Adaptive Immunotherapy of a Rat Glioma Using Lymphokine-activated Killer Cells and Interleukin 2

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ABSTRACT

The aim of the present study was to develop an animal model to test the therapeutic potential of purified adherent lymphokine-activated killer (A-LAK) cells against an intracerebrally implanted rat glioma, designated F98. Highly purified A-LAK cells demonstrated greater activity against F98 tumor cells than conventional lymphokine-activated killer cells, as determined by means of 3H-TdR-release and clonogenic assays. Therapeutic efficacy was evaluated by means of a Winn neutralization assay, in which F98 targets and A-LAK cells or control nonadherent mononuclear cells were incubated for 18 h in vitro and then implanted stereotactically into the right caudate nucleus of Fischer rats. Animals given injections of 4000 F98 cells alone or control nonadherent mononuclear cells had a mean survival time of 22.3 days, compared to 46.1 days (P < 0.001) for rats treated with A-LAK cells. Increasing the tumor inoculum to 12,500 cells reduced the survival time of A-LAK-treated animals to 27.8 days, compared to 20.8 days for untreated controls. Systemic administration of 50,000 units/kg of interleukin 2 every 12 h for 5 days failed to improve survival. The mean survival time of rats implanted with the F98 tumor ranged from 16 days for 10^5 cells to 29 days for 10^7 cells. Extrapolating from these survival data, treatment with A-LAK cells may have decreased the number of F98 cells to <10, but even this small number was still lethal. Supernatants from F98 cells had immunoinhibitory activity that, further, may have modulated the antitumor effects of A-LAK cells. Our results indicate that curative, adoptive immunotherapy of the F98 glioma by means of A-LAK/interleukin 2 is impossible to achieve and provide some explanation for the clinical failures that have been observed in the adoptive immunotherapy of malignant gliomas.

INTRODUCTION

Malignant gliomas (glioblastoma multiforme) are refractory to surgery, multidrug chemotherapy (1), radiation therapy (2), and adoptive immunotherapy with autologous lymphocytes (3) and tumor-specific cytotoxic T-lymphocytes (4). Over the past 5 years there has been increasing interest in the use of LAK cells for the treatment of a variety of solid tumors (5-7), but only more recently has this been extended to malignant gliomas. In a pilot study, Jacobs et al. (8) reported that there were no systemic or neurotoxic side effects following immunotherapy with LAK cells and IL-2 in 9 patients with glioblastoma. Mild but well tolerated side effects were reported by Merchant et al. (9) in 20 glioma patients treated by means of intralesional infusion of LAK cells and IL-2. In both of these studies, however, there was no evidence of therapeutic efficacy. In contrast, Yoshida et al. (10) reported tumor regression and some improvement in clinical status following the infusion of autologous LAK cells and IL-2 into the tumor bed following surgical resection of brain tumors in 23 patients, although there was no evidence of prolongation in survival time. Barba et al. (11) found that the therapeutic efficacy of intensive intratumoral LAK/IL-2 therapy of 9 glioma patients was limited by the cerebral edema-related neurological side effects.

The usual sequence of events in the development of a therapeutic modality is to demonstrate efficacy in an animal model and then to initiate human studies. Surprisingly, there were no preliminary animal studies to test the efficacy of LAK/IL-2 for the treatment of malignant brain tumors, and this prompted us to initiate the present study (12, 13). We have investigated the efficacy of immunotherapy with highly purified adherent LAK cells (14, 15), alone or in combination with IL-2, for the treatment of a well characterized rat glioma by means of a Winn neutralization assay (16). This assay was chosen in order to provide some indication of the maximum effects that might be achievable under in vivo conditions. In addition, since gliomas have been reported to produce a variety of factors that inhibit the function of immunologically active cells (17-19), we also have investigated how supernatants derived from the F98 glioma might affect LAK activity.

MATERIALS AND METHODS

Animals and Tumor Cells. Nine-week-old male Fischer rats weighing 170-200 g were purchased from Charles River (Wilmington, MA). Glioma clone F98 was derived from an undifferentiated neoplasia induced by N-ethyl-N-nitrosourea in an inbred CD Fischer rat (20). Its in vitro and in vivo morphology and growth have been described elsewhere (21, 22). Its in vivo biological behavior closely simulates that of human glioblastoma, in that it cannot be cured by either chemoradiotherapy and kills by virtue of the fact that it is an expanding intracranial mass.

