SUMMARY

The s.c.-propagated murine glioma, GL-26, was established in tissue culture. The tissue culture line, with a doubling time of 36 hr, was used as the common source for all tumor cells. Suspensions of the tumor cells were transplanted intracerebrally in mice to produce an anaplastic ependymoblastoma.

In vitro 51Cr cytotoxicity assays did not detect any cellular immunity against GL-26 tumor cells in animals bearing either s.c. or i.c. tumors, indicating that the tumor itself is not highly immunogenic. However, significant cellular cytotoxicity was elicited in non-tumor-bearing animals by immunization with *Vibrio cholerae* neuraminidase and mitomycin C-treated tumor cells plus complete Freund's adjuvant.

In vivo therapy studies revealed significant increases in survival of animals preimmunized with *V. cholerae* neuraminidase- and mitomycin C-treated cells plus complete Freund's adjuvant.

1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea, when given i.p. on Day 3 or 12 after tumor challenge, also resulted in significant increases in survival. Furthermore, the effects of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea and preimmunization were additive, with significant additional protection occurring in animals that had received preimmunization as well as 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea.

In contrast to results reported for several extracranial tumor systems, immunotherapy, using either *V. cholerae* neuraminidase- and mitomycin C-treated tumor cells, Bacillus Calmette-Guérin, or both, beginning 3 or 4 days after tumor challenge, did not produce any significant increases in survival.

INTRODUCTION

There has been considerable interest in recent years in the use of such agents as BCG and viable neuraminidase-treated tumor cells as immunotherapy in a variety of extracranial tumor systems (3, 4, 16, 19, 20, 26, 27). There has also been great interest in the development of brain tumor models for experimental chemotherapy (2, 4, 5, 6, 22, 24).

With this in mind, the investigators have developed, using an in vitro ependymoblastoma cell line, an i.c. glioma system in mice suitable for immunotherapy experiments, as well as for chemotherapy studies (15).

This report describes a series of in vivo therapy trials, using this i.c. glioma system, and evaluates the effects of neuraminidase-treated tumor cells, either alone or in combination with BCG or CFA when used as either preimmunization or immunotherapy, as well as the effects of CCNU, when used as systemic chemotherapy. In addition, in vitro 51Cr cytotoxicity data are reported for animals immunized with neuraminidase-treated tumor cells.

MATERIALS AND METHODS

Used throughout these experiments were C57BL/6 mice, weighing between 20 and 25 g, obtained from the Drug Research and Development Colonies of the National Cancer Institute.

The medium used in cell cultures and for cell manipulations was modified Eagle's Basal No. 2 medium (NIH Media Unit) containing penicillin, 62 mg/liter; streptomycin, 135 mg/liter; and amphotericin B, 2.5 mg/liter. The medium was either used alone (EM) or supplemented with heat-inactivated fetal bovine serum (EM-FBS).

Development of Experimental Model. The tumor used in this study was the methylcholanthrene-induced murine ependymoblastoma, GL-26 (2, 21), presently being propagated by serial s.c. transplantation in C57BL/6 mice. Tumor from this source was begun in monolayer tissue culture using 10% EM-FBS, where it grew rapidly, typically to a population of $1.4 \times 10^7$ cells/75-sq cm flask. The in vitro doubling time during exponential growth was 36 hr (15), and this growth rate permitted the rapid production of large numbers of viable cells. The in vitro cytology was that of an anaplastic glial tumor.

The GL-26 monolayers were then used as the sole source of tumor cells throughout the experiment. Cells were...
mechanically harvested from culture without the use of trypsin. Viability of the resultant cell suspensions, by trypan blue exclusion, ranged from 85 to 95%.

Intracranial tumors were then produced, using these tumor cells. The desired number of cells were harvested, washed, and suspended in 0.05 ml of EM and injected into the right parietal region of mice anesthetized with ether, using a 25-gauge sleeve needle to ensure a uniform penetration of 3 mm. The technique proved both rapid and safe. Over 100 injections/hr could be performed by 1 person, with an acute mortality for the procedure of under 5% in over 800 injections.

