Formation of Cytoxic Antibody against Leukemias Induced by Friend Virus*

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SUMMARY

A specific antigenic component in mouse leukemias induced by Friend virus has been demonstrated by cytotoxic tests in vitro. Serum from mice of several inbred strains immunized with histoincompatible Friend leukemia tissue contained antibody that was cytotoxic in vitro for leukemia cells induced by Friend virus in the strains providing the immune serum. Unexpectedly, a high proportion of mice from strains that are highly susceptible to leukemogenesis by Friend virus did not develop leukemia after immunization as adults with incompatible viable Friend leukemia cells or with large amounts of infective filtrate. Cytotoxic sera could therefore be prepared in mice of both resistant and susceptible strains. By direct cytotoxic tests, and also by absorption studies, the Friend and Rauscher viruses appeared to induce the same antigenic change in the spleen cells of infected animals. A similar antigen has not been found in a variety of normal and leukemia tissues, including leukemias induced by the Passage A Gross virus.

Two findings might suggest the intervention of a specific immune response during the induction of Friend virus leukemia (FVL). In the first place, treatment with formalinized filtrates of Friend leukemia tissue can protect mice against subsequent inoculation of active Friend virus preparations (6). Secondly, mice of the C3H strain that are resistant as adults to induction of leukemia by Friend virus appear susceptible when the virus is injected into newborn recipients (42). The possibility exists that the resistance of adults of the C3H and other strains is determined by immunological factors. This view is supported by the finding, reported here, that mice immunized against Friend leukemia tissue form antibodies that are specifically cytotoxic for Friend leukemia cells.1 Cytotoxic antisera have been obtained not only from mice of resistant strains but also from mice that are highly susceptible to the induction of leukemia by Friend virus in adult life.

MATERIALS AND METHODS

Mice.—Mice of the following strains were obtained from our own inbred colonies: BALB/c, DBA/2, C3H/An, C3Hf/Bi, C57BL/6, A, and I. These colonies are maintained by monogamous brother-to-sister matings from a single line of descent. Random-bred Ha/ICR Swiss mice were purchased from Millerton Farms, N.Y.

Viruses.—Friend virus (5): A frozen filtrate of spleen from mice infected with the Friend virus was provided by Dr. Charlotte Friend, Sloan-Kettering Institute. Routine passage was performed in Swiss mice by I.P. inoculation of 0.2 ml. of a 20 per cent leukemic spleen homogenate. Leukemias were induced in those inbred strains that are susceptible as adults, by I.P. injection of 0.2 ml. of a filtered (Selas 02) centrifuged 20 per cent homogenate of spleens from leukemic Swiss mice. Inbred mice of the two resistant strains, A and (C3H/An × D)F1, were given I.P. injections within the first 3 days after birth of 0.1 ml. or 0.2 ml. of this filtrate.

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Rauscher virus (17): a frozen centrifuged 10 per cent extract of spleen from BALB/c mice infected with the Rauscher virus was provided by Dr. F. J. Rauscher, National Institutes of Health. The spleen cells used in this study were all taken from A and BALB/c mice infected as weanlings with 0.2 ml. of this original material. Animals infected with Rauscher virus were maintained in quarters separate from mice infected with the Friend virus.

Gross virus, Passage A (12): C3H/Bi mice with leukemia induced by Passage A virus were obtained from Dr. L. Gross, Veterans Administration Hospital, Bronx, N.Y. A frozen filtrate (Selas 02) with an initial S.C. inoculum of $10 \times 10^6$ viable cells (unstained by trypan blue), followed by $55 \times 10^6$ cells and $100 \times 10^6$ cells intraperitoneally at intervals of 3 weeks.

BALB/c mice immunized with leukemia filtrate received 1 ml. standard filtrate (see above) intraperitoneally, followed by two injections of 1 ml. each at intervals of 10 and 7 weeks.

Serum was obtained by tail bleeding, 2 weeks after the final inoculation, excluding those mice that developed leukemia during the course of immunization.

