The Effect of a Leukemia Virus on Thrombopoiesis

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SUMMARY

The effect of virus titer and duration of infection with Rauscher virus on thrombopoiesis in BALB/c mice was investigated. Splenectomized mice were infected with $10^{-1}$ to $10^{-3}$ dilutions of Rauscher virus. Platelet counts were obtained at 2-week intervals. Two and then 10–24 weeks after infection mice with thrombocytopenia were killed and femur marrow imprints prepared. In a second group of mice platelet survival curves were obtained by cohort labeling with selenomethionine ($^{75}$Se) 4 and 7 days after infection with Rauscher virus and compared to uninfected controls. Two weeks after infection, Rauscher virus caused a virus titer-dependent thrombocytopenia which was potentiated by splenectomy. The platelet count rose 4–6 weeks after infection, reached a plateau, and again fell when the mice were dying. The degree of recovery in platelet count was greater and the severity of the secondary thrombocytopenia less with the lower viral doses. At 2 weeks the bone marrow was hypercellular with megakaryocytic hyperplasia, whereas later (10 to 24 weeks) the marrow was infiltrated with leukemic cells and megakaryocytes were absent. Platelet survival was decreased 4 and 7 days after infection. The early reversible thrombocytopenia is a preleukemic manifestation due to a direct effect of Rauscher virus on megakaryocytes, whereas the late thrombocytopenia is secondary to marrow infiltration with leukemic cells.

INTRODUCTION

Rauscher virus leukemia is associated with a preleukemic phase characterized by splenomegaly, anemia, thrombocytopenia, lymphocytosis, mononcytosis, and granulocytosis (5, 16). Splenectomy before infection modifies the disease in that the resultant thrombocytopenia and anemia are more severe and leukocytosis does not occur two weeks after infection (5). In nonsplenectomized mice cortisol therapy like splenectomy can temporarily suppress the early leukocytosis (6). Erythroid kinetic studies indicate that Rauscher virus infection causes a hemolytic anemia which, early in infection, is associated with a depression in erythropoiesis, particularly in splenectomized mice (5).

The pathogenesis of the early thrombocytopenia and its relationship to the ultimate development of leukemia has not been investigated. Electron microscopy studies have demonstrated that the murine leukemia viruses are characteristically present in the cytoplasm of megakaryocytes and platelets (8, 12). Thrombocytopenia is one of the most sensitive indices of early virus infection and can be used in dose-response curves to titer both Friend and Rauscher viruses (5, 9, 10). In the present study the effects of Rauscher virus infection on thrombopoiesis were investigated. The platelet count was followed serially in splenectomized BALB/c mice infected with ten-fold dilutions of Rauscher virus. Bone marrow preparations were obtained in the preleukemic phase and in the subsequent leukemic phase in order to determine the number of megakaryocytes and degree of infiltrations with leukemic cells. Additionally, in the preleukemic state, platelet survival was investigated by labeling of platelets with selenomethionine ($^{75}$Se) (7, 15).

The study demonstrates that thrombocytopenia following Rauscher virus infection is biphasic, i.e., an early spontaneously reversible preleukemic thrombocytopenia followed by a secondary thrombocytopenia associated with infiltration of the bone marrow by leukemic cells and death of the mouse.

MATERIALS AND METHODS

Virus

Rauscher virus has been maintained in the laboratory by serial passage of clarified spleen suspensions. The preparation and storage of the splenic suspensions were performed by methods previously described (5). The virus titer was determined by a graded assay technic. Using this method the expression of potency of virus preparations as determined by the dose-response assay titer was arbitrarily defined as the dilution of the virus preparation which would cause the platelet count to fall to 500,000 per cu mm 2 weeks after infection with Rauscher virus. This titer is referred to as the Platelet Depressant Dose 500,000 (PDD 500,000) (5, 10). In the present experiments the titer of the virus preparation was PDD 500,000 3.2.

Virus Inoculum

Preparation of virus inoculum was carried out as follows: A frozen fraction of virus concentrate was diluted with an equal
volume of sterile 0.9% saline. Serial ten-fold dilutions of virus concentrate were prepared in chilled 0.9% saline solution and the dilution tubes maintained in an ice bath. Two-tenths ml of the various dilutions (10^{-1} to 10^{-3}) were inoculated i.v. into the tail veins of BALB/c mice. Ten mice were used per dilution. In the platelet survival studies the virus inoculum was 10^9.