The i.c. injection of GL-26 tumor cells produced massive intracranial neoplasms which typically occupied the entire right hemisphere by the time of death (Fig. 1). Extracranial growth of the tumor through the injection site was negligible. Histopathology by light and electron microscopy was that of an anaplastic ependymoblastoma, similar to that of the parent in vivo line, except for having a higher mitotic index.

Quantitative survival data for the system were compiled, in order to determine the smallest i.c. cell dose that would produce an acceptably high level of tumor takes with adequately long survival times. The data indicated the optimal i.c. dose to lie between 10^6 and 10^7 cells, which would produce lethal tumors in 89 to 100% of animals, with MST’s of 19 to 25 days, and a narrow range of mortality (15).

During these studies, alterations in the survival data for i.c. injection-treated mice were noted, suggesting a trend toward longer MST’s and decreased percentage tumor takes with cells from older cultures. Because of this phenomenon, which has been reported by other investigators (5), all cultures were routinely discarded after a maximum of 7 months in passage and were replaced either with earlier cultures which had been kept frozen, or with monolayers freshly inoculated from the parent GL-26 tumor line. Within these limits, the in vitro kinetics and morphology, as well as the intracranial histopathology, remained stable.

**Treatment of Tumor Cells Used for Immunizations.**

Tumor cells were harvested and suspended in EM at a concentration of 10^6 cells/ml. MITO (Schwarz/Mann, Orangeburg, N. Y.) was incubated with the tumor cells in a concentration of 25 µg/ml/10^6 cells, at pH 7.1 to 7.4 and 37°C for 60 to 90 min, to stop cell division. Where noted, VCN (General Biochemicals, Inc., Chagrin Falls, Ohio; 500 units of enzyme per ml) was also added to the incubation mixture, in a concentration of 25 units/ml/10^6 cells, as described by Simmons and Rios (19). Under these conditions, VCN released at least 400 nmoles of sialic acid from 10^6 GL-26 tumor cells, as measured by the Warren thiobarbiturate assay (23). Following incubation, cells were washed 3 times in EM and diluted to the desired concentration for injection. Viability of cell suspensions following this treatment generally ranged from 60 to 80% of the total cell count, by trypan blue exclusion.

**Cytotoxicity Assay.**

1^1Cr cellular cytotoxicity assays were performed in quadruplicate for each group of animals, in the manner described by Canty and Wunderlich (8), using 20% EM-FBS throughout. Five x 10^4 ^1Cr-labeled GL-26 tumor cells per dish were used as target cells, with 10^7 lymphoid spleen cells from the experimental animals (pooled within each group) as attacker cells (an attacker-to-target cell ratio of 200:1). An 18-hr incubation at 37°C in 5% CO_2 was used. Preliminary experiments, including 4 hr of incubation time and attacker:target ratios of from 25:1 to 200:1, showed the chosen time and ratio to be optimal in this system. Cytotoxicity was calculated as a percentage of the maximal (freeze-thaw) ^1Cr release, and was expressed for each group as the percentage cytotoxicity above spontaneous (target cells plus medium) release, ± S.E. (p = 0.05).

**RESULTS**

**In Vitro ^5^1Cr Cytotoxicity Studies**

Untreated Tumor-bearing Animals. Forty mice underwent i.c. injection of 10^6 GL-26 tumor cells, as described above. Thirty additional mice received s.c. injections of 10^6 GL-26 tumor cells in the right inguinal area in a volume of 0.2 ml. Fifteen mice were designated as normal controls, and received no tumor cell injections. On Days 7, 14, 21, and 28 after tumor cell injection, 6 mice from each tumor group and 3 mice from the control group were sacrificed and their spleens were collected for cellular cytotoxicity assay. By Day 21, most of the unsacrificed animals that had received s.c. injections of GL-26 tumor cells had developed palpable s.c. tumors, and those that had received i.c. injections showed typical clinical signs of i.c. tumor growth. However, no significant cellular cytotoxicity above spontaneous release was seen in either intracranial or s.c. tumor-bearing animals up to 28 days following tumor implantation.

