Resistance to Erythroleukemia in Immunodeficient $\textit{xic}^-$ CBA/N Mice with Susceptibility after Immune Stimulation

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ABSTRACT

CBA/N ($\textit{xic}^-$) mice failed to develop erythroleukemia when inoculated with an NB-tropic, anemia-causing Friend virus stock (FVA), while Fv-2$^m$ mouse strains succumbed rapidly to the characteristic Friend disease, even after a virus dose 30-fold lower than that given to CBA/N mice. Immunization with bacterial antigens or with spleen cell allografts prior to FVA inoculation rendered CBA/N mice highly susceptible to FVA. Transplantation studies confirmed that non-immunized CBA/N mice were able to both support erythroleukemic cells and permit erythroleukemic transformation, thus arguing against host defense mechanisms as a cause of resistance. On the basis of early erythroid progenitor cell sensitivity to hydroxyurea $\textit{in vitro}$, the CBA/N strain appeared to have the FVA sensitive genotype (Fv-2$^m$). These results imply that CBA/N mice are not intrinsically resistant to FVA and that an as yet unknown type of immunological activity, evoked both by various immunizations and allelic geneic transplantation, is required for Friend leukemogenesis in this immunodeficient inbred strain. These findings further suggest that the erythroid target cells transformed by Friend viruses are influenced by immunological activity.

INTRODUCTION

The production of an important humoral regulator of early erythroid differentiation (IL-3) by mitogen-stimulated spleen cells suggests a regulatory link between lymphocyte function and erythropoiesis. We have been exploring this hypothesis by comparing the erythropoietic response of CBA/N mouse to immune challenge with (a) \textit{Bordetella pertussis} vaccine and (b) infectious Friend virus. We chose the CBA/N strain because of its well-described immune defects (1-3) resulting from the \textit{xic} mutation (1), including an undetectable T-cell response to \textit{MoLV} protein (4). Resistance among inbred strains of mice to NB-tropic FVA complex is thought to be regulated by a single autosomal gene, Fv-2 (5). Few inbred mouse strains possess the homozogous Fv-2$^m$ genotype (6), which completely blocks FVA pathogenesis. CBA/N has been listed (but not shown; Ref. 7) as Fv-2$^m$, in accordance with all other CBA strains typed at Fv-2 (6). We therefore anticipated that CBA/N would be susceptible to FVA. Immunizations with BPT (8) or LPS (9) induce splenomegaly with predominant splenic erythroid hyperplasia in mice. Given the immunogenicity of attenuated FVA (10), we reasoned that the initial phase of erythroid hyperplasia in FVA infected spleens might involve an immunologically induced component which precedes or accompanies the leukemogenic component. From Lee and Ihle's observations (4), we anticipated that CBA/N might provide a unique model of impaired immune responses to FVA in an environment permissive for the erythroleukemia.

MATERIALS AND METHODS

Mice. All mice except (BALB/c x DBA/2)F1 (i.e., CD2F1) were purchased from The Jackson Laboratory (Bar Harbor, ME), CD2F1 were purchased from Cumberland View Farms (Clinton, TN). All mice, unless otherwise noted, were female and between 7 and 10 weeks of age. All mice were housed and maintained by the Vivarium of Texas Tech University Health Sciences Center according to standard laboratory practices. Mice were sacrificed using carbon dioxide anesthesia. Viruses. FVA was generously provided by Dr. Michael Dewey, Dept. of Biology, University of South Carolina, Columbia, SC, who obtained it from Dr. Philip Furmanski. This FVA was originally N-tropic, but acquired NB-tropism by forced passage through BALB/c mice in Dr. Dewey's laboratory. The stock was passaged by injecting 10% \( v/v \), clarified spleen homogenates, diluted 1:10 in fresh phosphate buffered saline (10X; Grand Island Biological Co.) i.v. into DBA/2J, BALB/CJ, and SJL/J mice, 0.1 ml per mouse. Within 3-4 weeks, recipients demonstrated massive splenomegaly and anemic hematocrits. Mortality at four weeks ranged from 75-100%. This virus stock failed to form titratable macroscopic foci in spleens of sensitive animals, as has been reported for other FVA stocks (11).