Mice

BALB/c weanling females (16–18 gm) obtained from the Cumberland Farms, Clinton, Tennessee were maintained in plastic cages, 10 per cage and fed Purina laboratory chow, micromix pellets, and water ad libitum. Mice were splenectomized by previously described procedures (3, 5). The splenectomized mice were allowed to recover for 4 weeks before being used for experiments.

Hematologic Studies

Blood for determination of the hematologic data was obtained by puncture of the retroorbital venous plexus with a heparinized capillary tube (3, 4). Platelets were counted directly by the method of Brecher and Cronkite (2). In selected animals the total white blood cell count and differential were obtained.

Experiment No. 1: Effect of Virus Titer and Duration of Infection on the Platelet Count and Survival of Splenectomized BALB/c Mice. Ten nonsplenectomized and 10 splenectomized mice were inoculated with 10^{-1} dilution of Rauscher virus. Platelet counts were obtained by serial bleeding before and then 2, 7, and 14 days after virus inoculation.

Thirty splenectomized mice were inoculated with 10^{-1} to 10^{-3} dilutions of Rauscher virus. Ten mice were used per dilution and platelet counts were determined in all surviving mice at 2-week intervals. The experiment was terminated at 24 weeks, at which time there were no survivors for the 10^{-1} and 10^{-2} dilutions and only 4 survivors at the 10^{-3}.

Experiment No. 2: Bone Marrow Morphology. Forty-five splenectomized mice were inoculated with 10^{-1} to 10^{-3} dilutions of Rauscher virus (15 mice were used per dilution), and serial platelet counts, white blood cell counts and differentials were obtained. The 15 mice at the 10^{-1} dilution were killed 2 weeks after infection. Bone marrow imprints were prepared from the femur and stained with Wright’s-Giemsa. In this group all platelet counts were less than 100,000 per cu mm, and the leucocyte count was not in excess of 10,000 per cu mm. At the 10^{-2} and 10^{-3} dilutions 15 mice were sacrificed 10 to 24 weeks after infection. Femur bone marrow imprints were obtained as above. Those mice which were sacrificed had white blood cell counts greater than 50,000 per cu mm and platelet counts less than 400,000 per cu mm.

Experiment No. 3: Platelet Survival Studies. Platelet survival was determined in control noninfected nonsplenectomized and splenectomized mice and infected (10^9 Rauscher virus) nonsplenectomized and splenectomized mice. Platelets were labeled with selenomethionine (75Se) (7, 15). For each experiment 40 control and 40 experimental mice were injected i.v. with 1 μc in 0.25 ml of 0.9% saline of selenomethionine (75Se) (Sethotope, ® Squibb; specific activity 1 mc/mg). In the infected group the isotope was injected either 4 or 7 days after infection with Rauscher virus. Thereafter 3 mice were sacrificed daily from the control and experimental group by bleeding from the retroorbital venous plexus. The blood was pooled and the platelet volumes from the control and experimental groups obtained by the following method, which was modified from that of Cohen et al. (7). Three ml of whole blood were added to 2.5 ml of 1% ethylenediaminetetraacetic acid in 0.7% saline. The blood was then filtered through gauze and the total platelet count obtained. Platelet rich plasma was then obtained by centrifugation for 14 min at 200 × g in an International Centrifuge. The top 2 ml of this platelet containing plasma were then centrifuged for 5 minutes at 400 × g in order to remove contaminating red and white blood cells. The supernatant was then centrifuged for 30 minutes at 2000 × g. The platelets were washed 3 times with 0.9% saline and the platelet button was then resuspended and the radioactivity counted in a well-type scintillation counter. The cpm were always corrected to the total platelet count obtained 24 hours after injection of the isotope. The platelet survival curves were plotted on arithmetic paper with the percent reappearance of 75Se in the circulating platelets on the ordinate and time in days on the abscissa. Results were graphed as percent of highest platelet button radioactivity. The interval from the 50% point on the ascending to the 50% point on the descending slope were considered to represent the platelet survival time in days.