Immunized Non-Tumor-bearing Animals. Sixty mice, in 4 groups of 15 animals, were immunized with various combinations of treated GL-26 tumor cells and CFA. Fifteen additional animals, designated as normal controls, received no immunizations. Cells used for immunizations were treated with MITO, and where noted, also with VCN. Treated cells were diluted in EM, and a dose of 10^6 cells in 0.1 ml was injected s.c. into the right flank of each animal on Days 1 and 16 of the experiment. Where noted, the injections also included 0.1 ml of CFA. On Days 7, 15, 21, 28, and 49, 3 mice from each group were sacrificed and their spleens were collected for cytotoxicity assay. The results of the cellular cytotoxicity assays are summarized in Chart 1, which shows, for each mode of immunization, the percentage cytotoxicity above spontaneous release with the S.E. (p = 0.05) indicated in brackets. The spontaneous release ranged from 26 to 30% in all assays. The group immunized with tumor cells treated with MITO and VCN, plus CFA, showed significant cellular cytotoxicity on Days 15 and 21. No significant cytotoxicity was exhibited by animals receiving other modes of immunization.

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2 Immunotherapy refers to treatment after tumor implantation. Preimmunization refers to treatment before tumor implantation.
**In Vivo Therapy Studies**

**Therapy Study 1.** One hundred seven animals received i.c. injections of $10^4$ GL-26 tumor cells. This represented 11 groups of animals randomly designated to receive preimmunization, immunotherapy, or systemic chemotherapy, either alone or in various combinations, plus a tumor-control group which received no treatment. An additional group received sham i.c. injections of 0.05 ml of medium alone and no treatment.

Animals receiving preimmunization had been injected s.c. on alternate sides with $5 \times 10^5$ MITO- and VCN-treated GL-26 tumor cells in 0.1 ml of EM, combined with 0.1 ml of CFA, on Days 21 and 8 prior to i.c. tumor implantation. Animals receiving immunotherapy were given s.c. injections on alternate sides with $10^6$ MITO- and VCN-treated GL-26 tumor cells in 0.1 ml of EM combined with 0.1 ml of fresh BCG (Pasteur Institute, Paris, France), containing 1 to 2 million viable organisms per 0.1 ml dose, every other day, beginning 4 days after i.c. tumor implantation, and continuing for 45 days or until the animal's death. Animals receiving chemotherapy were given CCNU, i.p., in doses of either 15 or 30 mg/kg, dissolved in 10% ethanol and 10% Emulfor EL-620 (GAF Corp., New York, N. Y.), on Day 3 after tumor implantation (Table 1). Statistical analysis on the basis of the percentage of long-term (> 60 days) survival on the arcsine scale (1) was undertaken. In Therapy Study 1, the least significant difference between any 2 groups on the arcsine scale is 23.78 for $p = 0.05$, and 33.24 for $p = 0.01$. Ninety-eight % of all deaths in the experiment occurred by 60 days. The untreated tumor control group, Group 12, had an MST of 27 days; 1 of 9 was a long-term survivor. No deaths were noted among the 9 sham controls, Group 13. The group receiving preimmunization alone, Group 6, did significantly better than control ($p < 0.05$), with an MST of 59.5 days, and 4 of 8 long-term survivors. The group receiving the higher dose of CCNU alone, Group 10, did significantly better than control ($p < 0.01$), with an MST of >88 days and 8 of 10 long-term survivors. The group receiving the lower dose of CCNU alone, Group 8, showed intermediate values, with an MST of 34.5 days, and 3 of 10 survivors. The therapeutic effects of preimmunization and chemotherapy appeared to be additive within the dose range

