Anemia Virus as a Distinct Component of the Murine Leukemia-Sarcoma Complex of Viruses

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SUMMARY

The severe infectious anemia in rats previously associated with stocks of Abelson virus is demonstrated to be due to an infectious, replicating particle the size and density of a virus. This anemia virus is separable from Abelson virus by passage in rats.

INTRODUCTION

In a recent report, we described a fulminant anemia in rats that were inoculated at birth with our stocks of Abelson virus (3). This anemia was apparent within 2 weeks, and many rats died shortly thereafter with a red cell count as low as 1 x 10⁶ cells/cu mm. Passage of the anemia from rat to rat was accomplished with cell-free material, and electron micrographs of the erythroblasts showed numerous C-type particles characteristic of the murine leukemia-sarcoma complex (2). We report now evidence that the anemia-inducing principle is separable from that inducing stem-cell leukemia, that this anemia virus is a distinct entity, and that it is a member of the leukemia-sarcoma complex of murine viruses.

MATERIALS AND METHODS

Origin of Virus Stock. Our initial virus stock (designated as “Abelson virus”), was kindly supplied by Dr. Louise Rabstein, MBA, Walkersville, Md. Virus stock was passaged in our laboratory by inoculation into newborn random-bred albino mice. At about 25 days, most inoculated mice had developed tumors of the stem-cell Abelson type (2). A 20% w/v suspension of these tumors was prepared in 0.9% NaCl solution with a Teflon grinder. For passage, virus stock was clarified by centrifugation (10,000 x g for 5 min) and stored sealed in glass at —70°.

Source of the Anemia Agent. The anemia virus may be presumed to have been present either in the Abelson virus stock obtained from MBA or to have been present in 1 or more of our mice used for passage of the Abelson virus. It was previously reported by us that the Rabstein-MBA stocks of Abelson failed to produce anemia in rats (2). Since this anemia virus is present in only moderate titer compared to the Abelson virus or the thymic lymphoma virus, it was possible that the method of preparation of the virus used at MBA might have inactivated the anemia principle or lowered its titer sufficiently so that the anemia was no longer apparent. Accordingly, in order to compare the anemia-producing activity of our virus stocks directly with that of Rabstein-MBA origin, we prepared lots of virus stock by the same citrate concentration method.

RESULTS

Each newborn rat received 0.2 ml of 1 of the 2 citrate virus concentrates diluted 1:1 in 0.9% NaCl solution. At 20 days of age, all animals were bled, killed, and autopsied. In addition to a complete hemogram, the presence of lymph node cysts, splenomegaly, and histological evidence of erythroblastosis was noted. The results are given in Table I.

The data indicate that virus present in our citrate preparation was able to produce definite disease. The Rabstein-MBA stocks were uniformly negative in this respect by histological and hematological parameters, indicating that the anemia virus is not merely a contaminant of Moloney virus-related stocks. As a positive control for this experiment, both the MBA and CCRF stocks of virus were inoculated into mice to determine whether they still possessed Abelson leukemia activity. With the use of a 30-day end-point assay, 6 of 17 mice inoculated with the MBA virus were positive and 5 of 6 mice inoculated with the CCRF virus were positive, indicating that both stocks contained infectious Abelson virus.

In order to determine whether the anemia virus arose by...
passage in our strain of mice, we prepared virus from the 6 leukemic mice of 17 inoculated with Rabstein-MBA stocks. This mouse-passaged MBA virus was then inoculated into 17 newborn rats. At 25 days of age these and 7 uninoculated control rats were bled for a hemogram. These data, given in Table 2, indicate that a single mouse passage of Rabstein-MBA stock, which was hitherto free of anemia activity, now contained an agent that produced severe anemia.

These data indicate that the source of the anemia virus was more probably from our mice than from the Abelson virus stocks provided by MBA.

**Demonstration of Filterability.** Anemia virus of mouse origin was passed through Millipore filters of decreasing pore size. The integrity of each filter was demonstrated by the addition of 0.5 ml of a 24-hr broth culture of *Chromobacterium prodigiosum* (*Serratia marcescens*) and subsequent culture of filtrate. Only the 800-nm filter allowed passage of the test bacteria.

Rats 1 to 2 days old were inoculated i.p. with graded filtrates and bled at 20 days by severing the carotid artery. The blood was collected in a B-D Unopette and the cells were enumerated electronically in a Coulter counter. Controls included rats receiving clarified but unfiltered virus stock, uninoculated rats, and rats that received normal pooled mouse tissue. The results are reported in Table 3.

The data indicate that the virus passed through 220-nm pores but was largely retained by pores that averaged 100 nm.

