Effect of Antilymphocytic and Antithymocytic Sera on the Development of Mouse Lymphoma

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SUMMARY

Injections of antisera to lymphoid tissues of C3H/HeJ mice significantly increased the incidence of (a) lymphoma in normally resistant C3H/HeJ adult mice injected 4 times with Gross leukemia virus and (b) growth of allogeneic lymphomas in C3H/HeJ and AKR mice when compared to similarly treated animals injected with normal rabbit serum. The same antilymphoid cell sera were able to suppress sheep red blood cell hemagglutinin responses in parallel sets of mice.

Immunosuppressive drugs, 6-mercaptopurine (100 mg/kg of diet) and azathioprine (100, 150, or 200 mg/kg of diets or 150 mg/kg mouse injected intraperitoneally), did not increase the incidence of lymphoma in C3H/HeJ adult mice receiving multiple injections of Gross leukemia virus or depress the hemagglutinin response to sheep red blood cells.

There was a tendency towards prolongation of latent periods of spontaneous lymphoma in AKR mice and virus-induced lymphoma in C3H/HeJ mice injected with C3H/HeJ antithymocytic serum prior to development of lymphoma. Incidence and latent period of virus-accelerated lymphoma in AKR mice was not altered by administration of the antithymocytic serum during the latent period of lymphomagenesis.

INTRODUCTION

The purpose of this study was to investigate the effects of antilymphocytic and antithymocytic sera on the development of spontaneous and virus-induced lymphoma in mice. The selection of ALS and ATS was based on results of a comparative study, reported here, of effects of the sera and drugs, 6-MP and azathioprine, on immune responses of AKR and C3H mice. These agents have been shown to be effective in suppressing immune responses to a variety of antigens (15, 16, 19–21, 23).

Antilymphocytic sera have been shown to potentiate adenovirus and polyoma virus oncogenesis (1, 2) and Moloney and Rauscher virus leukemogenesis (2, 13), possibly by depression of antiviral immune mechanisms. On the other hand, in lymphocytic choriomeningitis virus infection in mice (11, 14), where virus infection has been postulated to be a result of host immunologic response, ATS prevented or delayed the disease. Tumor-specific antigens, which may be distinct from viral antigens (12, 17), arise on the neoplastic cells. The question then was how do antigenic tumor cells survive and grow without being eliminated immunologically by the host? Establishment and growth of the “foreign” neoplastic clone might be postulated to occur when host immune responses are depressed. Indeed, evidence is accumulating that there is a period of immunologic deficiency in preleukemic animals (6–8, 22). One could then ask whether experimental immunosuppression with antilymphoid cell serum during the prelymphoma period in mice would decrease the latent period or increase the incidence of lymphoma.

On the other hand, antilymphoid cell sera might inactivate prelymphoma or lymphoma cells, resulting in an increased latent period and decreased incidence of lymphoma. The effects of ALS and ATS in various host-virus and -tumor cell combinations were investigated here using strains of mice which were tolerant or reactive to viral and tumor-specific antigens.

MATERIALS AND METHODS

Mice. AKR and C3H/HeJ (referred to here as C3H) mice from strains originally obtained from The Jackson Laboratory and maintained by continuous, single-line, brother and sister mating, were used for experiments reported here. Mice received a standard pellet diet with water ad libitum. AKR mice have histocompatibility factors H-2k, H-7a; C3H/HeJ mice have factors H-1a, H-2k, H-5b, H-6a (24).

Experiments on adult animals were performed with mice of one sex. Experiments in which newborn animals were injected with virus were done with mixed groups of both sexes.

Lymphomas. A Gross virus-induced lymphoma obtained from a C3Hf/Bi mouse with primary lymphoma induced by passage A virus supplied by Dr. Ludwik Gross was used. AKR
animals from the 22nd to 25th passage of filtrates to newborn mice were used as sources of virus-induced lymphomas.