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**Table 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Preimmunization*</th>
<th>CCNU* (mg/kg)</th>
<th>Immunotherapy*</th>
<th>MST (days)</th>
<th>Long-term survivors/total</th>
<th>Arcsine scale</th>
</tr>
</thead>
<tbody>
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<td>15</td>
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<td>5/8</td>
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<td>15</td>
<td>-</td>
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<td>7/8</td>
<td>69.30*</td>
</tr>
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<td>30</td>
<td>+</td>
<td>&gt;88</td>
<td>7/8</td>
<td>69.30*</td>
</tr>
<tr>
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<td>&gt;88</td>
<td>8/8</td>
<td>79.82*</td>
</tr>
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<td>-</td>
<td>+</td>
<td>&gt;88</td>
<td>6/8</td>
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<td>4/8</td>
<td>45.00*</td>
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<td>15</td>
<td>+</td>
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<td>39.23</td>
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<tr>
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<td>-</td>
<td>15</td>
<td>-</td>
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<td>3/10</td>
<td>33.21</td>
</tr>
<tr>
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<td>-</td>
<td>30</td>
<td>+</td>
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<td>71.57*</td>
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<td>30</td>
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<td>8/10</td>
<td>63.43*</td>
</tr>
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<td>-</td>
<td>+</td>
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<td>0/10</td>
<td>9.10</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>27</td>
<td>1/9</td>
<td>19.47</td>
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<tr>
<td>13</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>27</td>
<td>1/9</td>
<td>19.47</td>
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</tbody>
</table>

* Preimmunization included the injection of $5 \times 10^5$ MITO- and VCN-treated GL-26 cells, plus 0.1 ml CFA, 21 and 8 days prior to intracerebral tumor implantation.

+ Injected i.p. 3 days after tumor implantation.

- Immunotherapy included the injection of $10^6$ MITO- and VCN-treated GL-26 cells, plus 0.1 ml BCG every other day, beginning 4 days after i.c. tumor implantation.

* Survival rate larger than control at a 5% significance level.

* Survival rate larger than control at a 1% significance level.

Tumor control group.

Sham control group.
tested. For example, Group 2, treated with the lower dose of CCNU plus preimmunization, did significantly better than the groups that had received either modality alone, i.e., Groups 6 and 8. The survival of Group 10, which received CCNU, 30 mg/kg, alone, was prolonged to such a degree that additive effects of preimmunization (Group 4) showed only a slightly higher value on the arcsine scale. In both Groups, p < 0.01. The survival data plotted on a probability scale (Chart 2) demonstrated a linear trend for the effect of CCNU within the range of the study, and also indicated the further increase in survival of groups receiving preimmunization.

However, Group 11, receiving immunotherapy alone, had an MST of 23 days with no survivors among 10, which indicated no therapeutic benefit. The reduction of MST suggested a negative effect but was not statistically significant. When immunotherapy was combined with either preimmunization or chemotherapy, a slight therapeutic effect was suggested, i.e., comparing Group 7 (CCNU, 15 mg/kg, plus immunotherapy) with Group 8 (CCNU, 15 mg/kg alone), or Group 5 (preimmunization plus immunotherapy) with Group 6 (preimmunization alone), but these differences were not statistically significant.

An analysis of variance on the overall effect of each treatment modality was performed, which compared the percentage survival among all groups receiving a modality, whether alone or in a combination, with that of all groups not receiving it. In this analysis, the overall therapeutic effect of either preimmunization or chemotherapy were highly significant (p < 0.01). On the other hand, no overall significant effect of immunotherapy was seen.

**Therapy Study 2.** One hundred twelve animals received i.c. injections of 4 × 10^4 GL-26 tumor cells. This represented 12 groups of animals randomly designated to receive either preimmunization, immunotherapy, or both, plus a tumor control group which received no treatment.

