Effect of Transient Immunosuppression on Host Response to Neonatally Introduced Oncogenic Virus

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SUMMARY

A significant incidence of humoral antibodies that react with the virion of the mammary tumor virus in immunodiffusion is found in adult mice, which had been infected neonatally with the virus, following their recovery from transient immunodepression induced by antithymocyte serum.

INTRODUCTION

There is considerable evidence that the use of immunosuppressive treatments that mainly affect cell-mediated immune reactivity, such as neonatal thymectomy or injection of ATS and ALS, increases the incidence of "spontaneous" or induced tumors in experimental animals (16, 21, 24). Nevertheless, there are some exceptions to this generalization. The effect of neonatal thymectomy on the incidence of viral-induced leukemias in mice is inhibitory (17). Immunodepression also inhibits the development of tumors of the mouse mammary gland induced by the MTV. There have been several reports that neonatal thymectomy decreases mammary tumor incidence or increases the latent period in MTV-infected mice (11, 22, 23), and this laboratory has recently reported that a similar decrease in the incidence of MTV-induced mammary tumors is observed after treatment with ALS (15).

It has been suggested (18) that the inhibiting effect of thymectomy on the development of MTV-induced mammary tumors could result from either an alteration in the hormonal milieu, the necessity of the thymus for the growth and multiplication of MTV, or a relationship between oncogenesis and the immunological responsiveness of the host. Evidence of such hormonal (22) and immunological (10) changes has been reported. This communication deals with the 1st part of an investigation of the possibility that the similar inhibiting effect of treatment with ALS or ATS on mammary tumor development might be the result of an enhanced immunological reactivity to MTV or to cells altered by MTV following recovery of the mice from the transient immunodepression. Treatment with ATS is herein reported to lead to the development of circulating antibodies against the MTV virion.

MATERIALS AND METHODS

The inbred virgin female C3H and BALB/cfC3H mice used in these experiments were derived from the colony of the Cancer Research Genetics Laboratory. Mice of both of these strains are infected with MTV soon after birth via their mother's milk. C3H mice normally carry MTV. BALB/c mice do not carry the MTV; our subline of BALB/cfC3H mice was originated by fostering newborn BALB/c mice on lactating C3H females.

Two experiments were carried out, 1 with C3H mice and 1 with BALB/cfC3H mice. BALB/cfC3H females received injections of either ATS or NRS. C3H females received injections of either ATG or NRG. In both experiments, 11 i.p. injections were given to each mouse. The first 7 injections were given daily, and the last 4 injections were given every 2nd or 3rd day thereafter; the 11th injection was given 15 days after the 1st injection.

Rabbits were immunized to provide the several pools of immunosuppressive ATS used in these and related experiments. For each pool of serum, 2 to 4 young adult, female, New Zealand rabbits were given monthly injections, in several s.c. sites, of a suspension of BALB/c thymocytes obtained from 7- to 10-day-old mice. The thymocytes were harvested by forcing the thymic tissue through a fine mesh; a total of \(1 \times 10^8\) cells emulsified in incomplete Freund's adjuvant were given to each rabbit each month. From 7 to 14 days after the 2nd injection of antigen, the rabbits were bled 3 times to obtain a total of approximately 75 ml serum per rabbit. This procedure was repeated after the 3rd and 4th injections of antigen. After each collection, the serum was separated and stored at \(-4^\circ\). After the collection of the last serum sample, the samples were unfrozen, decomplemented by heat inactivation, pooled, and refrozen at \(-4^\circ\) until needed. One such pool of rabbit ATS was used in the treatment of the C3H females, and a 2nd pool was used in the treatment of the BALB/cfC3H females. NRS for injection into the control mice was obtained by bleeding normal, uninoculated rabbits.

ATG and NRG were prepared from pooled serum samples by precipitating the globulins in saturated ammonium sulfate, pH 7.8 to 7.9, and redissolving them 3 times in 0.9% NaCl solution. The final solutions were extensively dialyzed against 0.9% NaCl solution in the cold and then stored at \(-4^\circ\) until needed. The amount of globulin given to each C3H female at...
Each injection was equivalent to that contained in 1 ml of initial ATS. The BALB/cfC3H females received in each injection 0.25 ml of either ATS or NRS.

The immunosuppressive activity of these and other ATS and ATG pools was routinely determined by testing the time that elapsed after initiation of the standard series of 11 injections before the test mice recovered from the immunodepression sufficiently to reject a skin allograft. Within a few days after the 1st ATS or ATG injection, full-thickness skin grafts were prepared and secured in place with Air-Vent tape (Johnson & Johnson, New Brunswick, N. J.). The tape bandages were removed 1.5 weeks after grafting, and the grafts were scored daily for signs of rejection. Most serum pools were tested by observing the survival time of DBA/2 skin allografts on BALB/c or BALB/cfC3H female mice. Average survival time of such allografts on control normal or NRS-treated mice is approximately 14 days. Recovery (measured by graft rejection) of the ATS-treated or ATG-treated mice occurred within 3 to 4.5 weeks after the initiation of ATS or ATG treatment. The ATG used for injection of the C3H females was also tested for immunosuppressive activity in C3H test mice, which then received allografts from mice of the A strain. Average survival time of these skin allografts on NRG-treated mice was approximately 14 days. Recovery (measured by graft rejection) of the ATG-treated mice given the same dose of ATG subsequently used for the experimental mice occurred 4 to 5.5 weeks after the initiation of the ATG injections.