**Cell-free Filtrates.** The thymus, lymph nodes, spleen, and a portion of the liver were removed from AKR mice which had developed Gross virus-induced lymphoma after inoculation of 0.1 ml of tumor filtrates as newborns. The organs were placed in preservative-free saline (20% w/v) and homogenized with a mortar and pestle. The homogenate was centrifuged at 3000 X g for 15 minutes, and the resulting supernatant was centrifuged at 7000 X g for 10 minutes. This final supernatant was passed through a 0.2 Selas filter. All procedures were carried out at 4°C. One-tenth ml of this filtrate, referred to here as virus, was used for injection into newborn (0–72 hour) mice.

Accelerated lymphoma developed in 100% of 100 newborn-injected AKR mice at an average of 83.5 ± 13.9 (standard deviation) days and in 65% of 100 C3H/HeJ mice at 144.1 ± 84.2 days. The latent period for spontaneous lymphoma in the AKR colony was 285.9 ± 77.1 (143 animals). Spontaneous lymphoma has not been observed in C3H mice less than one year of age.

**Tumor Passage.** Tumor cells derived from AKR or C3H mice injected with cell-free filtrates as newborns were passed to adult syngeneic mice for a minimum of two passages before experimental use. Following inoculation of 10⁶ to 10⁷ cells subcutaneously, recipients developed lymphoma resulting in death at 11–17 days.

**Hemagglutination Tests.** Blood was obtained from cut tails and collected in glass capillary tubes. Serum was collected following centrifugation of the tubes in a hematocrit centrifuge. Hemagglutination tests were performed on samples from individual mice with a Microtiter apparatus (Cooke Engineering Company) using serial dilutions of serum samples and a 2% suspension of washed SRBC as antigen. Plates were incubated 2 hours at room temperature. Titters are expressed as the log₂ of the last dilution of serum showing 1+ agglutination of erythrocytes.

**Sera.** White New Zealand rabbits were injected subcutaneously with a homogenate of tissue in equal parts of Freund’s complete adjuvant (Difco). C3H thymus was used for production of ATS, and pooled C3H axillary, brachial, inguinal, and mesenteric lymph nodes were used for production of ALS. Boosters were given at two-week intervals with homogenates of tissues in saline. Animals were bled 3 to 14 days after booster injections. Normal rabbit serum was obtained from New Zealand rabbits bled at intervals of 1 to 4 weeks. Sera were stored at −70°C for periods up to one month prior to injection.

**Titration of ALS and ATS by Leukoagglutination.** Titration of ALS and ATS was done according to the method of Monaco et al. (21) modified after a method of Amos and Peacocke (3). Rabbits were used as donors for ALS or ATS when leukoagglutination titters equaled or exceeded 1:64.

**Drugs.** 6-MP (Burroughs Wellcome and Co.) was mixed with Purina mouse diet in powder form and made into pellets with a final drug concentration of 100 mg/kg diet. Twenty-five-gram mice consuming 5 gm of diet per day thus received a dose of 20 mg drug per kg mouse. Bulk azathioprine (Imuran) was kindly supplied by Burroughs Wellcome Co. The drug was incorporated into Purina mouse diet at three concentrations: 100 mg/kg diet (20 mg/kg mouse/day), 150 mg/kg diet (30 mg/kg mouse/day), and 200 mg/kg diet (40 mg/kg mouse/day). Mice on drug diets were supplied with water ad libitum.

Azathioprine as the sodium salt for intraperitoneal injections was also supplied by Burroughs Wellcome Company. Injection doses were incorporated in sterile physiologic saline. New lots were made for each daily injection series.

**RESULTS**

Effect of Immunosuppressive Drugs and Antilymphocytic Serum on Adult C3H Mice Injected with Virus. A comparison of four agents potentially immunosuppressive was made in adult C3H mice: 6-MP, 100 mg/kg diet; azathioprine, 150 mg/kg diet; azathioprine i.p. injections, 150 mg/kg mouse/day; and ALS (Table 1). Agents were compared as to their effectiveness in suppressing (a) SRBC hemagglutinins and (b) host mechanisms involved in inactivation of lymphoma virus. C3H adult mice, normally resistant to lymphoma, received 4 weekly injections of CFF intraperitoneally. Introduction of drug diets and the injections are detailed in Table 1.

