Virus Induced Aplastic Crisis in Mice

By SENIH M. FIKRIG AND SUMNER BERKOVICH

APLASTIC CRISIS, a rare complication of the hereditary hemolytic anemias, is characterized by disappearance of reticulocytes from the peripheral circulation and maturation arrest of red cell precursors in bone marrow. Anemia develops due to both absence of red cell production and rapid elimination of defective cells. White blood cells and platelets remain normal. The process is self-limited and recovery is usually complete. A similar complication is erythropoiesis has also been observed in individuals without an underlying hematologic disorder. In these patients, erythropoiesis is basically normal; thus, the acute aplastic crisis is not associated with significant anemia and is rarely recognized.

The etiology of the aplastic crisis in people with hemolytic disease and in patients without a hematologic disorder is usually unknown. Since the changes are frequently preceded by non-specific infections thought to be viral, the question arises as to the role of viral agents in the pathogenesis of the aplastic crisis. An experimental animal model would add support to this viral hypothesis and could result in an understanding of the pathogenesis of the aplastic crisis.

For this purpose we have studied two inbred strains of mice, NZB/B1 with genetically determined autoimmune hemolytic anemia and CBA/T6 animals without hematologic disorders. Both strains have been investigated, following infection with various enteroviruses. One of the agents used, Coxsackie B virus, type 4, caused changes in peripheral blood and bone marrow similar to those described in humans with aplastic crisis. The findings indicate that viral infection can be etiologically related to the depression of red cell production in mice and may be similarly involved in the aplastic crisis observed in man.

MATERIALS AND METHODS

Experimental Animals

Mice from inbred strains NZB/B1 and CBA/T6 were used. All mice of NZB/B1 strain

From Department of Pediatrics State University of New York Downstate Medical Center, Brooklyn, N. Y.

This investigation was supported in part by USPHS Grant 5R01 AM10710-02 and in part by the Health Research Council of the City of New York, Contract No: U-1151

Presented in part at the Sixth Meeting of the Career Scientist of the Health Research Council of the City of New York, December 1967.

First submitted June 28, 1968; accepted for publication November 4, 1968.

SENIH M. FIKRIG, M.D.: Assistant Professor of Pediatrics, State University of New York, Downstate Medical Center, Brooklyn, N. Y. Recipient of Career Scientist Award, Health Research Council of City of New York, Contract I-330. SUMNER BERKOVICH, M.D.: Associate Professor of Pediatrics, State University of New York, Downstate Medical Center, Brooklyn, N. Y. Recipient of Career Scientist Award, Health Research Council of City of New York, Contract I-173.

582 BLOOD, VOL. 33, NO. 4 (APRIL), 1969
VIRUS INDUCED APLASTIC CRISIS

spontaneously develop several parameters of autoimmune disease during adult life. These parameters are Coombs positive autoimmune hemolytic anemia, presence of thymic germinal centers and kidney lesions similar to those seen in systemic lupus erythematosus. The test animals were obtained from established colonies maintained by proper inbreeding. Their ages ranged between 4-22 months. They were caged in a temperature-controlled room (72-76 F.) with 12 hour alternating cycles of light and darkness. No cage contained more than 3 animals of one sex. Unlimited quantities of food and water were provided.

Virus

The Coxsackie B4 virus was originally recovered from a newborn infant with encephalomyocarditis. The virus pool was prepared in rhesus monkey renal cell cultures maintained with medium 199 without added serum. Aliquots of cell free tissue culture fluid were stored at −5 C. The final product contained approximately $10^{5.5}$ TCID$_{50}$ per 0.1 cc. Control fluids were prepared from uninfected monkey renal cell monolayers maintained with medium 199. Each animal received an intraperitoneal injection of 0.1 cc. of the control fluid or the virus inoculum.

Hematologic Studies

Hematocrit (Hct), white blood count (WBC), and peripheral blood smears for differential and for reticulocyte counts were done according to standard hematological procedures. The smears were stained with 1 per cent solution of brilliant cresyl blue, dried and counterstained with Wright's stain. Platelets were evaluated directly from the smears. Bone marrow was exposed by splitting the femur longitudinally. Marrow smears were made with a 00 hair brush dipped in human or mouse serum. The fresh preparations were immediately dried and stained with benzidine and counterstained with May-Gruenwald-Giemsa. Five thousand cells were counted.

Virologic Studies

Stool specimens were examined in trypsinized cultures of primary rhesus monkey renal cells for presence of cytopathic agents. Neutralization tests were done by mixing serum dilutions in equal parts with a virus inoculum containing 100, 50 per cent tissue culture infectious doses (TCID$_{50}$).

Histologic Technic

Pancreatic tissues from virus infected and uninfected control animals were fixed in 10 per cent neutral formalin, embedded in paraffin, cut into 6 microns in thickness and were then stained with hematoxylin and eosin. All preparations were evaluated under the light microscope.