Cytotoxic test.—The method used is based on the technic of Gorer and O’Gorman (11). Leukemic spleens were obtained from mice infected with Swiss FVL filtrates 10–46 days previously. The cells were prepared as described for immunization and washed once in Medium 199. In order to remove a majority of the erythrocytes, the excised spleens from animals infected with Friend virus or Rauscher viruses were first perfused with 3–5 ml. of Medium 199 by means of a No. 27 needle inserted at several points through the intact capsule.

Equal volumes (0.1 ml.) of washed cells (10 $\times 10^6$/ml), antiserum dilution, and guinea pig serum (diluted $\frac{1}{2}$—complement source) were incubated for 45 minutes at 37°C. Per cent viability was determined microscopically after the addition of 0.2 ml. trypan blue solution prepared as described elsewhere (2). The diluent was Medium 199. In every test controls were included in which the cells were incubated with either guinea

### TABLE 1

<table>
<thead>
<tr>
<th>GENOTYPE OF MICE INOCULATED</th>
<th>SWISS FVL FILTRATE (0.2 ml. 20% SPLEEN HOMOGENATE I.P.)</th>
<th>VIALBLE SWISS FVL CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\varphi$</td>
<td>$\sigma$</td>
</tr>
<tr>
<td>DBA/2</td>
<td>4/9</td>
<td>5/9</td>
</tr>
<tr>
<td>BALB/c</td>
<td>22/34</td>
<td>6/9</td>
</tr>
<tr>
<td>C3H/An</td>
<td>20/31</td>
<td>8/10</td>
</tr>
<tr>
<td>(BALB/c×C3H/An)F$_1$</td>
<td>6/11</td>
<td>3/5</td>
</tr>
<tr>
<td>(C3H/An×1)F$_1$</td>
<td>0/9</td>
<td>1/5</td>
</tr>
<tr>
<td>A</td>
<td>1/8</td>
<td>0/12</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>0/12</td>
<td>0/12</td>
</tr>
</tbody>
</table>

* All mice were 2–3 months old at the time of first inoculation.
† Number of mice dying with splenomegaly/number of mice inoculated. (Period of observation > 3 months.)
‡ Figures in this column refer to mice dying with FVL during or following the course of immunization with viable FVL cells. See text for schedule of immunization.

source of virus for leukemia induction in newborn BALB/c and C3H/Bi mice of our colonies.

**Spontaneous and x-radiation-induced leukemias.**
—Eleven spontaneous or induced leukemias were used in control studies. These leukemias, which are named in the relevant sections of “Results” all arose in our inbred stocks within the past 2 years.

**Immunization procedure.**—Suspensions of viable cells were obtained from Swiss mice infected with Friend virus 2–6 weeks previously. The greatly enlarged leukemic spleens were minced with a fine, curved scissors in Medium 199 (Microbiological Associates, Bethesda, Md.). Tissue fragments were allowed to settle out and were discarded, leaving a suspension of single cells. Female mice of the strains listed in Table 1 (with the exception of those given filtrate) were immunized with Swiss FVL filtrates 10–46 days previously. The cells were prepared as described for immunization and washed once in Medium 199. In order to remove a majority of the erythrocytes, the excised spleens from animals infected with Friend or Rauscher viruses were first perfused with 3–5 ml. of Medium 199 by means of a No. 27 needle inserted at several points through the intact capsule.

Equal volumes (0.1 ml.) of washed cells (10 $\times 10^6$/ml), antiserum dilution, and guinea pig serum (diluted $\frac{1}{2}$—complement source) were incubated for 45 minutes at 37°C. Per cent viability was determined microscopically after the addition of 0.2 ml. trypan blue solution prepared as described elsewhere (2). The diluent was Medium 199. In every test controls were included in which the cells were incubated with either guinea...
pig serum or antiserum alone; these invariably contained no more than 10 per cent dead cells and are therefore omitted from the tables.

Absorption technic.—Each antiserum was diluted according to its cytotoxic titer (approximately two doubling dilutions below the end-point) and absorbed with an equal volume of packed washed cells for 1 hour at room temperature, followed by 1 hour at 4° C. The serum was recovered after centrifugation in the cold.

