Protection against Radiation Leukemogenesis by Repeated Injections of Immune and Nonimmune Foreign Sera

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SUMMARY

Significant inhibition of lymphoid tumor development was observed in X-irradiated strain C57BL mice treated with multiple injections of rat antiserum prepared by immunization of Wistar-Furth or (Wistar-Furth·BN) F₁ rats with histocompatible lymphomas induced by the radiation leukemia virus (RadLV). However, there was also significant inhibition, relative to phosphate-buffered saline controls, in C57BL mice that received injections of normal rat serum. This prompted additional experiments in which fetal calf serum or Freund's complete adjuvant were injected as nonspecific immunostimulants. Strong protection was observed with fetal calf serum, and a slight inhibitory effect (not significant) was also seen with Freund's complete adjuvant. The results are discussed in terms of the following alternative interpretations, which are not mutually incompatible: (a) the inhibitory effect of the rat anti-RadLV serum, as well as that of "normal" serum, which may have contained natural antibody to RadLV, is due to passive immunization by specific antiviral antibody, thus further implicating RadLV in the etiology of radiogenic lymphomas; (b) the protective effect is due to hyperimmunization of the mice against foreign (rat and bovine) sera, leading to nonspecific stimulation of the immune surveillance mechanisms of the host animals; and/or (c) prevention by the foreign sera is due to the radiation leukemia protection factor described by Berenblum et al.

INTRODUCTION

RadLV³ can be consistently recovered from the thymic lymphosarcomas and lymphatic leukemias induced in C57BL mice by whole-body X-ray exposure. It is an RNA-containing, C-type virus capable of inducing identical tumors in high incidence after neonatal inoculation into non-radiated mice of the same and other strains (15, 16, 18). All of the virus-induced tumors, and most of those developing after radiation, have been shown to contain a RadLV-specific cell-surface antigen (7). These and other findings constitute strong presumptive evidence that the leukemogenic effect of radiation, and probably also of chemical and other leukemogenic stimuli, is mediated by the activation of this latent virus (15). However, the alternative possibility has not been rigorously excluded that the presence of the virus and its associated antigen in such tumors is a secondary phenomenon without etiological significance. Although this possibility would admittedly entail a most remarkable coincidence, precedent for it exists in the demonstration that leukemic cells may be superinfected with unrelated leukemogenic viruses and thereby undergo "antigenic conversion," acquiring the specific antigens associated with these agents (26). To discriminate sharply between these 2 alternatives, experiments were designed to test the effect of specifically antiviral treatments, such as the injection of interferon or of virus-neutralizing antibody, on the incidence of radiation-induced lymphomas in C57BL mice. If RadLV were present in these tumors merely as a "passenger," specific antiviral treatments would not be expected to alter tumor incidence; conversely, if RadLV is the actual causative agent, significant inhibition of tumor development should occur.

One such approach has already been reported; it was found that multiple injections of interferon significantly reduced the incidence and delayed the onset of lymphomas in C57BL mice exposed to appropriate doses of whole-body X-radiation (19). Unfortunately, this result cannot be unambiguously interpreted as conclusive proof of the etiological role of RadLV in the induction of radiogenic lymphomas because interferon has also been shown to have other, presumably virus-unrelated effects, such as the inhibition of tumor cell growth (8, 17). The alternative approach of treating radiated mice with virus-specific immune sera was made possible by our earlier demonstration that the serum of W/Fu rats immunized with syngeneic RadLV-induced lymphomas contains virus-neutralizing antibodies specific for RadLV (7). This paper presents the results of 3 such experiments and of others stimulated by their unanticipated outcome. Although the original question which we sought to answer remains incompletely resolved, the significant degree of apparently specific, as well as nonspecific, inhibition of radiation leukemogenesis which these experiments have revealed seems worthy of publication.

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2Recipient of an Eleanor Roosevelt International Cancer Research Fellowship. Present address: School of Veterinary Medicine, University of Pennsylvania, New Bolton Center, Kennett Square, Pa. 19348.

3The abbreviations used are: RadLV, radiation leukemia virus; W/Fu, Wistar-Furth; FCA, Freund's complete adjuvant; PBS, phosphate-buffered saline; FCS, fetal calf serum; RLP, radiation leukemia protective factor.