Rat Brain Tumor Model. The implantation procedure of Kobayashi et al. (23) has been utilized. Forty thousand F98 glioma cells were injected in 10 µl of DMEM (Gibco, Grand Island, NY) containing 1% agarose at a gelling temperature of 33°C. A stereotactic method was employed, using a skull-embedded plastic screw, and tumor cells were implanted into the right caudate nucleus via a Hamilton syringe fitted with a 5-mm-long 26-gauge needle.

Preparation of Mononuclear Cells. Rat spleens were removed aseptically, crushed against a wire mesh, and dispersed into a single-cell suspension by repeatedly forcing them through a syringe fitted with a 25-gauge needle. Mononuclear cells were isolated after centrifugation on Histopaque-1077 gradients (Sigma, St. Louis, MO) at 200 × g for 20 min. Macrophages were removed by incubating the mononuclear cells in 100-mm Petri dishes at 37°C for 1 h. NMNC were used for control effector cells or for the generation of LAK cells. CM consisted of RPMI 1640 (GIBCO), 1 µM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 10% heat-inactivated FBS, and 5 × 10^7 cells/ml 2-mercaptoethanol.

Generation of Conventional LAK Cells and A-LAK Cells. In the dose-response study, NMNC were seeded at a density of 2 × 10^6 cells/ml of CM in a T25 flask. Cells were incubated in the presence of varying amounts of IL-2 (Kindly provided by Cetus Corporation, Emeryville, CA), ranging from 5 to 6000 units/ml of CM, for 4 days and their
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cytolytic activity against F98 targets was determined by means of an 18-h $^{51}$Cr-release assay. In the time-kinetis study, NMNC were incubated in the presence of 1000 units/ml IL-2 and their cytolytic activity was studied at daily intervals from day 1 to day 9, using the 18-h $^{51}$Cr-release assay.

A-LAK cells were generated as described by Vujanovic et al. (14) and Medler et al. (15). Briefly, NMNC were incubated in the presence of 1000 units/ml IL-2 for 24 h, following which the cell suspensions were decanted, collected, centrifuged to recover conditioned medium, and passed through a 0.22-μm filter. The plastic-adherent cells (A-LAK) were supplemented with medium containing 50% conditioned medium and 50% fresh medium containing 1000 units/ml IL-2. A-LAK cells were expanded over 7 to 10 days for further study.

$^{51}$Cr-release Assay. F98 target cells (3 × 10⁵) were labeled with 200 μCi of Na$_2$CrO$_4$ (specific activity, ≈10–35 mCi/ml; Amersham, Arlington Heights, IL) for 90 min at 37°C. Cells then were washed 3 times with DMEM containing 2% heat-inactivated FBS. Five thousand target cells were added to each well of 96-well flat-bottomed microtiter plates (Corning, Corning, NY). Following this, effector cells were added at varying E/T ratios, the volume was adjusted to 0.2 ml, and the mixture was incubated at 37°C in a humidified incubator containing 5% CO₂. Since F98 glioma cells had low spontaneous release of $^{51}$Cr, a more sensitive 18-h release assay was employed. At the end of this time, the plates were centrifuged at 300 × g for 30 s and 100 μl of the supernatants were removed from each well for counting in a Tracor Analytic model 1185 gamma scintillation counter. Spontaneous $^{51}$Cr-release was determined following incubation of targets in DMEM alone. Maximum $^{51}$Cr release was measured by lysis of targets with 0.1 N HCl. The ratio of spontaneous to maximum release was <30% in the 18-h assay. Percentage of specific lysis was calculated as [(experimental cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm)] × 100%.

Clonogenic Assay. The in vitro antiadherent activity of rat A-LAK cells was determined by cocultivating A-LAK cells with F98 glioma cells at varying E/T ratios, in the presence of 1000 units/ml IL-2, for 7 days. Following this, F98 colonies were fixed by adding 3 ml of 37% formaldehyde to medium containing plates and allowing them to stand for 3 min, washed with warm tap water, and stained with 1% crystal violet, and colony numbers were counted using an Artrek 880 image analyzer. Plating efficiency was calculated as (number of F98 colonies enumerated/total number of F98 cells plated) × 100%. Surviving fraction is [number of F98 colonies enumerated in the presence of effector cells/total number of F98 plated × plating efficiency] × 100%.