Study Plan

Mice were randomly assigned to a test group. Prior to infection with the Coxsackie B4 test agent, blood specimens were collected from the retro-orbital plexus using heparinized micro-hematocrit tubes. Second blood specimens were similarly obtained 2 to 3 weeks later. Neutralizing antibodies were determined on paired specimens from individual animals. Within the week prior to infection, Hct, WBC, and reticulocyte determinations were done on each mouse. Following virus inoculation, reticulocyte counts were done daily. Virus-free control animals were similarly tested. White blood counts, peripheral blood smears and Hcts were repeated at least once before the end of each experiment. Bone marrow preparations were done from various infected animals with reticulocytopenia and from a comparable number of normal controls. Stools for viral culture were collected 3 to 4 days after infection and again 6 to 7 days later. The experiment was terminated and the animals were sacrificed after the convalescent blood specimens were obtained.

RESULTS

Two to three days following infection with Coxsackie B4 virus, 7.3 per cent
of the NZB/B1 mice (average value for a total of 96 animals) and 60.3 per cent of CBA/T6 animals (average value for 118 mice), showed a precipitous drop in the number of reticulocytes in their peripheral blood (Tables 2 and 3). This drop ranged from an initial level of 20 to 50 per thousand in NZB/B1 mice and 15 to 25 per thousand in the CBA/T6 animals to 0 to 3 per thousand in both strains. The crisis lasted from 3 to 12 days and was followed by complete hematologic recovery (Figs. 1 and 2). No changes were observed in either the peripheral blood or platelet counts. If the crisis was only of short duration, hematocrit was not affected; if prolonged however, there was a significant drop. In uninfected CBA/T6 mice the hematocrit varied from 51 to 69 per cent (Mean: 58 per cent). Following infection the levels in animals with prolonged reticulocytopenia ranged from 31 to 49 per cent (Mean: 41 per cent). In the NZB/B1 strain the hematocrit varied between 34 to 60 per cent. On 2 out of 7 animals that developed aplastic crisis the hematocrit dropped from a preinfection level of approximately 50 per cent to a postinfection reading of 40 per cent.

At the height of the crisis, bone marrow from a representative group of 12 mice (8 CBA/T6 and 4 NZB/B1) was investigated and compared with bone marrow findings observed in 13 normals (7 CBA/T6 and 6 NZB/B1). Table 1 summarizes changes that occurred in the red cell precursors. The pronormoblasts and normoblasts did not completely disappear, but there was a four to sixfold drop in their total number (fourfold in CBA/T6 and sixfold in NZB/B1).

Of 118 infected CBA/T6 mice (61 females and 57 males) 71 (34 females and 37 males) had aplastic crisis. The incidence of crisis was independent of
both age and sex. None of the 35 control animals (19 females and 16 males) became sick. Results are summarized in Table 2. By contrast, among the 96 infected NZB/B1 animals (51 females and 45 males) only 7 females, all between the ages of 4 to 8 months, showed evidence of aplasia. Thirty-three comparable control animals (13 females and 20 males) and 38 infected animals aged 9 to 20 months remained hematologically normal. These findings are summarized in detail in Table 3.
Table 3.—Frequency of Aplastic Crisis in Coxsackie B<sub>4</sub> Infected and Uninfected NZB/B1 Mice

<table>
<thead>
<tr>
<th>Age in Months</th>
<th>No. of Animals</th>
<th>Aplastic Crisis</th>
<th>Per Cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4–8</td>
<td>Male 27</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Female 21</td>
<td>7</td>
<td>33.3</td>
</tr>
<tr>
<td></td>
<td>Male 18</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Female 30</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9–20</td>
<td>Male 15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Female 9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Male 5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Female 4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Uninfected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4–8</td>
<td>Male 15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Female 9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9–20</td>
<td>Male 5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Female 4</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The Coxsackie B<sub>4</sub> virus was cultured from stool obtained from 57 of 65 (87.7 per cent) of the animals tested. The cultures were positive by the third or fourth day, following the intraperitoneal inoculation of virus. An occasional stool yielded virus as late as the ninth day after infection. The test agent was never cultured from stool samples obtained from uninfected control animals.

A significant rise in Coxsackie B<sub>4</sub> neutralizing antibody from levels of less than 1:8 to 1:32 or above was demonstrated in convalescent sera obtained from 69 animals investigated (71 per cent). None of the control animals tested had measurable antibody at the lowest dilution tested (1:8).

Histologic studies of pancreas from virus infected animals revealed diffuse destruction and fibrosis. Lesions were limited to the acini; the islets of Langerhans were not affected.

**COMMENTS**

In 1948 Owren was the first to describe the acute aplastic crisis in association with congenital spherocytosis. His patient developed a sudden severe anemia of unknown etiology. Since the initial report, various authors have stressed the role of infectious agents in precipitating aplastic episodes in patients with chronic hemolytic disease. Some of these investigators implicated bacterial agents such as salmonella; others found a causal relation between acute erythroblastopenia and infections such as infectious mononucleosis and atypical viral pneumonia. Leikin observed two families in which several members had an aplastic crisis at the same time. Dameshek and Bloom made similar observations in a different family. These authors suggested the possible role of infection in triggering or precipitating the aplastic outbreaks they observed. In contrast, Chernoff and Josephson described a number of sickle cell patients with acute erythroblastopenia and failed to isolate or implicate a specific infectious agent.

