SUMMARY

Sera from BALB/c mice bearing progressively growing tumors induced by Moloney sarcoma virus (progressor serum) or from BALB/c × DBA/2 F₁ mice immunized with Moloney sarcoma virus-induced tumor cells of C57BL origin (allogeneic serum) were tested in immunotherapy experiments against a transplantable Moloney leukemia virus-induced lymphoma (LSTRA). The progressor serum had previously been reported to contain low antibody titers as determined in indirect membrane immunofluorescence and neutralization assays while the allogeneic serum contained high antibody titers in both assays. Multiple inoculations of the allogeneic but not the progressor serum into mice with systemic LSTRA disease resulted in a significantly increased median survival time over controls, but there were no long-term survivors. When single and multiple injections of allogeneic serum were administered to diseased mice following high-dose 1,3-bis(2-chloroethyl)-1-nitrosourea therapy (25 mg/kg), there were 79 and 95%, respectively, long-term survivors as opposed to 34% in the group treated with drug alone. Interestingly, multiple injections of normal serum also increased the percentage of survivors to 54%. Following low-dose 1,3-bis(2-chloroethyl)-1-nitrosourea therapy (15 mg/kg), administration of the allogeneic serum produced up to 40% survivors as opposed to none in the control groups. The results indicate that well-defined immune sera should be considered in experiments investigating combined chemoimmunotherapy as an approach to the control of cancer.

INTRODUCTION

Immunological factors such as immune lymphoid cells, serum, or immunological stimulators such as Bacillus Calmette-Guérin have been reported to be effective in preventing tumor production or inducing regressions of small palpable tumors (2, 7–9, 13, 14, 16). In general, however, the use of these factors alone in eradicating established tumors has met with little success. Immunotherapy as an adjunct to chemotherapy, X-irradiation, or surgery, however, has yielded promising results in the treatment of established animal tumors (3, 11). These results demonstrated the importance of reducing the tumor burden before applying specific immunotherapeutic measures to eliminate residual tumor cells.

We recently reported that certain immune sera were highly effective in preventing the growth not only of a transplantable Moloney virus-induced sarcoma but also of a highly lethal Moloney virus-induced lymphoma (LSTRA). This was true when the sera were given before tumor cell challenge or during the latent period after tumor inoculation but before tumor appearance (10). The results indicated that the effectiveness of a serum in preventing tumor growth correlated closely with the presence of antibody directed against Moloney virus-associated antigens as determined by the membrane immunofluorescence and neutralization assays. Sera with high antibody titers as determined by these assays prevented tumor induction while sera with low titers were ineffective.

The present study was designed to determine whether high-titered immune sera would be successful in eradicating established LSTRA disease when given either alone or after drug therapy. The results demonstrate that these sera were very effective in producing high percentages of long-term survivors when used as an adjunct to chemotherapy.

MATERIALS AND METHODS

Mice. Adult BALB/c × DBA/2 F₁ (CD2F₁) mice, males, 8 to 12 weeks old, were obtained from Charles River Breeding Laboratories, Wilmington, Mass. The animals were housed in plastic cages and fed Purina laboratory chow with water ad libitum.

Tumor. A Moloney lymphoid leukemia line (LSTRA), originally induced in BALB/c mice by the murine leukemia virus (Moloney), has been maintained and passaged routinely in our laboratory as a transplantable tumor line for over 300 generations in CD2F₁ mice. The ascites tumor is serially transplanted i.p. at weekly intervals.

Drug. BCNU¹ was kindly supplied by the Cancer Chemotherapy National Service Center, National Cancer Institute, NIH, Bethesda, Md. The alkylating agent was dissolved in a steroid-suspending vehicle and administered s.c. in a constant volume of 0.01 ml/g of body weight.

Immune Sera. The production and characterization of antisera to MSV-induced antigens have previously been described in detail (10). Two pools of immune sera prepared in different manners were used in the investigations reported here. The 1st pool referred to as “progressor serum” was prepared from BALB/c mice with progressively growing tumors induced by MSV. Serum was collected approximately 50 to 60 days after virus inoculation.

¹The abbreviations used are: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; MSV, Moloney sarcoma virus; MF, indirect membrane immunofluorescence; MST, median survival time.
The 2nd pool referred to as "allogeneic serum" was prepared in CD2F1 mice by multiple immunization with MSV-induced tumor cells of C57BL origin. The serum pools were heat inactivated at 56°C and then titrated for antibodies directed against MSV-induced antigens by MF, neutralization, and cytotoxicity as previously described (10). Three different pools of the "allogeneic serum" and 1 pool of the "progressor serum" were used in these experiments.