The C3H females were given injections of ATG or NRG when they were 8 to 9 months old. Serum samples were collected from the mice every other week thereafter for a total of 7 collections.

The BALB/cfC3H females were given injections of ATS or NRS when they were 3 months old. A total of 5 serum samples were collected from each mouse; the collections were spaced over a period of 14 weeks after the completion of the ATS or NRS injections.

Each mouse was bled from the retroorbital plexus. The serum was separated by incubation, and the individual serum samples were frozen at —4° until tested. Not all sera were tested in immunodiffusion on the same day, but all sera collected at 1 time (from both the ATS- or ATG-treated mice and the NRS- or NRG-treated controls) were tested at the same time.

During the experimental period, the C3H females received either 1 or 2 inoculations of isogenic mammary tumor cells to test their cell-mediated immune reactivity. Some of the C3H mice (17 ATG-treated and 11 NRG-treated mice) received tumor cells on the same day that the serum injections were initiated. Tumors from the 1st transplant generation of an MTV-induced mammary tumor arising in a multiparous C3H female were minced, and a suspension of single tumor cells was prepared and diluted so that each mouse received 10⁶ living cells in a s.c. inoculation. No tumor inoculations were given at this time to an additional 13 ATG-treated and 15 NRG-treated C3H females. After the 4th collection of serum, all the females, including those not previously inoculated, received similar inoculations of tumor cells. The cells were derived from the 2nd transplant generation of the same tumor that was used for the 1st inoculation.

Although the BALB/cfC3H females were inoculated with isogenic mammary tumor cells at a later time, they did not receive any inoculation of cells during the time in which the serum samples were collected.

The immunodiffusion assay for MTV was used to detect antibodies in the treated mice. The assay as developed and currently used in this laboratory detects antibodies directed against the MTV B-type virus particle (3). The virion itself is the antigenic unit; it can be detected by electron microscopy in the precipitate line that develops after reaction of the particles with specific antiserum (8). The participation of the virion in this precipitate line has been further documented in ferritin labeling studies (7).

The immunodiffusion plates were prepared with 0.6% Noble’s agar, 0.85% NaCl, and 1:10,000 Merthiolate in distilled water at pH 7. Wells were cut 4 mm apart in the agar with a metal tube (diameter, 4 mm). The antigen wells were filled on Day 1 with a preparation of MTV virions purified from BALB/cfC3H milk by sucrose-density-gradient centrifugation, and the individual, undiluted, unabsorbed mouse serum samples were added to the other wells on Days 1, 2, and 3. The plates were maintained in a closed container in a 37° incubator. Precipitate lines were usually visible within a few days, but the plates were routinely examined for approximately 10 days.

The x² test, in which Yates' correction is used where appropriate, or the Mann-Whitney U test were used to determine the statistical significance of the observed differences. Differences were considered significant only if $p \leq 0.05$.

RESULTS

The incidence of C3H females that produce antibodies against the MTV virion was calculated twice, once including only the test results of the 4 serum samples collected from some of the females prior to the inoculation of the tumor isograft (Table 1), and the 2nd time including the test results of all of the serum samples (Table 2). We have previously reported that C3H females can be induced to produce antibodies against MTV virions by the inoculation of an isogenic, MTV-induced mammary tumor (6); it was thus necessary in this experiment to separate the results of injection with ATG alone from the results of the combined treatment of ATG injection and tumor isograft inoculation.

In the series of 4 serum samples collected over a 10-week period from mice treated with ATG or NRG alone (no tumor inoculation), antibodies were detected only in the mice treated with ATG (Table 1). None of the 15 C3H females given injections of NRG developed detectable levels of circulating antibodies against the MTV virion during this time. On the other hand, 8 of the 13 C3H females given injections of ATG did produce such antibodies, an incidence of 62%. This difference is statistically significant. Sera positive for antibody were first detected in some of the females 3 weeks after the initiation of the globulin treatment; the incidence rose to 62% by 10 weeks.

In those C3H females inoculated with isogenic, MTV-
Production of antibodies against MTV virion antigens by female mice neonatally infected with MTV after treatment with ATS or ATG

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Samples of BALB/cfC3H sera were examined for antibodies periodically for 16 weeks after the beginning of serum treatment. Samples of C3H serum could be tested and used in this compilation of data for only 10 weeks after the initiation of serum treatment, since at Week 10 the mice received a tumor isograft. After inoculation of the tumor cells, an additional 2 ATG-treated mice developed detectable antibody titers, as did 5 of the NRG-treated mice (see Table 2).

### Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serum treatment</th>
<th>No. of mice</th>
<th>No. producing antibodies</th>
<th>% producing antibodies</th>
<th>Time of antibody appearance (wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/cfC3H</td>
<td>ATS</td>
<td>24</td>
<td>7</td>
<td>29</td>
<td>6–10</td>
</tr>
<tr>
<td>C3H</td>
<td>ATG</td>
<td>13</td>
<td>8</td>
<td>62</td>
<td>3–10</td>
</tr>
<tr>
<td>C3H</td>
<td>NRS</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2

Production of antibodies against MTV virion antigens by C3H female mice neonatally infected with MTV after injection of ATG or NRG and after inoculation of C3H mammary tumor isografts

<table>
<thead>
<tr>
<th>Serum treatment</th>
<th>Tumor isograft at time of serum injection</th>
<th>Tumor isograft 10 wk after serum injection</th>
<th>No. of mice</th>
<th>No. producing antibodies</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATG</td>
<td>+</td>
<td>+</td>
<td>17</td>
<td>15</td>
<td>88</td>
</tr>
<tr>
<td>ATG</td>
<td>0</td>
<td>+</td>
<td>13</td>
<td>10</td>
<td>77</td>
</tr>
<tr>
<td>NRG</td>
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<td>11</td>
<td>5a</td>
<td>45</td>
</tr>
<tr>
<td>NRG</td>
<td>0</td>
<td>+</td>
<td>15</td>
<td>5a</td>
<td>33</td>
</tr>
</tbody>
</table>

* No NRG-treated mice developed detectable levels of antibody against MTV prior to the implantation of the tumor isografts.

induced mammary tumor cells at the initiation of ATG or NRG treatment and receiving a 2nd inoculation of tumor cells 10 weeks later, both ATG-treated and NRG-treated mice developed antibodies against MTV as anticipated (Table 2). In those mice initially receiving only ATG or NRG injections but then receiving an inoculation of isogenic, MTV-induced mammary tumor cells 10 weeks later, an additional 2 ATG-treated mice developed detectable antibody levels following tumor inoculation, which increased the total incidence to 10 of 13 (77%). Further, whereas none of the 15 NRG-treated mice had developed detectable antibodies within the 1st 10 weeks after initiation of the globulin treatment, 5 of these females did so after the inoculation of the tumor cells (33%).

Little difference in final tumor incidence in females producing positive serum samples was observed between the ATG-treated mice receiving 1 as compared to 2 tumor cell inoculations (77% and 88%, respectively) or between the NRG-treated mice receiving 1 as compared to 2 tumor cell inoculations (33% and 45%, respectively). However, comparison of the incidence in ATG-treated mice with that in NRG-treated mice, regardless of the number of tumor cell inoculations, shows a considerable difference in the incidence of mice producing antibodies. Of the ATG-treated mice, 25 of 30 developed detectable antibody levels (83%), whereas only 10 of 26 NRG-treated mice did so (38%). This difference is statistically significant.

Similar results were observed in the treated BALB/cfC3H mice. Beginning 6 weeks after the initiation of the ATS or NRS treatment, antibodies against MTV virions could be detected in the sera of some of the ATS-treated females. Within 6 to 10 weeks after treatment, 7 of 24 females (29%) produced positive serum samples (Table 1). No NRS-treated females developed detectable antibody levels for 10 weeks after the initiation of the NRS injections; after that time, 2 of the 24 females (8%) produced positive serum samples. The difference between the ATS-treated and the NRS-treated groups is statistically significant by the Mann-Whitney U test.

**DISCUSSION**

These results demonstrate that mice neonatally infected with MTV can produce detectable quantities of antibodies against the MTV virion following treatment with an immunosuppressant.

Reports from this and other laboratories have clearly demonstrated that mice neonatally infected with MTV do not respond immunologically to MTV-associated cellular antigens following immunization and challenge with MTV-infected mammary tumors, although they can be induced to produce antibodies against the MTV virion (for reviews, see Refs. 2 and 5). Similar results demonstrating a relative deficiency in cell-mediated immunity as compared to humoral antibody response in the neonatally infected "tolerant" animal have been reported for other viruses (9, 13, 14).
One possible explanation for such observations is that mature virion antigens may not be available to immunologically competent cells during development (19, 20), so that tolerance is not induced or maintained, whereas cells altered by the viral genome and possessing distinctive antigenicity may be present early and commonly. If this is so, the effect of the transient immunodepression in the mouse neonatally infected with MTV may be to produce a viremia. After recovery from the immunodepression, the mouse may now respond by producing antibodies against the MTV virions.

The idea that immunodepression may activate latent viral infections or alter the type of pathology produced has been clearly established for other virus-host systems (1, 12). Such changes have been reported both for nononcogenic viruses such as canine distemper virus and LCM virus, as well as for oncogenic viruses such as polyoma, murine leukemia virus, and murine sarcoma virus.

Since the mammary gland tissue is already infected with MTV prior to the age at which circulating antibodies against the virion were detected in the treated females, the observations reported here do not provide a direct explanation for the fact that treatment with ATS prior to the age of MTV-induced mammary tumor onset is so effective in preventing the normal development of these tumors in the neonatally infected mouse (15).

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