RESULTS

The susceptibility of the various strains of mice used in the present study to the induction for testing the sera from these two resistant mouse strains were obtained by infecting mice within the first few days of birth. This invariably produced leukemia in these two strains. C57BL/6 mice, however, were resistant to the virus even as newborns.

It was also found that adult mice of the sensitive BALB/c strain given injections of 1 ml. of 20 per cent leukemic spleen filtrate, rather than the standard leukemogenic inoculum of 0.2 ml., displayed a striking reduction in leukemia incidence (5/14 mice injected with 1 ml. developed leukemia as compared with 29/31 mice injected with 0.2 ml.). Further immunization with filtrates was effective in inducing the formation of cytotoxic sera against isogenic2 FVL cells (Table 2).

The A and BALB/c antisera (Table 2) were also tested against a variety of isogenic leukemias and against normal spleen cells. These had no cytotoxic effect upon normal spleen cells, nor upon any of the following eight isogenic transplanted leukemias of recent origin:

A strain.—two spontaneous lymphatic leukemias (ASL1 and ASL3);—four leukemias induced by x-radiation (RADA1-4).

BALB/c.—one leukemia induced by Passage A Gross virus;—one granulocytic leukemia

2 The term isogenic indicates that the cells used for testing were derived from the same strain as that in which the antiserum was prepared.
(BALBL1) which originated in a mouse treated with dibenz[a,h]anthracene.

Both the A and the BALB/c antisera, however, were cytotoxic for isogenic spleen cells from mice infected with the Rauscher virus (Table 3). These cells were taken from mice during the early phase of infection when splenomegaly is the prominent feature. As yet no tests have been performed with the later lymphatic leukemias that are known to follow infection with this virus (17).

The specificity of the cytotoxic reaction against FVL cells was tested further with five hyper-immune sera prepared in A strain mice against the following five unrelated incompatible leukemias:

C57BL/6. —two leukemias induced by x-radiation (ERLA and ERLD). (C57BL/6 × A)F1. —two leukemias induced by x-radiation (EARAD1 and EARAD2). C57Bl/—one leukemia induced by Passage A Gross virus. None of these antisera was cytotoxic for A strain FVL cells.

Data from absorption experiments fully confirm the results obtained by the direct cytotoxic test.

### TABLE 3

**Cytotoxic Tests on Spleen Cells from Adult BALB/c or A Strain Mice Infected with Rauscher Virus**

<table>
<thead>
<tr>
<th>Antiserum and test cells*</th>
<th>Antiserum dilutions:</th>
<th>% Cells stained by trypan blue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1/8</td>
<td>1/4</td>
</tr>
<tr>
<td>BALB/c ♀</td>
<td>88</td>
<td>50</td>
</tr>
<tr>
<td>A ♀</td>
<td>45</td>
<td>55</td>
</tr>
</tbody>
</table>

* Antiserum prepared against Swiss FVL and titrated with isogenic Rauscher spleen cells. Spleen cells from mice infected with Rauscher virus 33 days (BALB/c) and 75 (A strain) days previously.

The only tissues capable of removing the specific cytotoxic activity for isogenic FVL cells were cells from the enlarged spleens of animals infected with either Friend or Rauscher virus. Table 4 shows one of several experiments with absorbed sera. Activity was removed by absorption with BALB/c or Swiss FVL cells and also by cells from the spleens of BALB/c mice infected with Rauscher virus. Neither normal tissues nor three unrelated leukemias, including one induced by Passage A Gross virus, produced any reduction in cytotoxic activity against FVL cells. Similarly, although the activity of an A anti-Swiss FVL serum against A strain FVL cells was completely abolished by absorption with spleens from A mice infected with Friend or Rauscher virus, it was not affected by absorption with the following three unrelated leukemias:

- A strain.—one spontaneous lymphatic leukemia (ASL1);—one leukemia induced by x-radiation (RADA1). (C57BL/6 × A)F1.—one leukemia induced by x-radiation (EARAD1).