RESULTS

Effect of Immune Sera on Established LSTRA Disease.

It was previously reported that the allogeneic serum (MF titer > 128) but not the progressor serum (MF titer = 8 to 16) or a 3rd pooled serum prepared from mice following the regression of MSV-induced tumors (regressor serum; MF titer = 64) prevented the development of this leukemia when given as early as 3 days before tumor challenge or during the latent period before tumor development (10).

It was of interest, therefore, to determine the effect of this particular serum when given at a time when the disease was systemic. The leukemia can be detected systemically as determined by tissue bioassy 7 days after inoculation of 1 x 10⁴ LSTRA cells (11).

CD2F1 mice were given inoculations s.c. of 1 x 10⁴ LSTRA cells. Starting at 7 days after LSTRA inoculation, the mice received single or multiple injections of normal, progressor, or allogeneic serum. The mice were monitored 3 times a week for tumor size and death, and the MST and percentage of survivors in each group were determined. The composite results of 2 experiments are presented in Table 1. Single or multiple inoculations of normal or progressor serum had no effect on the course of this leukemia when compared to the untreated controls. The MST in this group was 13 days as opposed to 14 days in the groups receiving normal or progressor serum. Interestingly, there was no evidence for tumor growth enhancement in the groups inoculated with the progressor serum as had been previously noted with MSV-induced tumor (10). There were no survivors in any of these groups. Likewise, a single injection of the allogeneic serum was ineffective against LSTRA disease. However, multiple injections of this serum on Days 7, 9, and 11 after tumor challenge significantly increased the MST from 13 and 14 to 18 days (p < 0.01).

All animals, however, succumbed to the disease.

Combined Serum Therapy with High-Dose Chemotherapy.

The effect of different doses of BCNU on the induction of remission in diseased mice was previously reported (11). Twenty-five mg/kg was determined as the highest effective dose that could be used without producing apparent signs of drug toxicity. This dose of drug usually produced 20 to 30% long-term survivors and doubled the MST as compared to untreated controls. Animals treated with higher drug concentrations occasionally succumbed as a result of the effects of the drug.

Since multiple injections of the allogeneic serum were found to prolong significantly the life of mice with systemic LSTRA disease although there were no survivors, it was decided to test the effectiveness of this serum when given following reduction of the tumor load with drug therapy. Mice given inoculations of 1 x 10⁴ LSTRA cells were treated with 25 mg of BCNU per kg 7 days later when the disease was known to be systemic. These mice were then treated with single or multiple injections of normal or allogeneic serum starting 3 days later (Day 10). The composite results of 3 experiments are shown in Table 2. Treatment with BCNU only (Group 2) produced 34% long-term survivors when the experiments were terminated at 100 days. The MST was increased from 13 to 26 days. A single inoculation of normal serum following drug therapy (Group 3) had no apparent effect on survivors or MST when compared to drug alone. Interestingly, multiple injections of normal serum following drug therapy (Group 4) increased the percentage of survivors from 34 to 54% (p < 0.07) and increased the MST from 26 to > 100 days. Both single and multiple inoculations of the allogeneic serum (Groups 5 and 6) were highly effective in increasing the number of long-term survivors when given following BCNU therapy. Seventy-nine % of the mice (p < 0.001) that received 1 injection and 95% (p < 0.001) that received 3 injections of the allogeneic serum were still alive when the experiments were terminated at 100 days.

In another experiment, not reported here, the progressor serum was ineffective in increasing the MST or the percentage of survivors when compared with the groups treated with drug plus normal serum.

Combined Serum Therapy with Low-Dose Chemotherapy.

Since the above results indicated that the allogeneic serum was highly effective when combined with high-dose drug therapy in producing long-term survivors, it was decided to determine the effectiveness of this serum when used following a lower dose of BCNU (15 mg/kg). The results of this experiment are presented in Table 3. Treatment of mice with leukemia with 15 mg of BCNU per kg (Group 2) increased the MST from 13 to 21 days but there were no long-term survivors. Single or multiple inoculations of normal serum following drug therapy (Groups 3 and 4) was no more effective than drug alone. However, inoculation of the allogeneic serum following BCNU therapy resulted in a significant number of long-term survivors and an increased MST. There were 40% survivors when the experiment was terminated at 100 days following 1 injection of serum (Group 5) and 29% survivors following 3 injections (Group 6). The difference in survivors in these 2 groups is not significant. The MST in both groups was 28 days.