Animals receiving preimmunization had been injected s.c. in alternate flanks with either 10^6 MITO- and VCN-treated GL-26 tumor cells in 0.1 ml of EM combined with 0.1 ml of CFA, or else with 0.1 ml of CFA alone, on Days 21 and 8 prior to i.c. tumor implantation. Animals receiving immunotherapy were given s.c. injections in alternate flanks with either 10^6 MITO- and VCN-treated GL-26 tumor cells in 0.1 ml EM, or with 0.1 ml of reconstituted lyophilized BCG (Institute for Tuberculosis Research, Chicago, Ill., containing 9 million viable organisms per 0.1-ml dose) or both combined, twice weekly, from Day 3 after tumor implantation to Day 27, followed by single injections on Days 41 and 46. Statistical analysis was performed as described for Therapy Study 1, and the results appear in Table 2. Ninety-one percent of all deaths in the experiment had occurred by Day 60.

The untreated tumor control group had an MST of 28 days. All groups that had been preimmunized with treated cells plus CFA, i.e., Groups 1 to 4, had uniformly high survivals, significantly better than the control group (p < 0.01). On the other hand, all groups that had been preimmunized with CFA alone, i.e., Groups 5 to 8, had uniformly poor survivals, not significantly different from control.

All groups treated with immunotherapy alone, whether with BCG (Group 9), treated cells (Group 10), or both treated cells and BCG (Group 11), had uniformly poor survivals, not significantly different from control. In addition, there appeared to be no therapeutic benefit obtained by adding immunotherapy to preimmunization, because the groups that had received preimmunization plus immunotherapy, i.e., Groups 2 to 4 and 6 to 8, did not do better than corresponding groups treated with preimmunization alone, i.e., Groups 1 and 5. In the case of Groups 3, 4, and 9, a negative effect of immunotherapy was suggested, but was not statistically significant.

An analysis of variance was performed, similar to that described for Therapy Study 1. The overall therapeutic effect of preimmunization was highly significant (p < 0.01), and the use of treated cells appeared necessary to achieve this effect. On the other hand, no significant overall therapeutic effect was indicated for immunotherapy, by either cells or BCG.

**Therapy Study 3.** Two hundred animals received i.c. injections of 5 × 10^4 GL-26 tumor cells. This represented 4 groups of 50 animals randomly designated to receive either chemotherapy, immunotherapy, or both, plus a tumor control group which received no treatment. Animals receiving chemotherapy were given i.p. injections of 20 mg CCNU per kg on Day 12 after intracerebral tumor injection. Animals receiving immunotherapy were given s.c. injections of 10^6 MITO- and VCN-treated GL-26 cells, plus 0.1 ml BCG (Institute for Tuberculosis Research, Chicago, Ill.) containing 2 to 8 × 10^6 organisms per 0.1 ml) on alternate sides, beginning 3 days after i.c. tumor injection and continuing twice weekly until the animal's death. Incomplete Freund's adjuvant, 0.1 ml, was combined with the 1st 2 immunotherapy injections.

Of the 50 animals in each group, 35 were segregated for determining group mortality, which was statistically analyzed on the basis of median survival time, using the \( \chi^2 \) method. The remaining 15 animals in each group were kept.
Table 2

Results of GL-26 Therapy Study 2

For each group, the mode of therapy, MST, number of long-term (beyond 60 days) survivors per total, and the statistical significance on the arcsine scale, on the basis of the percentage of long-term survivors, are indicated.

<table>
<thead>
<tr>
<th>Group</th>
<th>Preimmunizationa</th>
<th>Immunotherapyb</th>
<th>MST (days)</th>
<th>Long-term survivors/total</th>
<th>Arcsine scale</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Cells CFA</td>
<td></td>
<td>&gt;88</td>
<td>9/10</td>
<td>71.56</td>
</tr>
<tr>
<td>2</td>
<td>Cells CFA</td>
<td>BCG</td>
<td>&gt;88</td>
<td>9/10</td>
<td>71.56</td>
</tr>
<tr>
<td>3</td>
<td>Cells CFA Cells</td>
<td></td>
<td>&gt;88</td>
<td>7/10</td>
<td>56.79</td>
</tr>
<tr>
<td>4</td>
<td>Cells CFA Cells</td>
<td>BCG</td>
<td>&gt;88</td>
<td>7/10</td>
<td>56.79</td>
</tr>
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<td>5</td>
<td>CFA CFA</td>
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<tr>
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<td>5/20</td>
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</tbody>
</table>