**Buoyant Density of Anemia Virus.** For determination of the buoyant density, 30 ml of virus stock (20% w/v tissue homogenate in 0.9% NaCl solution) were partially purified by centrifugation (10,000 × g × 10 min) and the sediment was discarded. The supernatant was then centrifuged at 30,000 × g for 60 min at 4°C, and the pellet was resuspended in 3.0 ml of phosphate-buffered saline or prepared tissue culture fluids. The virus suspension was then layered onto a 10 to 65% continuous sucrose gradient and centrifuged to isopycnic equilibrium (5 hr at 25,000 rpm in a Spinco SW 25.1 rotor). The gradient was fractionated and the specific gravity was determined with a refractometer. Three 1-ml fractions with an average specific gravity of 1.17 g/ml were pooled and brought to 30 ml volume with diluent. The virus was pelleted and resuspended in 1.2 ml of diluent; 0.2 ml was inoculated into each of 6 newborn rats. Four uninoculated litter mates served as controls. A profound anemia was visually apparent in all 6 inoculated rats, 4 dying by Day 17 with estimated RBC levels of about 1 × 10⁶ RBC/cu mm. By Day 20, the 2 surviving rats had an average RBC count of 1.99 ± 0.39, compared to 4.32 ± 0.09 × 10⁶ cells/cu mm for 4 control rats. This indicated that a high concentration of infectious anemia virus had equilibrated at a specific gravity of 1.17 g/ml.

**Ether Sensitivity.** For determination of ether sensitivity of the virus, virus alone and virus plus ether (20% ether, v/v) were gently agitated for 16 hr at 4°C, following which the ether was removed by evaporation. The ether-treated and control virus samples were then inoculated into 2 groups of 4 newborn rats. At Day 20, the hemograms were determined. The red cell counts of the virus treated animals was 2.57 ± 0.14 × 10⁶/cu mm, whereas that of the ether-treated virus group was 4.99 ± 0.07. This indicates that the virus is ether sensitive.

**Virus Neutralization by Anti-Moloney Sera.** For determination of whether any antigenic relation exists between the anemia virus and Moloney leukemia virus, a neutralization study was performed. Anti-murine sarcoma virus (Moloney leukemia virus pseudotype) antiserum was obtained from Dr. Adi Gazdar, National Cancer Institute, Bethesda, Md. This antiserum has specific activity against the group-specific antigen as well as against the envelope antigen and has neutralizing activity against the Friend-Moloney-Rauscher viruses. Antiserum and normal rat control serum were heat inactivated at 56°C for 30 min, after which a 1:20 and 1:40 dilution of sera and virus stock were incubated for 1 hr at 4°C, with intermittent gentle swirling, following which 0.2 ml of each preparation was inoculated into newborn rats. At Day 20, each animal

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**Table 1**

*Comparison of anemia-producing effect of various virus stocks*

<table>
<thead>
<tr>
<th>Virus stock</th>
<th>No. of rats inoculated</th>
<th>RBC (× 10^6/ cu mm)</th>
<th>Hemoglobin (g/100 ml)</th>
<th>No. with gross or histological pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabstein-MBA</td>
<td>20</td>
<td>4.65 ± 0.56a</td>
<td>9.68 ± 1.13</td>
<td>0</td>
</tr>
<tr>
<td>Siegler-CCRF</td>
<td>7</td>
<td>3.09 ± 0.10</td>
<td>7.29 ± 0.36</td>
<td>7</td>
</tr>
</tbody>
</table>

*a Mean ± mean deviations.

**Table 2**

*Effect of a single mouse passage of Rabstein-MBA stock in producing anemia*

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>No. of rats inoculated</th>
<th>RBC (× 10^6/ cu mm)</th>
<th>Hemoglobin (g/100 ml)</th>
<th>Hematocrit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse passaged</td>
<td>17</td>
<td>2.17 ± 0.35a</td>
<td>5.4 ± 0.6</td>
<td>16.5 ± 2.5</td>
</tr>
<tr>
<td>Rabstein-MBA</td>
<td>20</td>
<td>4.65 ± 0.56a</td>
<td>9.68 ± 1.13</td>
<td>0</td>
</tr>
<tr>
<td>Uninoculated</td>
<td>7</td>
<td>5.39 ± 0.17</td>
<td>11.6 ± 0.3</td>
<td>34.9 ± 1.0</td>
</tr>
</tbody>
</table>

*a Mean ± mean deviations.*
was bled, and the hemogram was determined. The mean red cell count of 4 rats receiving virus reacted with 1:20 anti-murine leukemia virus (Gazdar) was $4.39 \pm 0.01 \times 10^8$ cells/cu mm, and $4.58 \pm 0.13$ for the 1:40 dilution. By contrast, the mean red cell counts of rats receiving virus reacted with control normal serum was $2.13 \pm 0.19$ and $2.83 \pm 0.66 \times 10^8$ cells/cu mm for the 1:20 and 1:40 dilutions, respectively. This indicates that antiserum with specificity against the group-specific Friend-Moloney-Rauscher antigens neutralizes the anemia virus.