DISCUSSION

The results reported here demonstrate that 1 of 2 immune sera tested in these experiments was effective against a transplantable Moloney virus-induced leukemia particularly when used as an adjunct to chemotherapy. This serum (designated "allogeneic serum") contained high antibody titers against Moloney virus-induced cell surface and envelope antigens. The other serum (progressor serum), which was ineffective against systemic LSTRA disease, contained low antibody levels as measured by the 2 tests. The effectiveness of the allogeneic serum in producing high percentages of long-term survivors following
Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mice</th>
<th>Serum</th>
<th>Days post-inoculation</th>
<th>MST (days)</th>
<th>Range of death (days)</th>
<th>% survivors</th>
</tr>
</thead>
<tbody>
<tr>
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<td>11–17</td>
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<td></td>
</tr>
<tr>
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<td>25</td>
<td>Normal</td>
<td>7</td>
<td>11–14</td>
<td>0</td>
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<tr>
<td>3</td>
<td>24</td>
<td>Normal</td>
<td>7, 9, 11</td>
<td>14</td>
<td>11–16</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>24</td>
<td>Progressor</td>
<td>7</td>
<td>14</td>
<td>11–23</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>Progressor</td>
<td>7, 9, 11</td>
<td>14</td>
<td>11–22</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>22</td>
<td>Allogeneic</td>
<td>7</td>
<td>16</td>
<td>12–25</td>
<td>0</td>
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<tr>
<td>7</td>
<td>24</td>
<td>Allogeneic</td>
<td>7, 9, 11</td>
<td>18 c</td>
<td>14–28</td>
<td>0</td>
</tr>
</tbody>
</table>

* LSTRA cell (1 x 10^6) inoculated s.c. on Day 0. Mice inoculations i.p. of 0.2 ml serum at different times after LSTRA inoculation.

b CD2F. p < 0.01 when compared with Group 3 as determined by Student’s t test.

c p < 0.01 when compared with Group 3 as determined by Student’s t test.

d p < 0.01 when compared with Group 4 as determined by Student’s t test.

e p < 0.01 when compared with Group 4 as determined by Student’s t test.

Table 2

Combined therapy on LSTRA disease with allogeneic immune serum following high-dose BCNU chemotherapy

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mice</th>
<th>LSTRA (Day 0)*</th>
<th>BCNU Day 7 (mg/kg)*</th>
<th>Serum</th>
<th>Days post-inoculation</th>
<th>MST (days)</th>
<th>Range of death (days)</th>
<th>% survivors 100 (days)</th>
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<tr>
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<td>40</td>
<td>1 x 10^6</td>
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<td>13</td>
<td>11–17</td>
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<td></td>
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<tr>
<td>2</td>
<td>38</td>
<td>1 x 10^6</td>
<td>25</td>
<td>Normal</td>
<td>10</td>
<td>26</td>
<td>19–30</td>
<td>34</td>
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<tr>
<td>3</td>
<td>39</td>
<td>1 x 10^6</td>
<td>25</td>
<td>Normal</td>
<td>10, 12, 14</td>
<td>&gt; 100</td>
<td>21–47</td>
<td>54</td>
</tr>
<tr>
<td>4</td>
<td>39</td>
<td>1 x 10^6</td>
<td>25</td>
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<td>10, 12, 14</td>
<td>&gt; 100</td>
<td>22–36</td>
<td>79</td>
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<td>Allogeneic</td>
<td>10</td>
<td>&gt; 100</td>
<td>21–27</td>
<td>95</td>
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<tr>
<td>6</td>
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<td>25</td>
<td>Allogeneic</td>
<td>10, 12, 14</td>
<td>&gt; 100</td>
<td>24–33</td>
<td></td>
</tr>
</tbody>
</table>

* LSTRA cells inoculated s.c. on Day 0.

b Mice received 1 treatment of BCNU on Day 7 when disease was systemic (11).

p Mice given inoculation i.p. of single or multiple injections of serum (0.2 ml/inoculation) at designated days after LSTRA inoculation.

p p < 0.07 when compared with Group 2 as determined by Fisher’s exact test.

p p < 0.001 when compared with Group 3.

p p < 0.001 when compared with Group 4.