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MATERIALS AND METHODS

Animals. The mice used were strain C57BL/Ka from our inbred colony. Inbred W/Fu rats were purchased from Microbiological Associates, Inc., Walkersville, Md., and were 2 to 3 months old when received. W/Fu female rats were crossed in our laboratory with inbred BN male rats, also obtained from Microbiological Associates, to produce (W/Fu x BN) F₁ hybrids. The rats were maintained in quarters separate from those housing mice.

Sera. Normal serum was obtained from the rats by bleeding on the day following their arrival in the laboratory. In this way, we hoped to avoid any contamination with RadLV and thus the induction of anti-RadLV antibodies in their serum. The serum was heated for 30 min at 56°C and absorbed with C57BL/Ka erythrocytes (there was no visible clumping) before storage at -70°C. For the production of antisera to RadLV, W/Fu or W/Fu x BN F₁ rats were immunized by repeated injections of cells from RadLV-induced W/Fu rat lymphomas maintained in continuous passage through radiated W/Fu rats (7). The immunization procedure described previously (7) was modified later in that FCA was mixed 1:1 with the cell suspension for the 1st injection of the series. The antisera were processed in the same manner as the normal sera. The presence of anti-RadLV antibodies in the antisera was measured by cytotoxicity tests against either W/Fu or C57BL/Ka lymphomas induced by virus. To obviate the observed fluctuation in titer between individual sera, we pooled them for use, so that each batch had a titer of no less than 1:32 and usually at least 1:64 in cytotoxic tests. Some sera were also tested for neutralization of RadLV activity by the plaque assay with XC rat cells (25) and were found to be effective. In contrast, the normal rat sera had no effect in this assay, nor were they cytotoxic to RadLV-induced lymphoma cells, except as noted below.

Radiation. Mice were given 4 weekly whole-body X-ray exposures, begun when they were 5 to 7 weeks old. The dose given at each exposure is specified in the individual experiments. Physical factors were: 250 kV, 1.0 mm A1 + 0.25 mm Cu filter, 83 R/min.

Serological Tests. Details of the techniques, as well as of the origin and characteristics of the lymphomas used in the in vitro cytotoxic and absorption tests, have already been described (6, 7).

RESULTS

In the 1st 2 experiments, mice were given a series of i.p. injections of 0.3 to 0.4 ml of immune serum, normal serum, or PBS, starting 3 days before the 1st X-ray exposure, continuing during X-ray treatment for a total of 5 injections (9 in the 2nd run), and ending 5 days after the last exposure. This schedule was chosen so that it would provide antibody to the animal during the period in which viral activation is assumed to take place, i.e., after each exposure to radiation (9, 10). X-ray dosage was 160 R/exposure. Since both runs yielded similar results, the data have been combined in Table 1, Experiments 1 and 2. There is clearly protection in the group receiving antisera; lymphoma incidence was reduced to 55%, as compared with 85% in the PBS-injected controls ($\chi^2 = 5.1$); and there was a 40-day increase in the median latent period to death. However, the observed incidence in the other control group receiving normal serum was slightly lower than that in the PBS controls, and their median latent period was intermediate between those of the other 2 groups; thus, the difference between the immune serum and normal serum groups was not statistically significant.

For this reason, we repeated the experiment a 3rd time, with 2 modifications: the dose of X-rays per exposure was reduced to 130 R, to avoid overwhelming a possibly relatively weak protective effect of antiserum by a too powerful leukemogenic stimulus, and the duration of passive immunization was extended. The 1st injection was given on the day of the 1st X-ray exposure and continued twice weekly until 6 weeks following termination of X-ray treatment. Under these conditions, the protective effect of normal serum was even more distinct; it was as active as immune serum in reducing lymphoma incidence, although the median latent period in the group so treated was intermediate between the other 2 groups (Table 1, Experiment 3).