* Preimmunization included injection of either 10^6 MITO- and VCN-treated GL-26 cells plus 0.1 ml CFA, or 0.1 ml CFA alone, 21 and 8 days prior to i.c. tumor implantation.

b Immunotherapy included injection of either 10^6 MITO- and VCN-treated GL-26 cells or 0.1 ml BCG alone, or both, twice weekly from Days 3 to 27 after i.c. tumor implantation, then 1 injection again on Days 41 and 46.

c Survival rate larger than control at a 1% significance level.

d Tumor control group.

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DISCUSSION

Several conclusions can be drawn from these experimental results. First, preimmunization with viable neuraminidase-treated GL-26 tumor cells plus CFA is effective in inhibiting i.c. GL-26 tumor growth. Furthermore, the use of tumor cells is required to achieve this effect, since preimmunization with CFA alone affords no protection. On the other hand, experiments in progress suggest that VCN treatment and CFA are not necessary for in vivo immunization, as GL-26 cells treated only with MITO are capable of inducing protection (D. P. Houchens, unpublished observation). Preimmunization with cells plus CFA appears to be innocuous, since preimmunized animals did not exhibit signs of neurological or systemic disease up to the time of tumor implantation 21 days after immunization, and following tumor implantation had high survival rates.

Second, an in vitro cellular cytotoxic response against GL-26 tumor cells can be produced by immunization in vivo with viable VCN-treated GL-26 tumor cells plus CFA. This experimentation was carried out by separating the others, and on Days 7, 14, 21, and 28, 3 mice from each group were sacrificed, their spleen cells were obtained and pooled within each group, and 51Cr cellular cytotoxicity assays were performed as described above.

The mortality data appear in Chart 3. The group receiving no therapy had an MST of 19.5 days, similar to the group receiving immunotherapy alone, which had an MST of 17.8 days. Groups receiving chemotherapy alone or chemotherapy plus immunotherapy had significant (p < 0.01) prolongations of MSTs, to 31.5 and 30.6 days, respectively. The shortening of MSTs of groups receiving immunotherapy, either alone or in combination with chemotherapy, is not statistically significant (p > 0.1).

The 51Cr assays revealed no significant cellular cytotoxicity above spontaneous release in either the untreated tumor control group, or in any of the treated groups.

Chart 3. Mortality curves for Therapy Study 3, with percentages of deaths expressed on a probability scale.
is in contrast to the absence of any cytotoxic response seen in GL-26 tumor-bearing animals, regardless of whether the tumor is i.c. or s.c. These data indicate that the GL-26 monolayer tumor itself is not highly immunogenic as evaluated by the in vitro cellular cytotoxicity assay. This is in contrast to positive cellular cytotoxicity reported by Levy et al., (14) in human glioblastoma patients.

Third, the systemic administration of CCNU, whether given on Day 3 or Day 12 after tumor implantation, effectively inhibits intracranial tumor growth in this system. This is in agreement with the findings of Shapiro (18) on the parent GL-26 tumor, and indicates continued in vivo sensitivity of the tumor to this agent after its establishment as an in vitro cell line. These findings also confirm recent reports by Barker et al. (5) and Tator et al. (presented at American Association of Neurological Surgeons, 1974) on the effectiveness of the nitrosoureas in their recently developed murine and rat glioma systems. Furthermore, CCNU does not appear to interfere with the protective effect of preimmunization, or vice versa, but rather the 2 appear to be additive (Chart 2). The significance of this is that an immune protective mechanism can be utilized to increase the survival rate of a given dose of chemotherapy.