Winn Neutralization Assay. A-LAK cells or control NMNC and F98 targets were incubated at an E/T ratio of 400/1, in the presence of 1000 units/ml IL-2, in 6-well tissue culture plates for 18 h. As determined by means of trypsin blue exclusion, the viability of A-LAK cells was >90% after incubation. The cytolytic activity of A-LAK cells was >90% specific lysis at an E/T ratio of 50/1, as defined by the $^{51}$Cr-release assay, on the same day as the implantation procedure. Cells were harvested and stereotactically implanted into the brains of rats sedated with a 1:2 mixture of ketamine/xylazine. IL-2 was given i.p. at doses of 50,000 units/kg every 12 h for 5 days to one group of animals. Animals were fed ad libitum and weighed 3 times per week. Rats were euthanized when they became ataxic or had periorbital bleeding. Survival times were determined by adding 1 day to the death date. Percentage of ILS was expressed relative to the survival time of untreated control animals as follows: %ILS = [(mean survival time of treated group – mean survival time of untreated group)/(mean survival time of untreated group)] × 100%. Rat brains were removed, fixed in 10% formalin, and cut into sections, and the tumor size was measured with calipers. The TSI was defined as the cube root of the product of the largest measurement of tumor height, width, and length.

Production of F98 SN. F98 SN were produced as described by Fontana et al. (17). Briefly, 5 × 10⁶ F98 glioma cells were incubated in 24-well tissue culture plates (Corning), containing DMEM and 10% FBS, for 24 h. Medium then was replaced with 0.5 ml serum-free DMEM and SN were collected 2 days later, centrifuged at 2000 × g for 30 min, and subjected to ultrafiltration by means of a Centricon-10 membrane (Amicon, Danvers, MA). The material deposited on the membrane, having a molecular weight of >10,000, was resuspended in RPMI 1640 and filtered through a 0.22-μm filter (Millipore, Bedford, MA).

Effects of F98 SN on Thymocyte Proliferation and the Generation of LAK Cells. Rat thymocytes at a concentration of 6 × 10⁶ in 0.2 ml of CM were incubated in the presence of 1 μg/ml Con A (Sigma) and serial dilutions of F98 SN for 72 h. One μCi of [³H]thymidine (40–60 Ci/ml; Amersham) was added to each well 16 h prior to harvesting. Percentage of suppression was calculated as [(control cpm – experimental cpm)/control cpm] × 100%. Control cpm is the Con A response of thymocytes treated with DMEM. Experimental cpm is the Con A response of thymocytes treated with F98 SN. The effects of F98 SN on the generation of LAK cells were determined by incubating 2 × 10⁶ NMNC, in the presence of 1000 units/ml IL-2 and varying amounts of F98 SN, for 4 days in a T25 flask. The cytolytic activity of LAK cells against F98 targets was quantified using an 18-h $^{51}$Cr-release assay.

RESULTS

Generation of Effector Cells. In the dose-response curve (Fig. 1), maximum cytolytic activity of LAK cells was observed when 2 × 10⁵/ml of NMNC were incubated with 1000 units/ml IL-2 for 4 days and was essentially unchanged with further amounts of IL-2 up to 6000 units/ml. In subsequent experiments, therefore, 1000 units/ml IL-2 were used for generating LAK cells. As defined by a time-kinetis study, LAK cells attained their maximum cytolytic activity after 4 days of incubation in the presence of 1000 units/ml IL-2 and fell precipitously thereafter (Fig. 2). In contrast, cytolytic activity of control NMNC ranged from <5 to 10% at E/T ratios of 3/1 to 50/1 (Fig. 3).