The aplastic crisis we have described in the NZB/B1 test mice infected with Coxsackie B<sub>4</sub> virus was very similar to episodes that have been described in patients with hemolytic anemia. Reticulocytes disappeared from peripheral smears and the hematocrit dropped appreciably. Recovery was spontaneous. However, in contrast to the human, normoblasts and pronormoblasts did not completely disappear from the bone marrow of the infected mouse. Also, the
changes in the test animals were both age and sex dependent; only females from 4 to 8 months of age had aplastic crisis. In humans, males and females of all ages were affected.

Gasser\textsuperscript{2,13} has described the aplastic crisis in normal subjects who had no underlying hematologic disease. He suggested that the causative factor could be any of a number of unrelated drugs or that the hematologic changes might follow either viral or bacterial infection or a surgical procedure. Such cases are seldom recognized because anemia may not develop.

The aplastic crisis we observed in our CBA/T6 mice occurred in animals that were known to be without hematologic disease. The findings, therefore, were analogous to those of Gasser for the human. Many of the animals had a reticulocytopenia and aplastic crisis of short duration which would not have been recognized if only the hematocrit values were measured. However, although diminished in number, normoblasts and pronormoblasts were still present in the bone marrow of animals with aplastic crisis. Moreover, giant proerythroblasts described by Gasser in the human subject were not present in the Coxsackie B\textsubscript{4} infected CBA/T6 mice. In contrast to NZB/B1 animals, the occurrence of aplastic crisis was independent of both sex and age.

The causal relationship between the Coxsackie B\textsubscript{4} virus infection and aplastic crisis in our experimental mice has been established beyond doubt. The crisis occurred only in infected animals. Moreover, mice that recovered from the initial Coxsackie B\textsubscript{4} infection, were not affected hematologically by reinoculation with the same agent, although careful studies were done to detect the reoccurrence of signs of aplasia. The mechanism by which the virus induced the aplastic crisis is unknown. Venezuelan equine encephalitis\textsuperscript{14} and dengue fever\textsuperscript{15} have been associated with pancytopenia with marked hypoplasia of the bone marrow. Both murine and human hepatitis have also been associated with bone marrow failure in both mice and man.\textsuperscript{16,17} In the former, the high virus concentration measured in bone marrow and in lymphopoietic tissues suggests that the hematopoietic changes may be a direct viral effect. On the other hand, it is possible that toxic metabolic products produced during viral multiplication could affect hematopoietic cells by depressing or blocking essential enzymatic pathways or accessibility to nutritional products essential for erythrocyte maturation.

**Summary**

Following infection with Coxsackie B\textsubscript{4} virus, 2 inbred strains of mice, NZB/B1 with autoimmune hemolytic anemia and CBA/T6 without hematologic disorder, developed changes in peripheral blood and in bone marrow similar to those described in aplastic crisis in man. A marked decrease or complete disappearance of reticulocytes from the peripheral blood was associated with a decrease in the total number of red cell precursors in the bone marrow. Both findings were temporary; recovery was spontaneous. The hematologic findings were independent of both age and sex in the CBA/T6 animals. By contrast, in the NZB/B1 strain only females between the ages of 4–8 months developed hematologic abnormalities.
The etiologic relationship that has been established between Coxsackie B4 virus infection and aplastic crisis in the test mice suggests that viral agents may play an etiologic role in the pathogenesis of aplastic crisis in man.

SUMMARIO IN INTERLINGUA

Post infection con virus Coxsackie B4, lineas inbred de muses NZB/B1 con autoimmun anemia hemolytice e de muses CBA/T6 sin disordine hematologic disveloppava alterationes in le sanguine peripheric e in le medulla ossee simile a illos describite in crise aplastic in humanos. Un marcate declino o le complete disparentia de reticulocytes ab le sanguine peripheric eseva associate con un declino in le numero total de precursors erythrocytic in le medulla ossee. Ambe iste constataiones eseva temporare, e le restablimento eseva spontanee. Le constataiones hematologic eseva independente de etate e sexo in le caso del muses CBA/T6. Per contrasto con isto, in le caso del linea NZB/B1, solo femininas de etates de inter 4 e 8 menses disveloppava anormalitates hematologic.

Le relation etiologic que ha esiste establite inter infection a virus Coxsackie B4 e crise aplastic in le muses experimental suggestiona que agentes viral ha un rolo in le pathogenese de crise aplastic in humanos.

ACKNOWLEDGMENTS

The authors wish to thank Mr. Kenneth Dummett, Miss Etta M. Jackson, and Mrs. Ruth Hyatt for technical assistance.

ADDENDUM

Since this report was submitted we have seen one patient with sickle cell anemia and aplastic crisis, whose stool specimen yielded an ECHO virus type 18. A significant increase in neutralizing antibodies to this agent was measured in his convalescent serum specimen. We believe this the first clear cut association of a specific viral infection with the aplastic crisis in man.

REFERENCES

12. Chernoff, A. I., and Josephson, A. M.:


Virus Induced Aplastic Crisis in Mice

SENIH M. FIKRIG and SUMNER BERKOVICH