Two alternative explanations of the protective effect of normal rat serum were considered. First, there might be natural antibodies to RadLV-associated antigens in the serum of some normal W/Fu rats; this would not be surprising, since antibodies to Gross leukemia virus-associated antigens, which completely cross-react with the RadLV-associated antigens in both cytotoxic (7) and virus neutralization (J. F. Ferrer, manuscript in preparation) tests, are reportedly present in such sera (23). Indeed, one of us (J. F. Ferrer, unpublished observation) has detected antibodies cytotoxic to RadLV lymphoma cells of C57BL/Ka mice and/or Gross virus-induced E7G2 lymphoma of C57BL mice in W/Fu rats of our colony at 4 or more months of age. Further evidence for the existence of natural antibodies to RadLV antigens comes from absorption experiments summarized in Table 2. The cytotoxicity of a serum pool from 4- to 8-month-old normal (BN x W/Fu) F₁ female rats for the highly antigenic, virus-releasing RadLV lymphoma line LW-27 was completely removed by absorption with these cells but not by its apparently virus- and antigen-free subline (LW-27)B (6). However, these rats were older than those used as normal serum donors in the present in vivo experiments. Moreover, 1 batch of normal rat serum failed to neutralize RadLV in vitro as measured by a modified plaque assay.

Alternatively, there might be a nonspecific stimulation of host immune defenses in general as a result of the immunizing course of treatment with a foreign serum. This hypothesis was tested in the following experiment: C57BL/Ka mice were given a series of injections of (a) FCS, which had previously been heated for 30 min at 56°C and absorbed with C57BL/Ka erythrocytes; (b) FCA; or (c) PBS, in conjunction with a leukemogenic course of whole-body X-radiation (140 R/exposure), begun when they were 7 weeks old. The injection series was begun 1 week before the 1st X-ray ex-
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### Table 1

**Lymphoma development in radiated C57BL/Ka mice receiving injections of rat anti-RadLV serum**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No./net total</th>
<th>%</th>
<th>$x^2$ Values</th>
<th>Median latent period (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiments 1 and 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immune rat serum</td>
<td>26/47</td>
<td>55</td>
<td>3.6</td>
<td>182</td>
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<tr>
<td>Normal rat serum</td>
<td>23/30</td>
<td>77</td>
<td>5.1</td>
<td>167</td>
</tr>
<tr>
<td>PBS controls</td>
<td>22/26</td>
<td>85</td>
<td>&lt;1</td>
<td>143</td>
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<tr>
<td>Experiment 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immune rat serum</td>
<td>9/34</td>
<td>26</td>
<td>&lt;1</td>
<td>217</td>
</tr>
<tr>
<td>Normal rat serum</td>
<td>6/27</td>
<td>22</td>
<td>4.9</td>
<td>187</td>
</tr>
<tr>
<td>PBS controls</td>
<td>18/32</td>
<td>56</td>
<td>5.7</td>
<td>148</td>
</tr>
</tbody>
</table>

### Table 2

**In vitro absorption of the cytotoxicity of pooled sera from normal (W/Fu x BN) F1 hybrid rats for a RadLV-induced lymphoma**

<table>
<thead>
<tr>
<th>Absorption with</th>
<th>% dead target cells in serum dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>LW-27 (RadLV-induced lymphoma in a W/Fu rat) (LW-27)B (apparently virus- and antigen-free subline of LW-27)</td>
<td>&lt;10 10 &lt;10 &lt;10</td>
</tr>
<tr>
<td>None</td>
<td>80 67 47 nr*</td>
</tr>
</tbody>
</table>

* nr, not read.

### Table 3

**Lymphoma development in radiated C57BL/Ka mice receiving injections of FCS or FCA**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No./net total</th>
<th>%</th>
<th>$x^2$</th>
<th>Median latent period (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCS</td>
<td>11/35</td>
<td>31</td>
<td>12.9</td>
<td>199</td>
</tr>
<tr>
<td>PBS controls</td>
<td>26/35</td>
<td>74</td>
<td>3.6</td>
<td>188</td>
</tr>
<tr>
<td>FCA</td>
<td>41/74</td>
<td>55</td>
<td></td>
<td>239</td>
</tr>
</tbody>
</table>

RadLV serum inhibits development of lymphomas in C57BL mice exposed to leukemogenic dose of X-radiation. Although pronounced and reproducible inhibition was observed, the fact that inhibition was also observed with normal rat serum and even with FCS clouds the interpretation of these results.