LAK versus A-LAK Cells. Table 1 summarizes the cytolytic activity of LAK cells versus A-LAK cells against F98 glioma targets, as determined in an 18-h $^{51}$Cr-release assay. The mean percentage of specific lysis of control NMNC, at E/T ratios ranging from 3/1 to 100/1, were 1.9 to 8.7%, 22.7 to 69.8% for LAK cells, and 64.1 to 88.5% for A-LAK cells. The in vitro antiproliferative and/or antiadherent activities of effector cells were assessed by means of a clonogenic assay. Fig. 4 summarizes the effects of LAK versus A-LAK cells in the clonogenic assay. The plating efficiency of F98 colonies was 37.5% in this study. The surviving fraction of F98 colonies, at E/T ratios ranging from 50/1 to 400/1, were 101.3 to 105.1% following
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The percentage of ILS was −3.4% for the control NMNC-treated group and 33.7% for the A-LAK-treated group (Table 2). These differences in survival times were significant at the level of $P < 0.001$, as determined by means of Wilcoxon rank sum and Kruskal-Wallis tests (24, 25). In experiment 2, animals given injections of 4,000 F98 glioma cells had a mean survival time of 22.3 ± 0.6 days, compared to 22.1 ± 0.5 days for control NMNC-treated animals, 46.1 ± 5.3 days for A-LAK-treated animals, and 44.1 ± 5.2 days for A-LAK plus IL-2-treated animals. The percentage of ILS was −0.9% for the control NMNC-treated group, 106.7% for the A-LAK-treated group, and 97.8% for the A-LAK plus IL-2-treated group ($P < 0.001$ compared to controls). A Kaplan-Meyer plot of the survival in experiment 2 is shown in Fig. 6. The $P$ value was <0.001 for animals treated with either A-LAK or A-LAK plus IL-2, as compared to the untreated control group. There were no significant differences between MSTs of animals treated with A-LAK and A-LAK plus IL-2 and between MSTs of animals treated with control NMNC and the untreated group.

Tumor Size Index. The TSI was 5.6 ± 0.6 for animals treated with F98 glioma alone, 5.6 ± 0.4 for control NMNC-treated animals, 8.3 ± 0.4 for A-LAK-treated animals, and 7.6 ± 0.3 for A-LAK plus IL-2-treated animals. Survival times versus TSI are presented graphically in Fig. 7. These show that the longer the animals survived, the larger the TSI was. The $P$ values were all <0.01 for the differences of animals treated with cocultivation with control NMNC, 92.5 to 30.4% with LAK cells, and 37.9 to 0% with A-LAK cells.

In Vivo Survival Study of F98 Glioma-bearing Rats. The MST of rats implanted intracerebrally with 100 to 100,000 F98 glioma cells are presented graphically in Fig. 5. These were 15.9 ± 2.3 (MST ± SD) days for 10$^3$ cells, 19.4 ± 2.9 days for 10$^4$ cells, 22.7 ± 2.4 days for 10$^5$ cells, and 28.6 ± 3.9 days for 10$^6$ cells. Extrapolating from these survival data, the mean survival time was ≈33 days for rats implanted with 10 F98 cells and ≈39 days for 1 cell.

Winn Neutralization Assay. In experiment 1, animals given injections of 12,500 F98 cells had a MST (±SE) of 20.8 ± 0.5 days, compared to 20.1 ± 0.7 days for rats treated with control NMNC and 27.8 ± 1.7 days for rats treated with A-LAK cells.

<table>
<thead>
<tr>
<th>Specific lysis (%)</th>
<th>E/T ratio</th>
<th>Control NMNC</th>
<th>LAK cells</th>
<th>A-LAK cells</th>
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<tr>
<td>3/1</td>
<td>0.4 ± 5.6</td>
<td>22.7 ± 5.4</td>
<td>64.1 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>6/1</td>
<td>−1.9 ± 2.4</td>
<td>25.6 ± 3.8</td>
<td>67.1 ± 4.3</td>
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<tr>
<td>12/1</td>
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<td>30.0 ± 3.9</td>
<td>78.3 ± 6.0</td>
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</tr>
<tr>
<td>25/1</td>
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<td>41.0 ± 4.0</td>
<td>83.2 ± 5.9</td>
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<tr>
<td>50/1</td>
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<td>69.8 ± 3.2</td>
<td>88.5 ± 1.5</td>
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</tr>
<tr>
<td>100/1</td>
<td>7.6 ± 2.2</td>
<td>68.4 ± 5.6</td>
<td>87.3 ± 1.7</td>
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</table>

Fig. 2. Time kinetics for the development of LAK cells. NMNC were incubated in the presence of 1000 units/ml IL-2 and the cytolytic activity was studied at daily intervals from day 1 to day 9, using an 18-h $^{3}$Cr-release assay. O, E/T = 3/1; □, E/T = 6/1; △, E/T = 12/1; ©, E/T = 25/1; ⃝, E/T = 50/1.