RESULTS

Effect of Virus Titer and Duration of Infection on Platelet Count and Survival

Rauscher virus infection (10^{-1}) caused a significant decrease in the platelet count in both nonsplenectomized and splenectomized BALB/c mice within 48 hours after infection. The platelet count reached its nadir approximately 14 days after infection. Prior to infection the platelet count was higher in the splenectomized mice, but after infection the count was significantly lower at all time intervals in the splenectomized group than in the nonsplenectomized group (Chart 1).

The effects of 10^{-1} to 10^{-3} dilutions of Rauscher virus on the platelet count and survival in splenectomized BALB/c mice are presented in Table 1. The median value for platelet counts are plotted graphically in Chart 2. The severity of the thrombocytopenia at 2 weeks was related to the virus titer and therefore to the virus dose. The thrombocytopenia was more severe with the higher viral doses. Similarly, survival time was related to viral dose in that the mice given the greater doses had shorter median survivals, i.e., 10^{-1}, 48 days; 10^{-2}, 82 days; 10^{-3}, 148 days (Chart 3).

Partial to almost complete recovery in the platelet count occurred 4 to 6 weeks after infection. The degree of recovery was greater with the lower virus doses (Table 1; Chart 2). At 10^{-1} titer all animals were dead 6 to 8 weeks after infection. At this time their platelet counts were higher than at 14 days but still significantly depressed when compared to normals (Table 1; Chart 2). The platelet counts of mice given 10^{-2} or 10^{-3} titer plateaued and then showed a secondary decline.
Chart 1. The effect of $10^{-1}$ dilution of Rauscher virus on the platelet count 0, 2, 7 and 14 days after infection. Nonsplenectomized and splenectomized BALB/c mice were serially bled, and each point represents the mean of 10 mice.

Chart 2. The effect of $10^{-1}$ to $10^{-3}$ dilutions of Rauscher virus 2 to 24 weeks after infection of splenectomized BALB/c mice. Mice were serially bled at 2-weeks intervals, and each point represents the median platelet count of the surviving mice at each interval.

Chart 3. The effect of virus titer on the survival of BALB/c mice infected with $10^{-1}$ to $10^{-3}$ dilutions of Rauscher virus (RV). The median survival for $10^{-1}$ was 48 days; $10^{-2}$ 82 days; and $10^{-3}$ 148 days.
prior to death (Table 1; Chart 2). At the $10^{-2}$ titer all mice were dead 12 to 14 weeks after infection. Although the data indicate a wide scatter in platelet counts with some mice developing thrombocytosis and other thrombocytopenia, the general trend is apparent from the median values, and approximately 50% of the mice developed a secondary thrombocytopenia 10 to 24 weeks after infection.

### Effect of Rauscher Virus on Bone Marrow Morphology

The bone marrows obtained from normal splenectomized mice were cellular, with approximately 2–4 megakaryocytes per low power field. The myeloid erythroid ratio was approximately 4:1. The predominant cell of the myeloid series was a mature granulocyte which in the mouse has a characteristic doughnut-shaped nucleus. Most of the cells of the erythroid series were late normoblasts with dark pyknotic nuclei. Lymphocytes numbered less than 10 percent of nucleated cells.

Two weeks after infection the bone marrow morphology was similar to the control marrow. The marrow was hypercellular. The morphologic appearance of megakaryocytes was normal, but their numbers appeared increased (4–6 megakaryocytes per low power field). The myeloid erythroid ratio remained the same. Myeloid and erythroid maturation was normal. There was no infiltration with either lymphocytes or abnormal mononuclear cells.

Bone marrows obtained from mice late in infection were dramatically different from controls and marrows obtained from mice two weeks after infection. The marrows were cellular. The megakaryocytes numbered less than one per low power field. The number of mature granulocytes was greatly reduced and more than 50 percent of cells were immature mononuclear cells having the appearance of lymphoblasts and prolymphocytes. Normoblastic erythroid activity was present but depressed.

