**CONCISE REPORT**

**Successful Transplantation of Friend Virus-Induced Preleukemia Into Stem Cell-Deficient Fetal Mice**

By Roger A. Fleischman

The leukemias induced by the Friend polycythemia virus and other leukemogenic retroviruses have previously not been transplantable until weeks or months after virus inoculation. Because tumor-specific immune mechanisms persist in both irradiated and nude mice, it has not been possible to determine if this result is due to rejection of cells already immortalized by retrovirus infection, or reflects an inherent limitation in the proliferative capacity and malignancy of these "preleukemic" cells. To clarify these issues, we have transplanted virus-infected bone marrow into mouse fetuses that are immunologically immature and thus incapable of graft rejection. We report here that within days of virus inoculation, transplantable cells capable of disease progression in certain fetal hosts can be detected with this technique. These results demonstrate that cancer cells, i.e., cells capable of indefinite proliferation, do not exist in the initial "preleukemic" phase of leukemogenesis. An alternative thesis holds that malignant cells are present from the outset, but are rejected by host immune mechanisms that recognize virus-specific antigens. Although previous experiments have used irradiated mice in an attempt to circumvent host immunity, partial host immunity persists in these recipients and is capable of rejecting even syngeneic tumor cells. To clarify these issues and define the role of immune mechanisms in the regulation of tumor progression, we have used mouse fetuses as model recipients that are incapable of graft rejection. Using a previously described technique for microinjection via the placental circulation, we transplanted bone marrow cells from virus-infected adults into fetal hosts at day 11 of gestation, before the development of immunity.

To examine the role of normal hematopoietic cells in tumor progression, we have also studied hosts of the mutant W/W genotype, which are deficient in hematopoietic stem cells and thus accept bone marrow grafts without the necessity for conditioning. Transplantation studies of tumor development in these fetal recipients therefore permit an experimental separation of the role of immune mechanisms from that of normal stem cells.

We report here that within days of virus inoculation, transplantable cells capable of disease progression can be detected by injection of FV-infected bone marrow into mouse fetuses, but only in mutants that are deficient in hematopoietic stem cells. These experiments demonstrate that certain preleukemic cells are capable of leukemic evolution within days of virus infection, albeit in an environment that lacks not only immune mechanisms but also competing stem cells.

**METHODS**

Recipent mice. To investigate the requirements for transplantation of FV-infected cells, recipient mice have been studied at two distinct stages of development: in fetal life before the development of the immune system, and in adult life after complete immunity has been achieved (Table 1). These normal recipients, however, do not accept grafts of bone marrow cells from virus-infected adults into fetal hosts at day 11 of gestation, before the development of immunity.

To examine the role of normal hematopoietic cells in the regulation of tumor progression, we have used mouse fetuses as model recipients that are incapable of graft rejection. Using a previously described technique for microinjection via the placental circulation, we transplanted bone marrow cells from virus-infected adults into fetal hosts at day 11 of gestation, before the development of immunity.

To investigate the requirements for transplantation of FV-infected cells, recipient mice have been studied at two distinct stages of development: in fetal life before the development of the immune system, and in adult life after complete immunity has been achieved (Table 1). These normal recipients, however, do not accept grafts of bone marrow cells from virus-infected adults into fetal hosts at day 11 of gestation, before the development of immunity.

The genotypic composition of RBCs, buffy coat cells, and tissue homogenates taken at autopsy were determined from electrophoretic separation of the strain-specific variants of GPI, performed according to the method of Eldridge and Dewey.17

**Histology.** Peripheral blood smears and imprints of hematopoietic organs were stained with Wright's stain according to standard methods.

**RESULTS**

**Adult recipients.** Because earlier studies used normal or lethally irradiated adult recipients to demonstrate the inability to transplant FV-infected cells in the first weeks after virus inoculation, initial experiments were performed to confirm these results in adult mice of the mutant W'/W' genotype. The results of these experiments are presented in Fig 1.

As expected, proliferation of both uninfected and virus-infected cells was undetectable after transplantation into wild-type (+/++) recipients. None of these mice developed Friend disease during a subsequent 6-month period of observation. On the other hand, all of the stem cell-deficient W'/W' recipients showed evidence of engraftment by the second week after transplantation. Complete replacement occurred by 6 to 8 weeks, although the FV-infected cells exhibited a slight but significant delay in the rate of repopulation.

However, despite complete reconstitution with donor erythroid cells, none of the W'/W' animals developed polycythemia, erythroblastosis, or enlarged spleens. When autopsied at 16 weeks after transplantation, serum and spleen lysates from these mice did not produce spleen foci in DBA/2 mice, suggesting that they were not viremic at the time of death. In addition, the bone marrow from these animals repopulated secondary W'/W' recipients without induction of leukemia and with kinetics similar to those observed in the primary recipients (data not shown). These preliminary studies demonstrated that preleukemic hematopoietic cells do not give rise to progressive leukemia after transplantation into immune-competent adult recipients, even when the recipients are mutants that lack normal hematopoietic stem cells.