Fig. 3. The cytolytic activity of control NMNC determined as a function of time was studied as in Fig. 2. O, E/T = 3/1; □, E/T = 6/1; △, E/T = 12/1; ©, E/T = 25/1; ⃝, E/T = 50/1.

Fig. 4. Effects of A-LAK cells versus LAK cells against F98 glioma in the clonogenic assay. Effector cells and 1000 F98 glioma cells were cocultivated in the presence of 1000 units/ml IL-2 for 7 days. Following this, F98 colonies were fixed by 37% formaldehyde and stained with 1% crystal violet, and the colony numbers were counted using an Artek image analyzer. Surviving fraction = [number of F98 colonies enumerated in the presence of effector cells/(total number of F98 cells plated x plating efficiency)] x 100%. O, LAK cells; △, A-LAK cells; ©, control NMNC.

Fig. 5. In vivo survival study. Increasing numbers of F98 glioma cells, from 100 to 100,000, were implanted stereotactically into the right caudate nuclei of Fischer rats. Rats were euthanized and survival times were determined by adding 1 day to the death date.
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Table 2 Effects of A-LAK cells against F98 glioma by means of a Winn neutralization assay

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Experiment 1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Experiment 2&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>MST (days)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>n&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>F98 glioma only</td>
<td>20.8 ± 0.5</td>
<td>9</td>
</tr>
<tr>
<td>F98 + control NMNC</td>
<td>20.1 ± 0.7</td>
<td>8</td>
</tr>
<tr>
<td>F98 + A-LAK cells</td>
<td>27.8 ± 1.7</td>
<td>8</td>
</tr>
<tr>
<td>F98 + A-LAK cells + IL-2</td>
<td>ND&lt;sup&gt;f&lt;/sup&gt;</td>
<td>8</td>
</tr>
</tbody>
</table>

<sup>a</sup> Experiment 1, 1.25 x 10<sup>6</sup> F98 target cells were used in the Winn assay; experiment 2, 4 x 10<sup>3</sup> F98 target cells were used in the Winn assay.

<sup>b</sup> Mean ± SE.

<sup>c</sup> Number of experiments.

<sup>d</sup> Differences in survival times were analyzed by means of Wilcoxon rank sum and Kruskal-Wallis tests.

<sup>f</sup> ND = not determined.

Fig. 6. Results of the Winn neutralization assay. Effector cells and F98 targets (4000) were cocultivated at an E/T ratio of 400/1 for 18 h in 6-well tissue culture plates, following which all cells were harvested and implanted into the right caudate nuclei of rats. Rats were euthanized and survival times were determined by adding 1 day to the death data. ●, F98 glioma only; △, F98 plus control NMNC; □, F98 plus A-LAK cells; ●, F98 plus A-LAK cells plus IL-2.

Fig. 7. Tumor size index. Rat brains were removed and fixed in 10% formalin, and the size of the tumor mass was measured by calipers. TSI = (ab<sup>c</sup>)<sup>1/3</sup>, where a, b, and c are three coordinate measurements. The mean survival times were plotted against the TSI. ●, F98 glioma only; △, F98 plus control NMNC; □, F98 plus A-LAK cells; ●, F98 plus A-LAK cells plus IL-2.

Fig. 8. Effects of F98 SN on thymocyte proliferation. Rat thymocytes (6 x 10<sup>6</sup>) were incubated in the presence of 1 µg/ml Con A and serial dilutions of F98 SN for 72 h. One µCi of [<sup>3</sup>H]thymidine was added to each well 16 h prior to harvesting. Percentage of suppression = [(control cpm − experimental cpm)/control cpm] x 100%. Control cpm = the Con A response of thymocytes treated with DMEM. Experimental cpm = the Con A response of thymocytes treated with F98 SN. Results were calculated from three independent experiments.