Drug diets and injections did not suppress SRBC hemagglutinins or increase the incidence of lymphoma in adult C3H mice injected with virus (Table 1). In further studies of effects of other dosages of 6-MP and azathioprine on hemagglutinin responses in AKR and C3H mice, groups of 3 to 24 animals were placed on 6-MP or azathioprine (100, 150, or 200 mg/kg diet) for periods of 0 to 31 days prior to SRBC injection. Hemagglutinin responses assayed 7 to 12 days after antigen injection did not differ significantly from those of animals on normal diets.

The immunosuppressive effect of C3H ALS on hemagglutinins to SRBC injected at the end of the treatment is clearly evident by the decreased titers. Five animals had titers of "0". Titters of mice injected with antilymphocytic serum were significantly different from those of mice on normal diets (t = 7.681, P < 0.001) or mice receiving normal rabbit serum injections (t = 2.755, P < 0.02). Controls injected with normal rabbit serum showed slight depression of the hemagglutination titers which was of marginal significance when compared to titers of mice on normal diets (t = 2.086, P = 0.05).

The most significant finding is the correlation of immunosuppression of SRBC hemagglutinins with the increased incidence of lymphoma in the animals injected with ALS (Table 1). Thus, the ALS in the course of injections used here may suppress the host’s immune mechanisms for elimination of the lymphoma virus.

Other work was done to see whether a single injection of virus could induce lymphoma in adult C3H mice when a course of ATS was given before or after virus injection. Three injection schedules of ATS were used: (a) a 6-day course of ATS started 9 days prior to injection of CFF, (b) a 4-day course of ATS initiated 1 day after injection of CFF, and (c) a 4-day course of ATS initiated 5 days after CFF injection. Lymphoma was not noted in control mice or in animals given ATS in any of the 3 schedules.

With this evidence (Table 1) that antilymphoid cell serum treatment was capable of altering host immune responses to
Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days treatment received</th>
<th>Incidence of lymphomas</th>
<th>Incidence of natural deaths&lt;sup&gt;a&lt;/sup&gt; (not due to lymphomas)</th>
<th>$\log_2$ hemagglutination titers; arithmetic mean ± S.D.&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-MP diet</td>
<td>0–32</td>
<td>0/15</td>
<td>5/15 (36.0 ± 1.9)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.7 ± 0.5</td>
</tr>
<tr>
<td>(100 mg/kg diet)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azathioprine diet</td>
<td>0–32</td>
<td>0/15</td>
<td>2/15 (84, 186)</td>
<td>6.8 ± 0.6</td>
</tr>
<tr>
<td>(150 mg/kg diet)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azathioprine injections</td>
<td>0–4</td>
<td>1/15</td>
<td>7/15 (20.6 ± 4.1)</td>
<td>7.0 ± 0.8</td>
</tr>
<tr>
<td>(150 mg/kg mouse)</td>
<td>7–11</td>
<td>(144)</td>
<td>1/15</td>
<td>6.2 ± 1.1</td>
</tr>
<tr>
<td>Normal diet</td>
<td>1/15</td>
<td>(200)</td>
<td>1/15</td>
<td></td>
</tr>
<tr>
<td>C3H ALS</td>
<td>0, 3–6, 11–14, 17–21, 24, 25</td>
<td>5/15</td>
<td>7/15 (87)</td>
<td>1.4 ± 1.6</td>
</tr>
<tr>
<td>Normal rabbit serum</td>
<td>0, 3–6, 11–14, 17–21, 24, 25</td>
<td>0/15</td>
<td>7/15 (16.6 ± 6.7)</td>
<td>4.2 ± 2.3</td>
</tr>
</tbody>
</table>

Lymphoma and other deaths in adult C3H mice injected with CFF on Days 0, 6, 14, and 21 of the experiment and placed on diet or injection treatment indicated. All mice were 76 ± 3 days of age at Day 0 of the experiment. Remaining mice in the experiment were alive without signs of lymphoma at 9 months postinitiation of treatment. See Footnote 2 for abbreviations.