Inoculations. Groups of two to four mice were inoculated i.v. for each observation, and each observation was repeated at least three times. Hematocrits. Portal blood was drawn immediately after sacrifice into microhematocrit capillary tubes containing heparin (Fisher, Pittsburgh, PA). This method, with no distress to the mouse, compared identically to retro-orbital bleeding for determination of hematocrit. Progenitor Cell Assays. Erythroid progenitors were assayed essentially as described previously (12). Briefly, 10$^3$ femoral marrow cells were plated per ml of medium containing IMDM (Grand Island Biological Co., Grand Island, NY), 20% fetal calf serum (HyClone, Logan, UT), 1% methylcellulose (Dow Chemical Co., Midland, MI), 10$^4$ mL 2-mercaptoethanol (Sigma, St. Louis, MO), and gentamycin (50 \( \mu \)g/ml) (Elkins Sinn, Cherry Hill, NJ). Erythropoietin was Connaught Step III (Swiftwater, PA). CFU-E cultures containing erythropoietin (250 \( \mu \)U/ml) were incubated for 2 days and then stained with benzidine reagent (12). BFU-E cultures contained erythropoietin (1 unit/ml) and were incubated for 8 days prior to staining with benzidine. Granulocyte-macrophage progenitors were plated at 10$^3$ cells/ml of the same medium without erythropoietin and contained 25 \( \mu \)l of conditioned medium from pokeweed mitogen-stimulated DBA/2 spleenocytes. Triplicate or quadruplicate cultures were placed into 35-mm polysyrte dishes (Falcon, Oxnard, CA) and incubated at 37°C and 5% CO$_2$. At time of assay, all colonies were scored in situ.

Immunogens. \textit{Bordetella pertussis} vaccine was purchased from the Dept. of Biologicals, Michigan Dept. of Public Health, Lansing, MI and was diluted in phosphate-buffered saline (10X; Grand Island Biological Co.) freshly diluted in sterile water for irrigation (Travenol, Deerfield, IL). Phenol-extracted lipopolysaccharides were purchased from Sigma and were dissolved in sterile, fresh, non-pyrogenic saline for irrigation (Travenol) immediately prior to injection.

RESULTS

As seen in Fig. 1, CBA/N failed to develop either anemia (Fig. 1a) or splenomegaly (Fig. 1b) when untreated mice were inoculated with FVA. FVA did cause a mild spleen enlargement (2-fold) in non-immunized CBA/N (Fig. 1b), a finding consistent with a splenic immune response to FVA antigens. In other experiments, resistance was not altered by the amount of virus injected (range, 10 to 300 \( \mu \)l per mouse), route of virus admin-
We tested the effect of acute immune stimulation on adult CBA/N resistance to FVA by injection with 2 × 10⁹ attenuated BPT organisms i.p. on two consecutive days. Two or three days after the final immunization, mice were inoculated with 100–200 µl of FVA i.v. Within 3 weeks after FVA, hematocrits of these CBA/N mice ranged from 22–36% (Fig. 1a), with spleens enlarged to 2000–4000 mg (Fig. 1b). CBA/N mice given injections of FVA at the same time but not immunized with BPT had spleen weights ranging from 90–118 mg and hematocrits of 48–52%. Thus BPT immunization rendered CBA/N mice susceptible to FVA leukemia.

The specificity of BPT-induced susceptibility to FVA in CBA/N was tested by immunizing CBA/N mice with LPS from either *Escherichia coli*, *Salmonella abortus equi*, or *Serratia marcescens*. Table 1 compares the response 3 weeks after FVA inoculation to single injections of LPS, at 100 µg per mouse, and to 2 × 10⁹ BPT, when these immunizations preceded FVA by 2 days. LPS immunization without subsequent FVA inoculation caused moderate spleen enlargement (100–200 mg) and mild anemia (45–50%) at 3 weeks. But when LPS preceded FVA treatment, these immunizations were as capable of inducing susceptibility to FVA as was BPT.

We questioned whether CBA/N target cells were reversibly resistant to FVA, or whether target cells which were transformed by FVA were subsequently (and reversibly) suppressed by the CBA/N immune system. As a test, normal CBA/N mice were engrafted with 10⁷ CBA/J spleen cells i.v. Two days later, the CBA/N hosts were inoculated with 200 µl FVA stock i.v. Within 4 weeks, the CBA/N hosts developed anemia (24–40%) and splenomegaly (1000–2300 mg). When BPT-immunized CBA/N mice developed erythroleukemia, their spleen cells also caused FVA when transplanted into normal CBA/N recipients. Thus cells transformed in immunized CBA/N mice could not be suppressed in non-immunized CBA/N. These results suggested that surveillance against transformed cells did not account for CBA/N resistance to FVA in non-immunized mice.