Fig. 9. Effects of F98 SN on the generation of LAK cells. Mononuclear cells (2 x 10<sup>7</sup>) were incubated in the presence of 1000 units/ml IL-2 and varying amounts [0 ng/ml (○), 10 ng/ml (♦), 100 ng/ml (△), 500 ng/ml (□)] of F98 SN for 4 days in a T25 tissue culture flask, following which cells were harvested and their cytolytic activity against F98 targets was determined, at E/T ratios ranging from 3/1 to 50/1, by means of an 18-h <sup>3</sup>Cr-release assay. In another experiment, LAK activity was already generated following 4 days of incubation with IL-2, following which 500 ng/ml F98 SN (▲) were added to the microtiter plates in the <sup>3</sup>Cr-release assay. Results were calculated from three independent experiments.

F98 glioma alone versus A-LAK-treated group, control NMNC-treated group versus A-LAK-treated group, and F98 glioma alone group versus A-LAK- plus IL-2-treated group, as determined by means of ANOVA.

Effects of F98 SN on Thymocyte Proliferation and the Generation of LAK Cells. The effects of F98 SN on the Con A-induced thymocyte proliferation were assessed by means of a <sup>3</sup>H-incorporation assay (Fig. 8). The percentage of suppression of thymocyte proliferation was 4.1 at 0.1 ng/ml F98 SN, 12.9 at 1 ng/ml, 24.5 at 5 ng/ml, 40.6 at 10 ng/ml, 54.1 at 50 ng/ml, 62.4 at 100 ng/ml, and 61.6 at 1000 ng/ml, in a dose-dependent fashion. In order to determine whether tumor-bearing rats still possessed precursor cells that could give rise to LAK cells, rat spleens and thymuses were removed from glioma-bearing animals at a point in time prior to death when they had developed symptoms of ataxia or periorbital bleeding.

LAK cells, generated in the presence of 1000 units/ml IL-2 as previously described, were shown to have cytolytic activity with 62.4 to 71.5% specific lysis at an E/T ratio of 50/1 (data not shown). The effects of F98 SN on the generation of LAK cells were determined by incubating NMNC in the presence of 1000 units/ml IL-2 and varying amounts of F98 SN for 4 days in a T25 tissue culture flask, following which cells were harvested and their cytolytic activity against F98 targets was determined, at E/T ratios ranging from 3/1 to 50/1, by means of an 18-h <sup>3</sup>Cr-release assay. In another experiment, LAK activity was already generated following 4 days of incubation with IL-2, following which 500 ng/ml F98 SN (▲) were added to the microtiter plates in the <sup>3</sup>Cr-release assay. Results were calculated from three independent experiments.
differences in LAK activity in the absence of F98 SN versus those in the presence of 10 to 500 ng/ml F98 SN, as determined by an ANOVA test. However, even at the concentration of 500 ng/ml, F98 SN did not significantly affect the cytolytic activity of LAK cells that already have been activated following 4 days of incubation with IL-2.

DISCUSSION

In the present study we have established that the intracerebrally implanted F98 rat glioma, which simulates human glioblastoma multiforme, is not curable by adoptive immunotherapy using A-LAK cells, either alone or in combination with IL-2. Although the A-LAK cells were highly cytotoxic/cytostatic, as determined by \(^{31}\)Cr-release and clonogenic assays, and had probably killed >99.9% of the F98 tumor cells, those surviving were sufficient to kill the host. Similarly, failure to cure human glioblastoma multiforme by all currently available therapeutic modalities most likely is due to an inability to eradicate every single tumor cell. In our study, 100% killing was not possible even under optimum experimental conditions, and it is not surprising, therefore, that the clinical studies of adoptive immunotherapy of glioblastoma, carried out by a number of investigators (9–11), were therapeutic failures.

IL-2 has been reported to enhance the in vivo proliferation and tumoricidal effects of LAK cells (26, 27). In the present study, however, i.p. administration of 50,000 units/kg IL-2 every 12 h for 5 days to glioma-bearing rats failed to improve survival rates. Animals treated with IL-2 were severely lethargic compared to untreated controls, at least suggesting that IL-2 had gotten to the brain. Dysfunction of the blood-brain barrier (28) and the resulting vascular leak syndrome (29–31) and compared to untreated controls, at least suggesting that IL-2 were sufficient to kill the host. Similarly, failure to cure human glioblastoma multiforme by all currently available therapeutic modalities most likely is due to an inability to eradicate every single tumor cell. In our study, 100% killing was not possible even under optimum experimental conditions, and it is not surprising, therefore, that the clinical studies of adoptive immunotherapy of glioblastoma, carried out by a number of investigators (9–11), were therapeutic failures.