**Fetal recipients.** To further elucidate the roles of immune mechanisms and endogenous stem cells, similar experiments were performed via placental injection of fetal recipients. As shown in Table 2, survival of the fetal recipients from injection to birth was approximately 80% for both uninfected control and FV-infected experimental groups. Under these conditions, then, the presence of virus-infected cells did not increase prenatal mortality, probably because the titer of free virus present at this stage of the infection was low.18 Similarly, the rate of initial engraftment for preleukemic bone marrow, 0% in the nonanemic mice and 35% in infected mice, was undetectable after transplantation into wild-type (+/++) recipients. None of these mice developed Friend disease during a subsequent 6-month period of observation. On the other hand, all of the stem cell-deficient W'/W' recipients showed evidence of engraftment by the second week after transplantation. Complete replacement occurred by 6 to 8 weeks, although the FV-infected cells exhibited a slight but significant delay in the rate of repopulation.

However, despite complete reconstitution with donor erythroid cells, none of the W'/W' animals developed polycythemia, erythroblastosis, or enlarged spleens. When autopsied at 16 weeks after transplantation, serum and spleen lysates from these mice did not produce spleen foci in DBA/2 mice, suggesting that they were not viremic at the time of death. In addition, the bone marrow from these animals repopulated secondary W'/W' recipients without induction of leukemia and with kinetics similar to those observed in the primary recipients (data not shown). These preliminary studies demonstrated that preleukemic hematopoietic cells do not give rise to progressive leukemia after transplantation into immune-competent adult recipients, even when the recipients are mutants that lack normal hematopoietic stem cells.

**Fig 1.** In vivo kinetics of erythroid replacement in adult +/+ and W'/W' mice injected with normal or preleukemic bone marrow. Donor C3H/HeJ mice were inoculated intraperitoneally with 5,000 focus-forming units of the FV complex. Uninfected controls were injected with normal saline. Two to four days after inoculation, suspensions of 2 x 10^6 nucleated bone marrow cells prepared from the killed donor mice were injected via the tail vein into adult recipients. At weekly intervals, hematocrits, blood smears, and analyses of the strain-specific hemoglobin variants were performed. The results were pooled from two separate experiments performed with four individual mice in each group. The vertical bar represents the standard error. Recipient mice were: [ ] + +, W'/W' homozygous mutants injected with normal bone marrow; [ ] W'/W' homozygous mutants injected with preleukemic bone marrow; [ ] + +, littermates injected with normal bone marrow; [ ] + +, littermates injected with preleukemic bone marrow.
the W'*/W mice, was not significantly different than that obtained with control bone marrow (0% and 45%, respectively). These results are consistent with our prior reports that demonstrate a 30% to 50% rate of successful engraftment with the placental injection technique in severely anemic mouse fetuses.

Friend disease in fetal recipients. Four of the initially engrafted W'*/W recipient of FV-infected bone marrow, however, rapidly developed typical FV-induced disease that proved to be donor in origin (Table 3). In three cases (A, B, D) smears of the peripheral blood revealed markedly increased numbers of immature "smudge" cells and erythroblasts. Analysis of the spleens and buffy coats, which contain the circulating nucleated cells, demonstrated that the leukemic cells were predominantly, if not completely, donor-derived. Mosaicism in the blood, liver, and spleen of case C was more limited; however, gross examination revealed a single, white 1 mm nodule within otherwise normal-appearing spleen. Imprints of the nodule showed immature blasts consistent with FV-induced disease, and electrophoretic analysis showed that the cells were more than 80% donor in origin. In contrast, the splenic foci induced in adult mice by FV-infected cells are exclusively host in origin.

The failure to observe, after more than 1 year of observation, any similar cases of donor-derived disease in the non-anemic genotypes (+/+, W'*/+, W'*/+; +/+) is statistically significant (χ² = 9.1; P < .01). Although virus-infected donor cells were unable to proliferate in the nonanemic fetuses, Friend disease of host origin was observed subsequently in 20 (12%) of the high-dose recipients at 2 to 4 weeks of age and in two of the low-virus group at 3 to 4 months of age, confirming the successful injection of these hosts with virus-infected cells. Apparently, in the fetal hosts, Fv-1 resistance provided incomplete protection from viral leukemogenesis.

DISCUSSION

The experiments described in this report demonstrate that within days of virus infection transplantable cells capable of disease progression can be detected by injection into immuno-logically immature mouse fetuses. However, this result was seen only in the W'*/W mutant fetuses that are deficient in hematopoietic stem cells, and not in their normal fetal littermates. The deficiency of host stem cells, although necessary, is not sufficient for successful transplantation. Donor-derived leukemias were not observed in the immune-competent W'*/W adult recipients, despite injection with cell inoculums that were ten to 20 times larger than the inoculums for fetal recipients.

