.0 ± 22.7	36.4 ± 9.5	14.7 ± 1.3/45.1 ± 3.5
BALB/c	5-8 (Group 1)	18	56.3 ± 7.9	70.2 ± 8.4	47.7 ± 3.9	15.6 ± 1.0/46.4 ± 3.3
	16-25 (Group 2)	15	59.8 ± 9.7	73.1 ± 15.5	38.1 ± 5.3	ND
	32-40 (Group 3)	20	61.7 ± 14.5	98.4 ± 17.9	42.3 ± 3.4	15.1 ± 1.1/45.3 ± 2.8

^a Mean ± S.D.

^b ND, not done.

Table 2
Statistical comparison of erythrokinetic data in AKR and BALB/c mice

Parameter	AKR		BALB/c		AKR vs. BALB/c	
	Group	SIG ^a	Group	SIG	Group	SIG
t ₁	1 vs. 2	NS	1 vs. 2	NS	1 vs. 1	p < 0.01
	2 vs. 3	NS	2 vs. 3	NS	2 vs. 2	NS
	1 vs. 3	NS	1 vs. 3	NS	3 vs. 3	p < 0.02
48-Hr reappearance	1 vs. 2	NS	1 vs. 2	NS	1 vs. 1	p < 0.05
	2 vs. 3	p < 0.010	2 vs. 3	p < 0.001	2 vs. 2	p < 0.003
	1 vs. 3	p < 0.001	1 vs. 3	p < 0.001	3 vs. 3	p < 0.005
Either RBC survival, Hgb concentration, or PCV	1 vs. 1	NS	1 vs. 2	NS	1 vs. 1	NS
	2 vs. 3	NS	2 vs. 3	NS	2 vs. 2	NS
	1 vs. 3	NS	1 vs. 3	NS	3 vs. 3	NS

^a SIG, statistical significance defined as p < 0.05; NS, not statistically significant (i.e., p > 0.05).

RESULTS

Effect of Aging on Death Rate

Chart 1 represents the death rate in the serially maintained AKR and BALB/c mice. In the AKR group, deaths were noted beginning at approximately 32 weeks. The death rate peaked between 44 and 48 weeks. By 50 weeks, almost one-half (60 of 125) of the animals had died. Autopsy study of these mice demonstrated involvement of liver, spleen, and thymus with lymphoma. We presume that death in these animals was related to this neoplastic process. The death rate in the BALB/c animals was much lower and, at the conclusion of the experiment, virtually 90% of the BALB/c mice were still alive. The deaths in the BALB/c group were caused, for the most part, accidentally during bleeding.

Erythrokinetic Data in AKR and BALB/c Mice

Table 1 lists erythrokinetic data in both AKR and BALB/c mice, and Table 2 gives a statistical comparison of these data. Both sets of animals were arbitrarily divided into 3 groups according to age: Group 1, 5 to 8 weeks; Group 2, 16 to 25 weeks; and Group 3, 32 to 40 weeks.

Within and between both the AKR and BALB/c groups, no differences were noted in RBC survival, Hgb concentration, or PCV.

The $t_{1/2}$ ranged between 64.9 and 74.0 in the AKR mice and 56.3 and 61.7 in the BALB/c mice. There were no significant differences among the 3 AKR groups or among the 3 BALB/c groups. However, when the youngest AKR group was compared with the youngest BALB/c group (Group 1 AKR versus Group 1 BALB/c), the $t_{1/2}$ was significantly longer in the AKR mice. Similar significant differences were noted in the older mice (Group 3 AKR versus Group 3 BALB/c). There was no significant difference between the $t_{1/2}$ values in the mice aged 16 to 25 weeks (Group 2 AKR versus Group 2 BALB/c). This resulted from the fact that the $t_{1/2}$ in the AKR mice shortened to 64.9 min, while the $t_{1/2}$ in the BALB/c mice remained fairly constant at 59.8 min. RBC obtained from mice during this period demonstrated increased *in vitro* glucose utilization and decreased ATP levels during incubation (see below).