Although CBA/H (the superstrain of CBA/N) and CBA/J have the same H-2 haplotype (H-2k) (6), a transplantation barrier exists between them (13). Thus it was possible that an allograft response to CBA/J rendered the CBA/N mice susceptible to FVA. We therefore engrafted CBA/N mice with 10⁶ spleen cells from Fv-2sr C57BL/6 (H-2k) donors. These CBA/N recipients also developed the erythroleukemia when inoculated with FVA 2 days after transplantation. Since C57BL/6 bone marrow may be intrinsically resistant to FVA (14, 15), our results suggested that the immune mechanism involved in overcoming CBA/N resistance is part of the allogeneic transplant response.

Lee and Ihle (4) demonstrated persistent viremia in MoLV-infected CBA/N mice despite the absence of leukemic pathology. To test for the persistence of infectious virus in apparently disease-free CBA/N mice, we prepared 10% w/v spleen homogenates of CBA/N females 5 weeks after inoculation with FVA (100 µl). These homogenates were then injected (100 µl i.v.) into Fv-2sr (SJL, C3H/He) mice. In addition, 10⁶ spleen cells from Fv-2sr females were subsequently (and reversibly) suppressed by the CBA/N immune system. As a test, normal CBA/N mice were engrafted with 10⁷ CBA/J spleen cells i.v. Two days later, the CBA/N hosts were inoculated with 200 µl FVA stock i.v. Within 4 weeks, the CBA/N hosts developed anemia (24–40%) and splenomegaly (1000–2300 mg). When BPT-immunized CBA/N mice developed erythroleukemia, their spleen cells also caused FVA when transplanted into normal CBA/N recipients. Thus cells transformed in immunized CBA/N mice could not be suppressed in non-immunized CBA/N. These results suggested that surveillance against transformed cells did not account for CBA/N resistance to FVA in non-immunized mice.

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>S. marcescens</th>
<th>S. ab. equi</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPT Bone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen weight (mg)</td>
<td>1100–2300</td>
<td>2400*</td>
<td>1700–1800</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>36–36%</td>
<td>36%</td>
<td>38–40%</td>
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| *One mouse of the pair died with splenic hemorrhage prior to 3 weeks.
from the FVA-inoculated CBA/N mice were transplanted into normal CBA/J recipients. Four weeks later, all animals were sacrificed, and all showed normal spleen weights (80–110 mg) and hematocrits (49–51%). Therefore, we conclude that neither persistent viremia nor virus-producing cells occur in CBA/N mice 5 weeks after injection with FVA.

We next tested for the presence of infectious virus in mice immunized prior to FVA inoculation. Whole spleen cell preparations and spleen homogenates were made from BPT-immunized, FVA-infected CBA/N mice. Spleen cells (10⁶) were injected into untreated CBA/N hosts, and DBA/2 and BALB/C mice received 10 μl of spleen homogenate per mouse. By 4 weeks, mortality among CBA/N recipients of leukemic spleen cells exceeded 50%. Surviving CBA/N mice had spleen weights of 1300 to 1700 mg and hematocrits between 32 and 37. The leukemia was serially transplanted for three passages, causing the same pathology and death in all CBA/N recipients. By 3 weeks, the DBA/2 and BALB/c recipients of cell-free spleen homogenates had spleen weights of 900–1100 mg and hematocrits of 38–43%. Thus the spleens of BPT-immunized, FVA-infected CBA/N were producing infectious erythroleukemia virus.

Strong evidence indicates that the genotype at the Fv-2 locus determines the proportion of BFU-E synthesizing DNA in normal, non-infected mice (16, 17). In order to determine whether the CBA/N and CBA/Ca strains had a high or low percentage of BFU-E in S-phase (16), we cultured their bone marrow after in vivo hydroxyurea treatment. The CBA/Ca inbred strain was considered to be isogenic with CBA/H (18). The data in Table 2 show that the BFU-E population of the two strains were equally sensitive to hydroxyurea, and their rate of S-phase killing was consistent with the Fv-2-M phenotype (16, 17). For both strains, most of the CFU-E and about one-half of the granulocyte-macrophage progenitors were judged to be in S-phase.