The most cogent explanation for the experimental data is that, early after virus infection, the fate of the transformed cells is determined not only by immune mechanisms but also by the hematopoietic environment of the host. Within the hypoplastic environment of stem cell-deficient fetuses, certain virus-infected cells are capable of extensive proliferation and progression to leukemia. The very early presentation of these donor-derived leukemias, as compared with host-derived leukemias, strongly suggests that viral transformation occurred before, rather than after, transplantation. Moreover, the results are not attributable to differences in the incidence or numbers of bone marrow cells and stem cells

Table 2. Frequency of Engraftment and Donor-Derived Friend Disease in Recipients of Normal and Preleukemic Bone Marrow

<table>
<thead>
<tr>
<th>Donor Cells</th>
<th>No. Injected</th>
<th>No. Born (%)</th>
<th>Engraftment*</th>
<th>Donor Disease†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no FV)</td>
<td>133</td>
<td>105 (79)</td>
<td>0/74</td>
<td>0/74 (Case A)</td>
</tr>
<tr>
<td>Virus (low FV)</td>
<td>140</td>
<td>102 (73)</td>
<td>0/81</td>
<td>0/81 (Case B)</td>
</tr>
<tr>
<td>Virus (high FV)</td>
<td>248</td>
<td>221 (89)</td>
<td>0/166</td>
<td>0/166 (Cases C, D)</td>
</tr>
</tbody>
</table>

The results are expressed as the number of animals initially engrafted or developing donor leukemia over the numbers of animals injected.

*All animals with detectable donor cells (> 1%), as determined by analysis of blood from one-day-old newborn mice for strain-specific variants of GPI, were considered to be engrafted.

†Friend disease was considered donor in origin if the GPI enzyme activity from the spleen, buffy coat cells, or tumor nodules was more than 80% donor-derived.

Table 3. Blood Analysis and Percent Tissue Mosaicism in W'*/W* Mice With Friend Disease of Donor Origin

<table>
<thead>
<tr>
<th>Case</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus dose</td>
<td>Low</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Age at death</td>
<td>23 d</td>
<td>3 d</td>
<td>1 d</td>
<td>4 d</td>
</tr>
<tr>
<td>WBC</td>
<td>115 K</td>
<td>&gt;500 K</td>
<td>&gt;10 K</td>
<td>&gt;500 K</td>
</tr>
<tr>
<td>Blood smear</td>
<td>Leukemia</td>
<td>Leukemia</td>
<td>Normal</td>
<td>Leukemia</td>
</tr>
<tr>
<td>Spleen†</td>
<td>8 x</td>
<td>10 x</td>
<td>1 x</td>
<td>5 x</td>
</tr>
<tr>
<td>Percent donor GPI</td>
<td>Blood</td>
<td>50</td>
<td>75</td>
<td>10</td>
</tr>
<tr>
<td>Buffy coat</td>
<td>ND</td>
<td>&gt;95</td>
<td>ND</td>
<td>&gt;95</td>
</tr>
<tr>
<td>Spleen</td>
<td>90</td>
<td>50</td>
<td>10</td>
<td>70</td>
</tr>
<tr>
<td>Liver</td>
<td>40</td>
<td>75</td>
<td>30</td>
<td>ND</td>
</tr>
<tr>
<td>Kidney</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>Other</td>
<td>40</td>
<td>—</td>
<td>80</td>
<td>—</td>
</tr>
</tbody>
</table>

*At three to four days of age, coat color differences, due to homozygosity for the W alleles, allowed the unequivocal identification of cases A, B, and D as severely anemic mutants. The W'*/W genotype of case C, which was severely anemic and recognizable paler than its nonanemic littermates, was confirmed by the marked macrocytosis of its peripheral RBCs (mean cell volume 120 μm²), calculated from the hematocrit and RBC counts.

†Relative spleen size was determined by dividing the wet weight of the experimental spleen by the average weight of four to five normal age-matched W'*/W* control spleens.
shown that normal virus. More recent experiments have demonstrated the ability of a normal embryonic environment to suppress growth by teratocarcinoma cells or tumor induction by Rous sarcoma virus.\textsuperscript{19,20} More recent experiments have shown that normal cells can also inhibit the growth and tumorigenicity of cells transformed by oncogenes in vitro.\textsuperscript{21-23}

In conclusion, transplantation of transformed cells into mouse fetuses represents a useful model system for the analysis of viral leukemogenesis in vivo. In addition, unlike irradiation of adult recipients, the present approach readily discriminates the role of host stem cells from that of host immunity. The experimental results suggest that growth of preleukemic hematopoietic cells is subject not only to rejection by host immune mechanisms but also to suppression by normal endogenous hematopoietic cells. In the absence of both constraints, donor-derived cells capable of transplantation and leukemic evolution can be detected within days of virus infection.

\textbf{REFERENCES}

Successful transplantation of Friend virus-induced preleukemia into stem cell-deficient fetal mice

RA Fleischman