There were significant differences in the comparison of the 48-hr reappearance results (Table 2). In both AKR and BALB/c mice, 48-hr reappearance increased as the mice aged. The differences became significant in Group 3, as contrasted to Group 2 or Group 1, in both the AKR and BALB/c mice. When each group of AKR mice was compared with a similar group of BALB/c mice, the 48-hr reappearance was always significantly lower in the AKR mice than it was in the BALB/c mice (Table 2) (48-hr reappearance, AKR versus BALB/c).

RBC Metabolic Studies (AKR and BALB/c)

In these experiments, all results represent the results of pooled blood from 10 or more mice per point.

Glucose Utilization. Over the course of these experi-

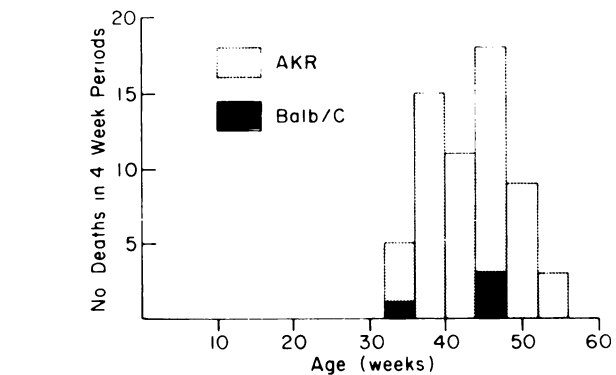


Chart 1. The number of deaths of AKR and BALB/c (Balb/c) mice in 4-week intervals throughout the length of the study.

ments, the glucose utilization ranged between 0.40 and 0.89 $\mu\text{mole/ml RBC/hr}$ in the AKR group and 0.35 to 0.74 $\mu\text{mole/ml RBC/hr}$ in the BALB/c group (Table 3). In the BALB/c group, glucose utilization tended to fall as the animals aged, but in the AKR group at 31 weeks, glucose utilization peaked at 0.89 $\mu\text{mole per ml RBC per hr}$.

ATP Levels during Incubation. These data are given in Table 3 and Chart 2. ATP levels ranged between 0.70 and 1.10 $\mu\text{moles/ml RBC}$ at the start of incubation (*i.e.*, t_0 values) in the AKR and BALB/c group over the course of these experiments. At all ages, during incubation with glucose, ATP levels were maintained in RBC's from both groups of mice (data not shown).

Incubation of RBC's in the absence of glucose showed a distinctly different pattern in the AKR group, compared with the BALB/c group. In either AKR or BALB/c mice, ATP levels remained fairly constant after 1 hr of incubation (Chart 2), and there was a fall in ATP levels after 4 hr of incubation. However, the fall in the AKR mice was much more pronounced in all age groups than it was in the BALB/c mice. The most striking differences in ATP levels were noted during the 25- to 35-week period. By 31 weeks, the ATP level was virtually 0 at 2 hr in the AKR mice as opposed to a level of approximately 50% of initial value in the BALB/c mice (Chart 2d).

DPG Levels. In Table 3, the DPG levels at 1 hr and at the end of 4 hr of incubation with glucose are presented. In the AKR mice, DPG levels rose during the 4-hr period, while there was a fall in DPG levels in the BALB/c mice. DPG levels ranged between 0.81 and 1.06 $\mu\text{moles/ml RBC}$ after 4 hr of incubation in the AKR mice, while the range was 0.41 to 1.13 $\mu\text{moles/ml RBC}$ after 4 hr of incubation in the BALB/c animal. Blood obtained from mice 18 to 31 weeks of age revealed that the 4-hr DPG level was consistently higher in the AKR group, in contrast to the BALB/c group. The DPG levels in the absence of glucose fell similarly in both groups of animals over the 4-hr period (data not shown).

Osmotic Fragility. Osmotic fragility studies showed no consistent differences between the AKR and BALB/c mice (curves not shown).