Table 3

Effect of allogeneic immune serum on LSTRA disease following low-dose BCNU therapy

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mice</th>
<th>LSTRA (Day 0)*</th>
<th>BCNU Day 7 (mg/kg)*</th>
<th>Serum</th>
<th>Days post-inoculation</th>
<th>MST (days)</th>
<th>Range of death (days)</th>
<th>% survivors 100 (days)</th>
</tr>
</thead>
<tbody>
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<td>15</td>
<td>1 x 10^6</td>
<td></td>
<td></td>
<td>13</td>
<td>11–17</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>1 x 10^6</td>
<td>15</td>
<td>Normal</td>
<td>10</td>
<td>21</td>
<td>19–27</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>1 x 10^6</td>
<td>15</td>
<td>Normal</td>
<td>10, 12, 14</td>
<td>19</td>
<td>16–37</td>
<td>7.0</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>1 x 10^6</td>
<td>15</td>
<td>Normal</td>
<td>10, 12, 14</td>
<td>19</td>
<td>16–37</td>
<td>7.0</td>
</tr>
<tr>
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<td>15</td>
<td>1 x 10^6</td>
<td>15</td>
<td>Allogeneic</td>
<td>10</td>
<td>28</td>
<td>19–29</td>
<td>40.0</td>
</tr>
<tr>
<td>6</td>
<td>14</td>
<td>1 x 10^6</td>
<td>15</td>
<td>Allogeneic</td>
<td>10, 12, 14</td>
<td>28</td>
<td>17–33</td>
<td>29.0</td>
</tr>
</tbody>
</table>

* LSTRA cell inoculated s.c. on Day 0.

b Mice received 1 treatment of BCNU on Day 7 when disease was systemic.

p Mice given inoculations i.p. of single or multiple injections of serum (0.2 ml/inoculation) at designated days after LSTRA inoculation.

Successful combined chemoimmunotherapy against LSTRA disease has previously been reported using immune lymphoid cells or immunological stimulators such as Bacillus Calmette-Guérin and Corynebacterium granulosum as adjuncts to chemotherapy (4, 5, 11). The results reported here are comparable to those obtained with these other forms of immunotherapy against this leukemia. Interestingly, multiple inoculations of normal serum following high-dose drug therapy also resulted in increased MST's and a higher percentage of long-term survivors when compared to drug-treated-only animals. A likely explanation for this is that serum components helped to counteract toxic factors due to the drug treatment. Although there were no apparent drug-induced...
deaths noted following BCNU therapy, the animals usually showed some body weight loss following drug inoculation indicating some toxicity. This explanation gains further support from the fact that multiple inoculations of normal serum in the absence of prior drug therapy or following low-dose chemotherapy was ineffective in increasing MST's.

When used as an adjunct to drug therapy, single injections of the allogeneic serum appeared to be as effective as multiple injections in increasing the percentage of long-term survivors when compared with the appropriate control groups. Following high-dose chemotherapy, there were approximately twice as many survivors in each of the groups treated with the allogeneic serum when compared with the control groups that received the same number of injections of normal serum. Serotherapy following low-dose BCNU therapy produced 40% survivors in the group that received 1 injection of allogeneic serum as opposed to 29% in the group receiving multiple inoculations of the same serum. These findings suggest that the time of administration of immune serum in relation to prior chemotherapy is the important factor not the number of serum administrations in producing maximum effects.

The active factors in the allogeneic serum that are responsible for the therapeutic effects of the serum are still unknown. It is apparent, however, from these results as well as from previously reported findings (10) that only those sera with high titers of antibody as detected by the MF and neutralization assays were effective against LSTRA disease, suggesting that antibody might be the active element. If this is the case, there are a number of possible explanations on how antibody might function: (a) neutralizing antibody present in the serum could prevent the induction of new tumor cells by residual virus, a possibility that cannot be ruled out at this time; (b) antibody may act directly in destroying residual tumor cells left in the host following drug therapy; (c) factors akin to unblocking antibodies (1, 6) may be responsible for the antitumor effect of the serum; and (d) antibody in the serum may act synergistically with the reticuloendothelial cells of the host in rejecting residual and/or newly formed tumor cells. Explanations c and d are not necessarily mutually exclusive. Explanation d is an attractive one and receives support from the observations that immune and normal lymphocytes show enhanced in vitro cytotoxicity in the presence of certain immune sera (12, 15). It is also possible that factors unrelated to antibody may be present in the immune serum that activates the reticuloendothelial system of the host. Serum fractionation studies are now in progress attempting to isolate and characterize this immune factor in the allogeneic serum. Whatever the explanation, it is apparent that well-defined immune sera should be considered in experiments investigating the use of combined chemoimmunotherapy as a potential means of therapy against established tumors.

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Serochemotherapy against a Moloney Virus-induced Leukemia

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