Fourth, in contrast to the in vivo protection and in vitro cytotoxicity achieved with preimmunization using treated tumor cells plus Freund’s adjuvant, immunotherapy using the same treated tumor cells, either with or without BCG or Freund’s adjuvant, was without significant in vivo therapeutic benefit and failed to elicit any in vivo cytotoxic response. These in vivo data are in contrast to results reported by Simmons and Rios (19) and Wepsic et al. (26) in their extracranial tumor systems.

It is unlikely that this was due either to insufficient release of cell surface sialic acid by the neuraminidase, or to a primary absence of sialic acid on GL-26 monolayer tumor cells, since assays have confirmed the release of at least 400 nmoles of sialic acid per 10^8 GL-26 cells by this treatment. This exceeds the sialic acid release of 250 nmoles/10^8 cells reported by Simmons and Rios (19) for the MC-42 sarcoma. It is possible that too much sialic acid was removed, thus destroying the antigenicity; however, preimmunization with a smaller total cell dose and the same VCN treatment produced significant in vivo protection and in vitro cytotoxicity. It is also unlikely that insufficient numbers of cells were given as immunotherapy, since the treatment schedules included injections totaling 2 to 4 x 10^4 cells/week/animal, continuing for up to 6 weeks, which was similar to cell doses reported by Simmons and Rios (19) and Wepsic et al. (24).

Immunotherapy itself appears to cause no deleterious effects in non-tumor-bearing animals. For example, a group of 6 non-tumor-bearing animals were given biweekly injections of 0.1 ml of BCG for 3 weeks and showed no ill effects over several months of observation. Likewise, a group of 10 non-tumor-bearing animals were given biweekly injections of 0.1 ml of BCG plus 10^8 VCN- and MITO-treated GL-26 tumor cells (combined with 0.2 ml of incomplete Freund’s adjuvant on the 1st 2 injections), for 6 weeks, and showed no ill effects over several months of observation.

Several possibilities might explain the absence of in vivo therapeutic benefit from immunotherapy in our studies, as well as the small negative effects seen in certain groups. For example, the therapeutic effect of immunotherapy may simply vary inversely with tumor burden, as recently reported by Rios and Simmons (17). A 2nd possibility is that antigenic competition may be occurring, so that tumor cells given as immunotherapy may be competing for the effenter portion of the immune response. Third, the continued presence of tumor cells, either those of the tumor or those injected as immunotherapy, may produce a state of acquired tolerance by exhaustive differentiation (25). Baldwin and Pimm (3) noted tumor enhancement when weekly antigenic tumors were used in immunotherapy. Finally, immunotherapy, whether with cells or BCG, may stimulate humoral as well as cellular immunity, which could result in the formation of blocking factors (9).

The results of these experiments are of importance in the light of recent reports by Lemonde (13), Baldwin and Pimm (3), Rios and Simmons (16), and Bansal and Sjögren (4), documenting absent or negative effects of active immunotherapy with VCN-treated cells, or BCG, or both, in certain extracranial tumor systems and of the lack of effect of active immunotherapy with irradiated autologous tumor cells in human glioblastoma as reported by Bloom et al. (7).

ACKNOWLEDGMENTS

The authors gratefully thank Dr. Leslie Cahan for performing the Warren barbiturate assays for sialic acid release, and Dr. David Young for performing the animal pathology. We also wish to thank Maurice Banks for his excellent technical assistance in all phases of these experiments.

We are also grateful for the invaluable assistance of Dr. Ta-Chuan Chen for preparing the statistical analysis.

REFERENCES

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Fig. 1. Photomicrograph of anterior view of decalcified coronal section of brain from a mouse dying 28 days after i.c. injection of $10^6$ monolayer cells. Note the massive intracranial tumor (darker staining area) and the minimal extracranial growth at the site of injection (arrow). H & E, × 25.
Therapy in an Intracerebral Murine Glioma Model, Using Bacillus Calmette-Guérin, Neuraminidase-treated Tumor Cells, and 1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea

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