RBC Metabolic Studies in C3H Mice. A group of 30 C3H mice were studied to determine whether another low leukemia mouse strain would have results similar to the BALB/c controls.

ATP levels following 4 hr of incubation without glucose ranged between 35 and 60% of t_0 values. These patterns were similar to those of BALB/c mice (compare with Chart 2, a and d).

DPG levels were also measured in these mice. At 35 and 40 weeks, DGP levels after 4 hr of incubation with glucose ranged between 55 and 65% of initial value. These data are similar to the BALB/c group at 31 and 36 weeks (Table 3), where DPG levels fell approximately 50% after 4 hr of incubation while, in the AKR mice aged 31 and 36 weeks, DPG levels rose after 4 hr of incubation with glucose.

DISCUSSION

The results reported herein demonstrate that in AKR mice RBC metabolism is different than that resulting from similar

studies of BALB/c and C3H mice. Inability to maintain ATP levels in RBC during incubation may be caused by a block in production or excessive utilization or hydrolysis of ATP. The glucose utilization and DPG production data suggest that there is a no block in production of ATP. Rather, the hyperutilization of glucose suggests an attempt by the cell to compensate for an increased hydrolysis of ATP. When RBC's from AKR animals are incubated with glucose, ATP levels remain stable. Also, these changes in RBC metabolism were not secondary to anemia or hemolysis *in vivo*, since Hgb concentration, PCV, and survival of RBC were similar in both groups. Taken together, therefore, the data suggest an increase in ATP turnover in AKR RBC. Similar results have been found in RBC from Rauscher-infected BALB/c mice (6).

Altered ferrokinetics in the AKR mice were also noted.

Table 3
RBC metabolic studies in AKR and BALB/c mice at selected times during study

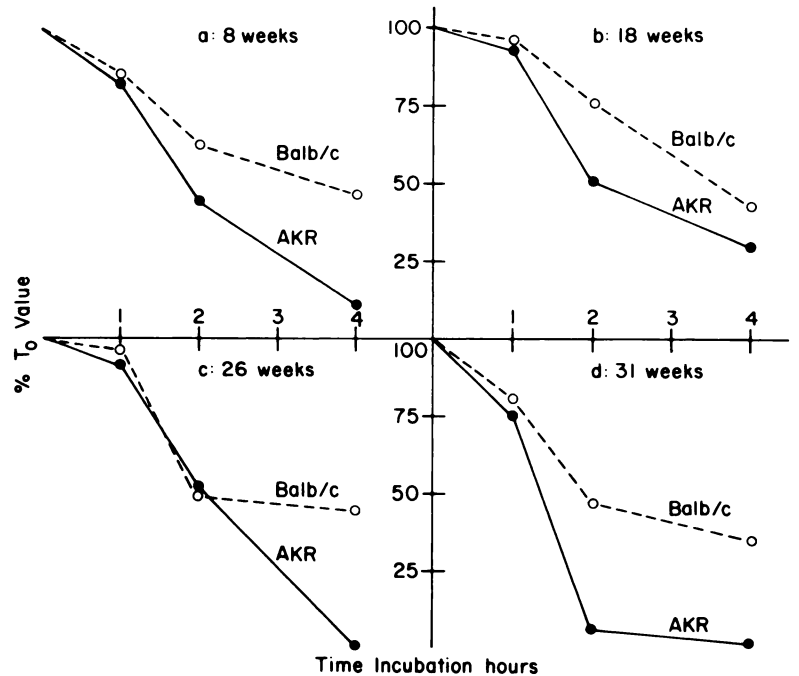
Age (wk)	Glucose utilized (μ mole/ml/hr)	AKR				BALB/c				
		ATP level ^a (μ mole/ml) at		DPG level ^b (μ mole/ml) at		ATP level ^a (μ mole/ml) at		DPG level ^b (μ mole/ml) at		
		1 hr	4 hr	1 hr	4 hr	1 hr	4 hr	1 hr	4 hr	
8	ND ^c	0.83	0.10	0.98	1.03	ND	0.84	0.46	1.08	1.13
10	ND	0.69	0.26	0.75	0.82	ND	0.76	0.23	1.06	0.89
12	ND	0.69	0.26	0.75	0.81	0.74	0.93	0.40	1.03	1.1
14	ND	0.61	0.31	0.75	0.87	ND	1.00	0.33	0.96	0.87
18	0.55	0.93	0.31	0.99	1.03	ND	0.95	0.40	0.99	0.94
26	0.89	0.93	0.05	0.93	1.02	0.58	0.99	0.47	0.96	0.87
31	0.40	0.65	<0.01	1.00	1.06	0.35	0.82	0.35	0.88	0.41
36	ND	0.93	0.29	0.84	0.94	ND	0.71	0.38	0.93	0.52