<sup>a</sup>None of the animals was sacrificed. Autopsy disclosed no evidence of lymphoma.

<sup>b</sup>0.2 ml of 20% SRBC was injected intraperitoneally on Day 25 of the experiment. Mice were bled 8 days later.

<sup>c</sup>Days (mean ± standard deviation, or raw data) postinitiation of treatment at which death occurred.

Injections of lymphoma virus or SRBC, further work was initiated to determine the most efficient course of serum injections in C3H and AKR mice. C3H antithymocytic serum was chosen for test since previous work had indicated that this serum was a more effective immunosuppressive agent than antilymphocytic serum (9).

Effect of ATS on Allogeneic Tumor Grafts. Adult AKR and C3H mice were placed on a course of 5, 7, or 10 injections of ATS (Table 2). The 7- and 10-day courses were interrupted by a 2-day rest beginning at Day 5; this resulted in a total time for the injection course of 9 and 12 days for the two groups. On the last day of treatment, parallel sets of mice were challenged either with SRBC intraperitoneally or allogeneic tumor cells subcutaneously.

Again depression of SRBC hemagglutinin titers was used to assay immunosuppressive activity of the serum. Mice treated with ATS showed consistently lower hemagglutination titers than mice injected with NRS. Correlating with the depressed hemagglutinin titers is the growth of allogeneic tumor cells. Only those recipients dying of the transplanted tumor are reported here. It is apparent that a 5-day course of ATS is immunosuppressive to SRBC hemagglutinins and allogeneic tumor cell rejection in both AKR and C3H mice. This course of ATS injections was used for subsequent experiments on immunosuppression in prelymphomatoid animals.

Effect of ATS on Syngeneic Tumor Cell Growth. It was necessary to see whether ATS had a direct effect on the growth of large doses of syngeneic tumor cells. Mice were

Table 2

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Serum</th>
<th>Number of days</th>
<th>Incidence allogeneic tumor growth resulting in death</th>
<th>$\log_2$ hemagglutination titers</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKR</td>
<td>C3H ATS</td>
<td>5</td>
<td>4/4</td>
<td>4, 4, 4, 6</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>5</td>
<td>0/4</td>
<td>7, 8, 8</td>
</tr>
<tr>
<td></td>
<td>C3H ATS</td>
<td>7</td>
<td>2/4</td>
<td>2, 2, 3, 5</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>7</td>
<td>0/4</td>
<td>6, 7, 7</td>
</tr>
<tr>
<td></td>
<td>C3H ATS</td>
<td>10</td>
<td>3/4</td>
<td>4, 5, 5, 5</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>10</td>
<td>0/4</td>
<td>4, 8</td>
</tr>
<tr>
<td>C3H</td>
<td>C3H ATS</td>
<td>5</td>
<td>2/4</td>
<td>1, 1, 2</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>5</td>
<td>0/4</td>
<td>6, 8, 9</td>
</tr>
<tr>
<td></td>
<td>C3H ATS</td>
<td>7</td>
<td>2/2</td>
<td>not done</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>7</td>
<td>0/4</td>
<td>7, 7, 7</td>
</tr>
<tr>
<td></td>
<td>C3H ATS</td>
<td>10</td>
<td>4/4</td>
<td>3, 4, 4, 5</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>10</td>
<td>0/4</td>
<td>6, 6</td>
</tr>
</tbody>
</table>

Incidence of allogeneic tumor growth and hemagglutination titers in AKR or C3H mice injected with C3H ATS or control NRS. Tumor challenge and SRBC injection were done in parallel sets of mice on last day of serum injection series. Hemagglutination titers were tested 8 days later. See Footnote 2 for abbreviations.
Effect of ATS on Virus-induced Lymphoma. Newborn AKR and C3H mice were injected intraperitoneally with Gross leukemia virus. At 28 to 36 days of age parallel sets of mice were injected with either C3H ATS or NRS (Table 3). The incidence and latent period for the development of lymphoma in the AKR mice injected with ATS did not differ significantly when compared to controls injected with NRS. In tests with C3H mice there did not appear to be a trend toward a shorter latent period in the mice injected with ATS when compared to animals injected with NRS. Rather, a prolongation of the latent period in mice injected with ATS was indicated.