If specific antibodies were, at least in part, responsible for the protection afforded by the immune rat serum, it seems unlikely that this effect is due to the killing of incipient tumor cells by anti-RadLV cytotoxic antibodies which are also present in such sera (7). Such cytotoxic antibodies usually require complement as a cofactor for their cytocidal action, and mice are known to be deficient in complement. In addition, whereas rat serum and guinea pig serum have adequate complementary activity to support the in vitro cytotoxicity of the RadLV antiserum, serum from C57BL mice does not. It seems unlikely that the rat antiserum also supplied complement to the C57BL recipients, since it had been heated to 56° for 30 min. Thus, it seems reasonable to ascribe the antileukemogenic effect of the immune rat serum to its specific virus-neutralizing activity.

It is well established that natural antibody to the Gross

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leukemia virus develops spontaneously in several low-leukemia strains of mice (1). Normal rats several months of age also possess natural antibodies to the antigens specifically associated with the murine leukemia viruses (23); direct evidence for this phenomenon in the case of RadLV-associated antigen is presented in Table 2. If this is the correct explanation, as seems likely, the observed inhibitory effect of normal rat serum, like that of antiserum, would be ascribable to specific RadLV-neutralizing antibody. However, we have no evidence that natural antibody actually existed in the normal sera used here; the rats used as normal serum donors were only 2 to 3 months old when bled, and 1 batch of their serum failed to exhibit neutralizing activity for RadLV in a modified XC cell plaque assay (25). Moreover, this possibility cannot in any case explain the observed inhibitory effect of FCS, which would not be expected to contain such antibodies.

An alternative interpretation, which is not mutually incompatible with passive antibody action, is that repeated injections of these foreign sera may have been a sufficiently intense immunogenic stimulus to result also in the nonspecific augmentation of the immune defense mechanisms of the host animals in general. Consistent with this possibility is the reported observation that AKR mice pretreated with FCA (12) or chronically infected with intracellular parasites (11, 21) exhibit significantly increased incidence resistance to the development of spontaneous thymic lymphomas. The relevant mechanisms may involve the activation of macrophages (5, 13) or lymphocytes (4). Berenblum et al. (2, 3) have described a subcellular, nondialyzable factor from sheep spleen capable of inhibiting radiation leukemiaogenesis in C57BL mice. This inhibitory substance, designated RLP, has also been found by Hodes et al. (14) in bovine spleen extracts. The observation that RLP is precipitable between 0 and 60% saturation with ammonium sulfate and that the globulin fraction but not the albumin fraction of adult bovine serum also protects mice from radiation leukemiaogenesis suggested that this factor is a globulin (3, 14). It is entirely conceivable that the effect of RLP may also be mediated through the nonspecific stimulation of host immune surveillance mechanisms. The failure of the bovine albumin to afford protection could then be attributed to insufficient immunogenicity at the dose level utilized. On the other hand, it is also possible that RLP is a specific factor that is also present in the rat sera and that exerts its action through a nonimmunological mechanisms.

The fact that injection of normal foreign sera may inhibit leukemiaogenesis is important per se, since some investigations have used normal horse serum as the control injection against which the effect of other substances has been tested (24). The role of nonspecific immune surveillance processes in protecting C57BL mice against radiation leukemiaogenesis is under continuing study in this laboratory.

In view of the unanticipated finding of these nonspecific protective effects of foreign sera, it is now clear that an unambiguous test of the hypothesis that RadLV is the actual etiological agent in radiation leukemiaogenesis will require the use of anti-RadLV sera prepared in syngeneic mice. Although it has not been possible to immunize C57BL mice against “wild-type” RadLV freshly extracted from lymphoid tumors in vivo, preliminary evidence indicates that this may be accomplished with attenuated RadLV harvested from supernatant culture fluids of chronically infected C57BL mouse embryo fibroblasts in vitro (20). There is precedent for such an effect in the demonstrated immunogenicity of tissue culture-passaged Rauscher leukemia virus or Moloney leukemia virus in strains of mice known to be susceptible to Moloney leukemia virus (22).

REFERENCES

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