### DISCUSSION

These observations establish the fact that immunization with incompatible Friend leukemia tissue gives rise to antibody that is cytotoxic for
leukemia cells induced by Friend virus. Genetic heterogeneity as an explanation of these findings appears to be excluded on the following grounds: (a) intrastain skin grafting in mice from our colonies has given no indication of histoincompatibility; (b) specific cytotoxic antiserum has been found in mice of a variety of different unrelated inbred strains, (c) no other normal or neoplastic tissue, with the exception of spleen cells from mice infected with the Rauscher virus, has shown any sensitivity to antisera against FVL; these other tissues could neither absorb specific cytotoxic activity nor induce the formation of specific cytotoxic antiserum.

In the case of mice that are resistant as adults to the Friend leukemia virus it was necessary to induce the disease in newborn hosts in order to obtain a source of isogenic leukemic cells for testing. It was first thought that in susceptible strains it would be impossible to obtain immune serum, since the immunizing material might be expected to contain sufficient virus to induce leukemia in all recipients. However, it was found that immunization with progressively larger inoculations of incompatible viable cells was not accompanied by the development of leukemia in the majority of mice—in fact, these animals similarly formed cytotoxic antibodies against isogenic Friend leukemia cells.

Evidently the method of immunization with intact cells favors the development of resistance, even in highly susceptible strains, such as DBA/2. The C3H and BALB/c strains, although now sensitive to filtrates of Friend leukemic tissue (4), originally appeared resistant to leukemia induction (7). It is probable, therefore, that all strains possess a substantial degree of resistance, capable of expression under appropriate circumstances.

A high proportion of BALB/c mice given injections of 1 ml. of a 20 per cent Swiss FVL filtrate did not develop leukemia. This is in contrast to the 90–100 per cent incidence of leukemia in BALB/c mice receiving 0.2 ml. of a similar filtrate. Mice immunized with filtrate, like those immunized with viable cells, produced antiserum with cytotoxic activity against isogenic FVL cells. A reduction in the incidence of leukemias or solid tumors in mice, as a result of inoculating unusually large quantities of infective material, has been observed with other oncogenic viruses (1, 10). It is possible that initial exposure of the host to large amounts of antigen, whether this be virus or new components of cells transformed by virus, facilitates the development of an early effective immune response.

The demonstration of cytotoxic antibody, appearing in the course of immunization with Friend leukemia tissue, follows the findings of Slettenmark and Klein (21) with Gross leukemia. In both instances, serological data point to the presence of a common antigen in different leukemias induced by the same agent in mice of both similar and dissimilar genotypes. The same conclusion is indicated by cross-immunity in vivo among leukemias induced by the Gross virus (15) and among other classes of virus-induced tumors in the mouse where no cytotoxic antibody has as yet been demonstrated (13, 14, 18–20). In the case of tumors induced by polyoma virus, there is evidence that the immunity is directed against a new cellular antigen and is not mediated by antibody against the virus itself (14, 19). This will be particularly difficult to determine for Friend virus leukemia. In the first place, resistance to challenge with filtrates would not distinguish between immunity directed against the virus itself and immunity against a new cellular antigen. Second, transplantation of isogenic Friend leukemia cells apparently gives rise to new virus-induced leukemias (16). However, variant lines of the Friend leukemia that will grow as solid tumors on serial transplantation (3, 8, 9) may be of use in distinguishing between these two possibilities.

The direct cytotoxic test with mouse antiserum indicated a common antigenic component shared by spleen cells of mice infected by the Rauscher or Friend virus. This cross-reaction was fully confirmed by absorption experiments, in which all cytotoxic activity of sera prepared against FVL cells was removed by spleen cells from mice in the early phase of Rauscher disease. Rauscher has reported that rabbit antiserum against Friend virus apparently did not inactivate the Rauscher virus (17). Although it is possible that two unrelated viruses might induce the same cellular change, it seems more probable that the antigenic similarity revealed by the cytotoxic test indicates a close relationship between the Friend virus and the agent responsible for the splenomegalic phase of the Rauscher disease. It remains to be seen whether cells from the lymphatic leukemias induced by the Rauscher virus also contain the same antigenic component as FVL cells.

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