In further work on AKR virus-accelerated lymphoma, AKR mice were made “tolerant” to ATS by injection of the serum within 48 hours of birth (Table 4). The assumption was made that the immunosuppressive action of ATS would persist longer, i.e., the rabbit antiserum would not be eliminated as rapidly in the tolerant recipients. In one group of animals, no further injections of serum were given to test the effects of the tolerance-inducing serum injection during the neonatal period on lymphomagenesis. In a second set of animals, a second injection of ATS was given 23 days after birth to induce immunosuppression. A parallel set of control animals received NRS. Again, no significant difference in the incidence or latent period of lymphoma could be demonstrated when this group of animals was compared to the previous group injected with ATS at 28 to 36 days of age (Table 3).

Throughout these experiments on virus-induced lymphoma in AKR and C3H mice, comparisons may be made to the latent period of lymphoma in large groups selected at random from the colony mice injected with CFF as newborns and held until death with no further treatment. One hundred animals from the AKR and 65 animals from the C3H colonies, all of which lived until development of virus-accelerated lymphoma, had the following latent periods: AKR, 83.5 ± 13.9; C3H, 144.1 ± 84.2 days.

Effect of Combined Serum-Drug Immunosuppression on Virus-induced Lymphoma in AKR Mice. Newborn AKR mice were injected with CFF. At 33 days of age animals were placed on a diet incorporating 100 mg/kg diet of azathioprine; this was continued throughout the experiment. Beginning at Day 6 of postinitiation of diet, mice were further subjected to alternating 5-day courses of ATS and ALS during the period ranging between Days 6 and 35 postinitiation of diet. Four animals died of causes other than lymphoma at Days 46, 49, 52, and 66 of age. The remaining 9 animals developed lymphoma at 82.5 ± 10.1 days of age. There was a depressed hemagglutinin response to SRBC injected at 29 days postinitiation of drug diet (arithmetic mean ± S.D. of log2 titer = 1.5 ± 1.3). Thus, in spite of intensive drug–ALS-ATS immunosuppression, the latent period for lymphoma did not differ significantly from that of 100 mice selected at random from the colony with a latent period of 83.5 ± 13.9 for virus-accelerated lymphoma.

Effect of ATS on Spontaneous Lymphoma. AKR or C3H mice 2 to 2.5 months of age received 14 injections of ATS over an 18-day time period (Table 5). Mice were then held without further treatment until death or sacrifice. All living animals were sacrificed at 300 days. The incidence of lymphoma was lower in the AKR animals receiving ATS when compared to the NRS-injected controls. The latent period for spontaneous lymphoma in the AKR colony for nontreated animals is 285.9 ± 77.1 days (143 animals). These data indicate that ATS might decrease the incidence or prolong the latent period of lymphoma in the AKR mice.
DISCUSSION

A comparative study of antilymphocytic serum and the drugs, 6-MP and azathioprine, demonstrated that, in the doses and manner of administration used here, ALS is the most effective immunosuppressive agent to SRBC hemagglutinins in AKR and C3H mice. Furthermore, a prolonged course of ALS given simultaneously with a course of 4 Gross leukemia virus (CFF) injections resulted in a significant increase in the development of lymphoma in adult C3H mice. This may be due to the immunosuppressive action of ALS during the critical period of induction of immune response to the foreign viral antigens. A single injection of CFF, preceded or followed by treatment with ATS, did not result in lymphoma in adult C3H mice.