Effective immunotherapy with LAK cells requires the infusion of large numbers (>10⁸) of cultured lymphocytes. The use of purified populations of A-LAK cells, as reported by Vujnovic et al. (14) and Medier et al. (15), has allowed us to use lower numbers of highly cytotoxic effector cells. We have confirmed the culture conditions developed by them for the generation of A-LAK cells and demonstrated that A-LAK cells are more active against F98 glioma cells than conventional LAK cells, as determined by means of \(^{31}\)Cr-release and clonogenic assays. Takai et al. (33) also have reported on adoptive immunotherapy of rat brain tumors with LAK cells administered either intratumorally or i.v. The MST was 21 days for controls, 24 days for rats treated intratumorally, and 25 days for rats treated i.v. In their study, rat LAK cells were generated by using supernatants of Con A rather than recombinant IL-2 and had relatively low cytolytic activity against T9 tumor targets, compared to the much higher levels of cytolytic activity that we observed. Nevertheless, even with these higher levels of activity, intracerebral glioma was not curable by adoptive immunotherapy.

McCutcheon et al. (34) recently have studied the effects of systemic administration of LAK cells and IL-2 on lung and brain metastases of the KHT sarcoma in C3H mice. Although pulmonary metastases were reduced, there was no reduction in the number of intracerebral metastases or evidence of tumor-infiltrating lymphocytes or cytolytic activity. It was concluded that an insufficient number of LAK cells had either localized or been activated at the site of the tumor. This conclusion is supported by the report of Itoh et al. (35), who have studied the distribution of \(^{111}\)In-labeled LAK cells in patients with advanced malignant brain tumors. Scintigraphy revealed that large numbers of LAK cells had localized in the liver and spleen and only very small numbers were within the intracranial brain tumors, thereby explaining the failure of adoptive immunotherapy.

In the present study, we found that glioma-bearing rats treated with A-LAK or A-LAK plus IL-2 had larger TSIs than tumor-bearing controls or controls treated with NMNC (Fig. 7). Histological examination of hematoxylin- and eosin-stained tumor sections revealed larger numbers of tumor-infiltrating lymphocytes and more extensive necrosis in A-LAK- and A-LAK- plus IL-2-treated rats, compared to controls. Although the brain has been considered to be an immunologically “privileged” site (36), infiltration of malignant gliomas with lymphocytes has been correlated clinically with increasing survival (37, 38). Enhanced host immune responses following LAK/IL-2 therapy (39), as evidenced by denser infiltrates of cytotoxic/suppressor T-cells, also have been observed in s.c. implanted tumors (40) in animals treated with LAK cells. In our study, the breakdown of the blood-brain barrier may have been further enhanced by the growth of the tumor itself and the trauma associated with the intracerebral implantation procedure. These factors may have augmented the ability of adoptively transferred LAK cells, as well as host-derived cytotoxic lymphocytes, to infiltrate the tumor.

We also observed that the F98 glioma secreted an immuno-suppressive factor(s) that inhibited Con A-induced thymocyte proliferation and blocked the generation of LAK cells. TGF-β, which is produced by a variety of normal cells (41–43), virus-transformed cells (44, 45), and malignant cells (46–48), can affect T-cell function. Fontana and colleagues (17–19) have reported that human glioblastoma cells secreted a TGF-β-like factor that inhibited T-cell activation and LAK cell generation. Since several other immunosuppressive factors, distinct from TGF-β and secreted by tumors, have been identified (49–52), the exact nature of F98 SN needs to be clarified. Nevertheless, immunosuppressive factor(s) may further limit the therapeutic efficacy of adoptive immunotherapy of malignant gliomas by means of LAK/IL-2. The principal limitation of this form of treatment, however, appears to be the impossibility of eradicating every single tumor cell, which appears to be the sine qua non for the cure of malignant gliomas both in experimental animals and in humans. In combination with surgery, chemotherapy, and radiotherapy, however, immunotherapy may be able to eliminate tumor cells that otherwise would have survived. This should be sufficient reason to continue to explore the potential use of biological response modifiers for the treatment of malignant brain tumors.

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Adoptive Immunotherapy with LAK Cells and IL-2


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