Table 2: Phenotype of CBA/N and CBA/Ca hematopoietic progenitors: percentage killed by hydroxyurea (HOU) treatment in vivo

<table>
<thead>
<tr>
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<th>BFU-E</th>
<th>CFU-E</th>
<th>CFU-GM</th>
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<tbody>
<tr>
<td>No. of colonies/10⁵ cells plated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBA/N (+HOU/−HOU)</td>
<td>16 ± 3/50 ± 4*</td>
<td>8 ± 0/40 ± 8</td>
<td>51 ± 5/115 ± 8</td>
</tr>
<tr>
<td>Percentage killed</td>
<td>68</td>
<td>80</td>
<td>56</td>
</tr>
<tr>
<td>CBA/Ca (+HOU/−HOU)</td>
<td>16 ± 2/42 ± 3</td>
<td>8 ± 0/139 ± 12</td>
<td>42 ± 2/110 ± 1</td>
</tr>
<tr>
<td>Percentage killed</td>
<td>62</td>
<td>94</td>
<td>62</td>
</tr>
</tbody>
</table>

* Mean ± SD.

DISCUSSION

Our report of CBA/N resistance to FVA and its reversal by immune stimulation is original and unexpected. Yet other groups have reported results consistent with our findings. Steeves and Grundke-Iqbal (19) showed that endotoxin can overcome Fv-1 restriction of FVA disease. Rauscher et al. (20) reported that prior immunization increased chick susceptibility to Rous sarcoma virus. Hirano et al. (21) and Floersheim (22) have shown that BPT immunization accelerated the growth of tumor isografts derived from the Rauscher and MoLV lymphomas, respectively. Yet it seemed paradoxical that immunodeficient animals resisted viral leukemia prior to acute stimulation of the immune system. In order to explain the apparent paradox of impaired immune surveillance after immune stimulation, Elves (23) suggested that acute immunological mobilization temporarily depletes certain effector cell populations, rendering the host transiently immunodeficient. Increased sensitivity to FVA in CBA/H and other Fv-2 strains following T-cell depletion with anti-thymocyte serum (24) indirectly supports this notion. Thus acute immune activation may deplete CBA/N T-cell subsets which otherwise would resist early FVA infection.

However, several lines of evidence argue against this hypothesis. First, FVA has a direct immunosuppressive effect on T-cell dependent but not on thymus-independent responses in CBA and DBA/2 mice (25). Thus transient T-cell deficiency in CBA/N mice following FVA inoculation should be expected. Second, CBA/N mice already lack a T-cell response to the purified MoLV proteins gp71 and p12, in contrast to other Fv-2 mice (4). In order for an impaired surveillance hypothesis to explain the reversal of CBA/N resistance to FVA, the degree of acute immunosuppression during immunological activation would have to exceed the combined effects of Friend virus complex-induced immunosuppression and the genetic immune defect in CBA/N. Specific pharmacological inhibition of T-cell function in these strains with substances such as cyclosporin A may help to clarify this hypothesis.

The above discussion assumes that the immune system of CBA/N mice, in spite of significant immunological malfunction, protects other cells, presumably erythroid, from transformation by the virus. An alternative hypothesis is that in this strain, target cells for FVA infection and/or expression are not constitutively present in sufficient numbers, perhaps as a result of the xid mutation. Resistance to FVA would thus be a result of target cell numbers below a threshold necessary for leukemogenesis. Challenge with potent immunogens may then invoke a cohort of target cells surpassing the threshold for FVA pathogenesis. This concept adapts the ideas of Lee and Ihle (4), regarding the dependence of MoLV lymphomagenesis in CBA/N on a threshold of immunological activation, to the case of FVA erythroleukemia. It is possible that stimulation of immunological activity in CBA/N affects a compartment of erythroid-committed, or partially committed cells, which become the infected and transformed target cells of FVA. In support of this interpretation is the work of Steeves et al. (26), showing that endotoxin treatment during post-radiation hematopoietic regeneration greatly potentiated SFFV replication in Swiss mice. These authors concluded that endotoxin increased the number FVA target cells. A possible mechanism connecting immune activity with erythropoiesis is that immunological phenomena might regulate cell cycle parameters and hence population dynamics of an erythroid target cell compartment via interleukin-3. CBA/N may then provide a model system wherein steady-state immune deficiency restricts the size of the putative sensitive cell compartment.

Experimental immune deficiencies have been shown to increase susceptibility but not resistance, to oncogenic and leukemogenic viruses (24, 27). FVA resistance in immunodeficient CBA/N mice with susceptibility after immune activation raises questions about the relationship between immunological mobilization and viral infection resulting in leukemia. Our results call for reappraisal of regulatory mechanisms governing target cells for FVA transformation and of the relationship between immune activity and erythropoiesis in general. The reversible resistance of CBA/N mice to FVA erythroleukemia suggests that there exists an important regulatory linkage between immune function and erythropoiesis.

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REFERENCES


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