**Evidence That the Anemia is Not Cause by Other Microbiological Agents**

Because rats are especially susceptible to an infectious anemia due to latent endemic infection with species of *Hemobartonella*, the possibility that this agent was present was investigated.

**Culture.** Ten 21-day-old inoculated anemic rats were bled by aseptic carotid laceration into Noguchi semisolid medium used for isolation of *Hemobartonella* species and related microorganisms. The culture tubes were incubated for 10 days at 28°C and examined. Occasional contaminant mixtures of Gram-negative rods were encountered, but no colonies of *Hemobartonella* developed.

**Splenectomy.** Latent *Hemobartonella* are known to multiply rapidly and cause fatal anemia in the rat following splenectomy (4). Accordingly, 10 rats were splenectomized at 2 weeks of age and followed for 3 months, during which time the animals remained in good health with normal red cell counts. At autopsy no pathology except the absence of a spleen was noted. An additional 21 rats were splenectomized at 2 weeks of age and following for 1 month with weekly hemograms and smears stained with Giemsa. The average red cell count for the group was $4.69 \pm 0.18$ 1 week after splenectomy, $5.7 \pm 0.31$ after 2 weeks, $5.62 \pm 0.34$ after 3 weeks, and $6.70 \pm 0.25 \times 10^8$ cells/cu mm after 5 weeks. The smears were negative for parasites.

**Direct Observation of Erythrocytes.** The membranes of circulating red cells were examined for the presence of *Hemobartonella* microorganisms. Direct air-dried smears of anemic peripheral blood stained by Wright-Giemsa and May-Grünwald stains were examined. Also, red cells of anemic rats were fixed in 2% glutaraldehyde and prepared for transmission electron microscopy by conventional means. In no instance was a microorganism of the morphology described for *Hemobartonella* (1) observed.

**Passage in Rats**

As documented previously (3), anemia is produced when extracts of anemic rat organs are inoculated into newborn rats. Generally, the titer produced by such passage is low and, unless concentrated by centrifugation, rat passage yields progressively less and less infectious virus, which is finally lost after the 3rd or 4th passage.

In the primary or secondary rat passage, however, the thymuses and spleen contain significant amount of virus, as demonstrated by passage of these extracts. In one such
study, newborn rats were inoculated with 0.2 ml of mouse virus stock and bled and killed at 30 days. The thymuses and spleen of these anemic rats were prepared as a clarified 20% w/v NaCl homogenate, and 0.2 ml was inoculated into litters of newborn rats. At 20 days of age, the rats were bled and their hemograms were determined. The results are given in Table 4. These data indicate that anemic rat thymus contains significant amounts of anemia virus infectious for rats.

Effect of Inoculation of Rat Anemia Virus Back to Mice

For determination of whether infectious Abelson virus was still present in anemic rats, pooled tissues of such rats were prepared as given above and inoculated into newborn mice. The mice were observed for 45 days and then autopsied. All tissues were studied histologically to determine that type of neoplasia, if any, developed. Of 30 mice inoculated with anemic rat thymus homogenate, 29 were negative and 1 developed Abelson leukemia. Of 38 mice given anemic rat spleen, none developed Abelson leukemia, 1 developed thymic lymphoma, and 37 were negative. These data indicate that, whereas anemic rat thymus and spleen contain sufficient anemia virus to induce the disease in new rats, they contain only negligible amounts of infectious Abelson leukemia virus.

DISCUSSION

This report confirms our earlier study (3) of an infectious anemia in rats which is transmitted by cell- and bacteria-free material. Our data indicate that the agent has the properties of a virus and that this virus is probably a member of the leukemia-sarcoma complex of murine viruses.

In the recent paper of Taylor et al. (5), a similar anemia-inducing virus is described. The virus of Taylor et al. was derived from Moloney sarcoma virus stock and was physically and morphologically indistinguishable from the murine leukemia-sarcoma complex of viruses. Of special relevance to this report is the fact that those authors were able to separate the anemia-inducing activity from the sarcoma-inducing activity and concluded that they were dealing with a separate, distinct virus. Studies are now in progress in our laboratory to characterize this virus and the disease it produces, and to determine what role, if any, this agent may have in leukemogenesis.

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REFERENCES


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