### Platelet Survival

Table 2 and Charts 4–7 show platelet survival data. The platelet survival of normal nonsplenectomized and splenectomized mice varied between 3.0 and 5.75 days (Table 2). In nonsplenectomized mice 4 days after infection, platelet survival was reduced with results varying between 1.5 and 2.5 days (Table 2; Chart 4). In splenectomized animals, a similar

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*Platelet count x $10^{-3}$ per cu mm.*
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Survival in days of platelets labeled with 75Se 4 and 7 days after infection with Rauscher virus.
a 75Se injected 4 days after infection with Rauscher virus.
b 75Se injected 7 days after infection with Rauscher virus.
c N.C., not calculated; see text.
d Data presented graphically in Charts 4—7.

reduction in survival was seen when compared to control (Table 2; Chart 5). Seven days after infection platelet survival curves in nonsplenectomized mice were difficult to interpret because in all experiments a plateau in radioactivity appeared for the first 3 to 4 days which was followed by a rise and then a rapid decline in radioactivity (Chart 6). These data suggested a multicompartamental curve and made mathematical calculation of survival time impossible. Decrease in platelet survival was most marked in splenectomized-animals 7 days after infection. Survival time in these animals varied between 1.5 and 2.0 days (Table 2). The plateau noted in the intact animals was not seen in the splenectomized group (Chart 7).

DISCUSSION

The results indicate that the early preleukemic thrombocytopenia is dose (virus titer)-dependent, reversible, and associated with megakaryocytic hyperplasia without evidence of leukemic infiltration of the bone marrow. Platelet survival studies at this time indicated a decrease in survival.

Platelet survival studies were performed by the in vivo labeling of platelets with selenomethionine (75Se). The survival in normal mice is approximately 4 days. Using radioactive sodium sulfate (Na235SO4) as the platelet label, Odell and McDonald obtained a platelet survival of 4 days in BDF1 mice (14). With 75Se labeling the platelet survival in human beings is 9 to 11 days and is in the range of platelet survivals obtained with other radioisotopes, i.e., 51Cr and 32P-labeled diisopropylfluorophosphate (13). Therefore, our data indicate that selenomethionine is an excellent platelet label and will provide a valid estimate of platelet survival in mice.

The results with 75Se in infected nonsplenectomized and splenectomized mice demonstrate a consistent decrease in platelet survival 4 days after infection. A similar decrease in survival in splenectomized mice 7 days after infection is also present. The platelet survival curve in nonsplenectomized mice 7 days after infection is difficult to interpret. The percent reappearance of 75Se in platelets plateau for 3 to 4 days, then rises sharply to the peak level, after which rapid decline of radioactivity occurs. This early plateau may represent a decrease in release of platelets from the megakaryocytes or platelet sequestration by the enlarged spleen with subsequent release of the sequestered platelets. Following splenectomy in the thrombocytopenia is more severe, and the early plateau in the platelet survival curve is abolished. There appears to be no interference with the incorporation of 75Se into the megakaryocytes of infected splenectomized mice because the peak radioactivity of platelets is similar to that of controls. This implies that the megakaryocytes are capable of producing platelets but that the platelets produced have a decreased survival.

At this time adequate numbers of megakaryocytes are present in the bone marrow. The platelet survival studies are analogous to red blood cell survival and ferrokinetic studies following infection with either Rauscher virus or Friend virus (3, 5). The early anemia is primarily due to a decreased survival of red blood cells and this anemia, like the thrombocytopenia, is potentiated by splenectomy (3, 5).

Splenectomized mice rather than nonspienectomized mice were used in the serial platelet and bone marrow studies in order to avoid the early mortality peak associated with splenic rupture and the modifying influence that the spleen has on the course of Rauscher leukemia (5, 17). This and previous studies suggest that the enlarged spleen can ameliorate the severity of

Platelet Survival
Nonsplenectomized Mice

3.5 Normal

2.5 6-DAY-INFECTED

Chart 4. The effect of 10⁶ Rauscher virus infection on platelet survival in nonsplenectomized BALB/c mice. Platelets were labeled with 75Se 4 days after infection. Each point represents the percent reappearance of 75Se in the platelets obtained from the pooled blood of three mice. Results were compared to normal uninfected controls.
Leukemia Virus and Thrombopoiesis

There is no evidence of leukemic cell infiltration of the bone marrow. The later thrombocytopenia is associated with leukemic cell infiltration of the marrow and reduced numbers of megakaryocytes. Presumably this secondary thrombocytopenia is due to destruction of the megakaryocytes by the infiltrating leukemic cells. The possibility exists that persistent Rauscher virus infection of megakaryocytes may also cause megakaryocytic destruction. In human leukemia thrombocytopenia is generally associated with leukemic cell infiltration of the marrow (11). Human preleukemic states may be associated with thrombocytopenia with normal to increased numbers of megakaryocytes in the bone marrow (1).