^a No glucose added.

^b Glucose added.

^c ND, not done.

Chart 2. ATP levels in RBC from AKR and BALB/c (Balb/c) mice incubated *in vitro* without glucose over 4 hr. The ages indicated at the top of a to c indicate the age of the mice from which the RBC were obtained. Each experiment represents the pooled blood from 10 to 20 mice. The results are expressed as a percentage of the t_0 value.



These ferrokinetic differences were not due to differences in the exchangeable iron pool, since serum iron levels in AKR and BALB/c mice were the same (S. B. Kahn and I. Brodsky, personal observations). The ferrokinetic patterns became most dissimilar between the ages of 24 and 32 weeks. These altered ferrokinetic patterns suggest suppression of erythropoiesis.

The above-described abnormalities occurred during the period before overt lymphoma was noted. Prelymphomatous changes in thrombopoiesis and thymus morphology and metabolism in AKR mice have been reported previously (2, 5). Therefore, the RBC alterations noted herein and the platelet abnormalities noted previously are also prelymphomatous manifestations.

Gross leukemia virus is known to be necessary for the development of AKR lymphoma (17). The prelymphomatous changes in RBC metabolism noted herein suggest infection of a stem cell by the virus. Infection of the RBC precursors and subsequent budding of the virus from the RBC membrane might lead to increased membrane permeability (19, 20). This might stimulate ATP utilization, resulting in increased ATP turnover. Stimulation of glycolysis and increased DPG synthesis would then follow.

An alternative explanation might be that these differences could be ascribed to differences in mouse strain rather than an effect of viral infection. However, the results of RBC studies in C3H mice, a low leukemia mouse strain, are similar to those of BALB/c, not AKR, mice.

These data provide new insight into the mechanism by which a life-long viral infection may give rise to leukemia, lymphoma, or perhaps to no disease at all. Rowe *et al.* (24) have demonstrated that type C viral particles in the AKR mouse are most heavily concentrated in the bone and uterus. The presence of virus in bone is either the result or the cause of infection of a primitive bone marrow cell, which is capable of differentiating into lymphocytic, granulocytic, megakaryocytic, and erythrocytic precursors. Early in the life-time of the host, a response to infection occurs. This response is manifested not only in WBC but also in RBC, platelets, and perhaps in other cells and organs. The neoplasm that appears may represent the response of the immune system to the virus or to the altered cells (21). For example, in 1961, Gross (17) reported that thymectomized C3H mice infected with Passage A virus developed granulocytic leukemia, whereas intact C3H mice developed lymphoma.

The data reported herein and the work mentioned above suggest that viral infection of hematopoietic stem cells occurs early in the pathogenesis of murine leukemia. In the AKR mouse, subtle alterations in RBC and platelet metabolism occur many weeks before lymphoma can be diagnosed. The lymphoma that occurs in the older animal may be a reaction to this infection. Were the lymphoma prevented, then the ultimate expression of leukemogenesis

might be granulocytic leukemia. By analogy, the data suggest that the search for putative leukemia viruses in human beings should be directed to cells other than WBC and at times other than the final expression of the disease.

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