In a study of this nature, the antilymphocytic sera should not include antibodies which could inactivate lymphoma virus and increase the latent period of lymphomagenesis. Even AKR thymus tissue studied before lymphoma develops has been shown to contain Gross leukemia virus antigens (25); thus, AKR tissue could not be used as the antigen for production of ATS. Hence, C3H/HeJ animals, sharing the major histocompatibility antigen with AKR mice (24), were used as donors of thymus tissue for immunization of rabbits.

In using a reagent such as ATS in prelymphomatoid mice, several types of host-serum reactions might occur. It has been shown that leukemia in the high leukemia mouse strain Ak originates in the thymus (10). The leukemia cells then colonize in numerous organs. Whether ATS actually reacted with lymphoma cell precursors or thymic lymphoma factor was not tested directly in work reported here; however, virus-accelerated lymphomagenesis was not affected by injection of ATS beginning at 28 to 36 days of age.

As a first consideration, ATS might be viewed as an immunosuppressive agent, active against immunologically reactive cells but not against tumor cells. The finding that virus-accelerated lymphomagenesis was not affected by ATS would be expected since AKR mice are already tolerant to the Gross antigen (4). Work by Wahren (25, 26) and Hays (12) has shown that AKR animals are also tolerant to the tumor-specific antigen developing on lymphoma cells. If tolerance exists to viral as well as cellular antigens, further immunosuppression should have no effect on lymphomagenesis. However, this does not explain the increased latent period of lymphomagenesis in AKR mice developing spontaneous lymphoma. A direct action of ATS on preneoplastic and neoplastic cells, resulting in a decreased incidence or increased latent period of lymphomagenesis, should be considered. The prolongation of the latent period in spontaneous lymphoma in AKR mice might be due to the stage during which ATS was administered. In the virus-accelerated lymphoma, more precursors of lymphoma cells or neoplastic cells themselves might have been present during the period 28 to 36 days of age, when ATS treatment was initiated, than during the period 2 to 2.5 months of age period, during which ATS administration was initiated in spontaneous lymphoma. Tumorcidal action of the ATS might not have been apparent in the larger cell population. Indeed, data reported here indicated that growth of \(1 \times 10^7\) syngeneic tumor cells was not affected by injections of ATS to recipients. It is also possible that ATS does not affect tumor cells directly but acts on thymus cells or factors essential in lymphomagenesis.

Careful consideration must be given to the time when ATS administration was initiated. Berenbaum showed that humoral antibody mechanisms are most sensitive to ATS when the serum is given simultaneously with or before antigen (5). Thus, further studies with the virus and mouse strain systems used here should be done using different times of ATS administration.

One experiment reported here suggested that virus-induced lymphoma in C3H mice might not be accelerated by ATS, but actually curtailed. C3H mice receiving ATS prior to development of virus-induced lymphoma developed the disease later than controls injected with NRS. This pointed again to a direct or indirect action of the ATS on the preneoplastic or neoplastic cell. This effect might occur most readily early in lymphomagenesis.

On the other hand, if ATS had acted primarily as an immunosuppressive agent in C3H prelymphomatoid mice, another line of reasoning should have been considered. There is good evidence that transplantation resistance can arise in C3H mice against lymphomas induced by Gross virus by pretreating them with allogeneic grafts of Gross lymphomas or subthreshold doses of cells from the same lymphoma (18). Thus, normal adult C3H mice are not tolerant to the tumor-specific antigens of Gross virus-induced lymphomas. C3H mice could be made tolerant to virus by neonatal injections but could remain immunologically reactive to tumor-specific antigens arising later. Therefore, immunosuppression of this strain during the prelymphoma stage might result in latent periods and incidences of lymphoma in C3H mice comparable to AKR mice with virus-accelerated lymphoma. Evidence to support this line of reasoning was not presented here.

These experiments indicated that C3H ATS may inactivate prelymphoma or lymphoma cells during the latent period of spontaneous lymphoma in AKR mice or virus-induced lymphoma in C3H mice. ATS may also suppress mechanisms involved in elimination of multiple doses of lymphoma virus injected intraperitoneally in C3H mice and rejection of allogeneic tumor tissue.

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REFERENCES

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