Reversible thrombocytopenia is an important and sensitive preleukemic manifestation of infection with Rauscher virus (5, 9, 10). Many human virus infections and such disease states as idiopathic thrombocytopenic purpura are also associated with reversible thrombocytopenia. Is it possible that in some instances thrombocytopenia is caused by a leukemogenic agent? In human beings the latent period between this initial, apparently benign, thrombocytopenia and the subsequent development of leukemia may be long, extending over a period of months or perhaps years rather than weeks as in

the initial anemia and thrombocytopenia by increasing its own production of red blood cells and platelets (5). The above is not surprising since in the mouse the spleen is normally an active hematopoietic organ analogous to bone marrow (4). Splenectomy also modifies the white blood cell response to infection by delaying the early development of leukocytosis, lymphocytosis, monocytosis, and granulocytosis (5).

In splenectomized mice the early thrombocytopenia is spontaneously reversible with the platelet counts returning to normal limits 28 to 42 days after infection with 10^{-2} or 10^{-3} dilutions of Rauscher virus. A correlation was noted between severity of thrombocytopenia 2 weeks after infection and length of survival. The higher virus doses caused a more severe thrombocytopenia with a shorter survival time. If early death does not occur, surviving mice develop leukocytosis with white blood cell counts frequently in excess of 50,000 per cu mm. The majority of these cells are immature lymphocytes. The bone marrow shows infiltration with immature mononuclear cells and a decrease in mature granulocytes and megakaryocytes. At this point thrombocytopenia again develops followed by death.

The thrombocytopenia after Rauscher virus infection is biphasic in nature. The initial reversible thrombocytopenia is apparently due to a direct effect of Rauscher virus on megakaryocytes and platelets producing shortened platelet survival. There is no evidence of leukemic cell infiltration of the bone marrow. The later thrombocytopenia is associated with leukemic cell infiltration of the marrow and reduced numbers of megakaryocytes. Presumably this secondary thrombocytopenia is due to destruction of the megakaryocytes by the infiltrating leukemic cells. The possibility exists that persistent Rauscher virus infection of megakaryocytes may also cause megakaryocytic destruction. In human leukemia thrombocytopenia is generally associated with leukemic cell infiltration of the marrow (11). Human preleukemic states may be associated with thrombocytopenia with normal to increased numbers of megakaryocytes in the bone marrow (1).

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The thrombocytopenia after Rauscher virus infection is biphasic in nature. The initial reversible thrombocytopenia is apparently due to a direct effect of Rauscher virus on megakaryocytes and platelets producing shortened platelet survival.

Chart 5. The effect of 10^9 Rauscher virus infection on platelet survival in splenectomized BALB/c mice. Platelets were labeled with ^75Se 4 days after infection. Each point represents the percent reappearance of ^75Se in the platelets obtained from the pooled blood of three mice. Results were compared to normal uninfected controls.

Chart 6. The effect of 10^9 Rauscher virus infection on platelet survival in nonsplenectomized BALB/c mice. Platelets were labeled with ^75Se 7 days after infection. Each point represents the percent reappearance of ^75Se in the platelets obtained from the pooled blood of three mice. Results were compared to normal uninfected controls. In this group the platelet survival of infected mice was not calculated because the survival curve was unsuitable for analysis. See text.
Chart 7. The effect of 10^6 Rauscher virus infection on platelet survival in splenectomized BALB/c mice. Platelets were labeled with 75Se 7 days after infection. Each point represents the percent reappearance of 75Se in the platelets obtained from the pooled blood of three mice. Results were compared to normal uninfected controls.

mice. Careful epidemiologic studies, particularly in children with thrombocytopenia following virus infections, may now be indicated.

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