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Rat Mitogen-Stimulated Lymphokine-Activated T Killer Cells: Production and Effects on C_6 Glioma Cells In Vitro and In Vivo in the Brain of Wistar Rats

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Summary: An in vitro technique was developed to generate activated rat T cells, with antitumor activity. Splenic mononuclear cells (SMC) from outbred Wistar and inbred Wistar-Munich rats were stimulated with Concanavalin A and recombinant human interleukin-2 (rIL-2) in vitro for 48 h. After 2 days, the nonadherent cells began proliferating and were maintained in rIL-2 for up to 18 days in vitro. FACScan analysis revealed that SMC was a mixture of cell types; however, $CD5^+$ T cells rapidly increased and became the predominant cell type after 5 days in culture. SMC induced cytolysis of YAC-1, but not C_6 glioma cells in 4 h ^{51}Cr release assays. In contrast, 5- and 9-day T cells lysed C_6 glioma and YAC-1 cells. The C_6 cells were admixed with cultured effector cells at various effector-to-target (E:T) ratios and were injected into the right cerebral hemisphere of Wistar and Wistar-Munich rats for a Winn assay. Histopathologic evaluations revealed that a) SMC had no effect; b) 2- and 5-day T cells, injected at E:T ratios >5:1, caused significant reduction in tumor size; and c) 2- or 5-day T cells, at a 40:1 E:T ratio, resulted in little or no histologic evidence of tumor. Eighty-three percent of animals receiving C_6 and 5-day mitogen-stimulated lymphokine activated killer cells at an E:T ratio of 40:1 were alive 120 days postinjection ($p < 0.05$). **Key Words:** Recombinant human interleukin-2—Lymphokine-activated killer cells—Concanavalin A—Splenic mononuclear cells—Glioma cells.

Gliomas account for ~50% of the 15,000 brain tumors, which are diagnosed each year in the United States. Even with surgical tumor debulking followed by chemotherapy or radiation therapy, most patients with grade IV glial tumors will die within one year of recurrence (1). The possibility of employing one or more immunotherapies for this histologic type of deadly cancer is being examined by a number of investigators. One approach has been the use of autologous lymphocytes, activated to become cytolytic for tumor cells by one or more methods in vitro (2–9). Several laboratories have shown that human lymphokine activated killer (LAK) and mitogen-stimulated lymphokine activated killer (MAK) cells are capable of lysing autologous and allogeneic glioma cells on a panel of continuous cell lines in vitro (10,11). Human LAK

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cells are a heterogenous population of "natural killer (NK)-like" effector cells, which develop after coculture with interleukin-2 (IL-2) for 3–5 days (12,13); in contrast, MAK cells are T cells generated by 48-h coculture with lectin (phytohemagglutinin) and IL-2, and subsequently cultured with IL-2 alone for 10–14 days (7,9,11). Phase I trials of intratumoral LAK and MAK cells in human patients with recurrent gliomas have been conducted and, while safety has been established, clinical efficacy has yet to be proven. We believe that basic studies are needed with MAK cells to establish the mechanisms involved, improve present methods, and develop new approaches to immunotherapy of these deadly tumors.

Animal studies of the mechanisms involved in intracranial therapy of gliomas with autologous MAK cells may lead to possible new approaches to clinical trials. There are few animal studies employing LAK cells and no studies with MAK cells. Takai et al. (14) have shown that treatment of rats, bearing experimental gliomas, with syngeneic LAK cells can prolong animal survival.

This is the first report in a series of basic studies, examining the plausibility of immunotherapeutic treatment of experimental brain tumors in rats. We report methods of generating nonadherent MAK cells of T cell phenotype from the spleen of Wistar and Wistar-Munich rats in vitro. We also show that these MAK cells cause cytolysis of C_6 glioma cells in vitro and can destroy glioma cells in vivo, when implanted into the rat brain in a Winn-type assay.

MATERIALS AND METHODS

Animals

Outbred male Wistar and inbred Wistar-Munich rats, 180–200 g, were obtained from Simonsen Laboratories (Gilroy, CA, U.S.A.). They were housed in the Biological Sciences vivarium and provided food and water *ad libitum*.

MAK Media

MAK media consists of RPMI 1640 media (Gibco Laboratories, Grand Island, NY, U.S.A.) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco), 2 m*M* glutamine (Sigma Chemicals, St. Louis, MO, U.S.A.), 10 U/ml penicillin (Sigma), 0.1 mg/ml streptomycin (Sigma), 0.1 m*M* nonessential amino acids (Gibco), 1.0 m*M* sodium pyruvate (Gibco), and 5×10^{-5} *M* 2-mercaptoetanol (J. T. Baker, Phillipsburg, NJ, U.S.A.).

Biological Response Modifier

Recombinant human interleukin-2 (rIL-2) (Ro 23-6019, Teceleukin) was kindly provided by Hoffman-La Roche (Nutley, NJ, U.S.A.).

In Vitro Cell Lines

Two continuous cell lines were employed in these studies: A) C_6, a clone of a rat glial cell tumor originally induced in outbred male Wistar rats by the intravenous injection of N-nitrosomethyl/urea (15); and B) YAC-1, a NK-sensitive murine lymphoma (16). These cell lines were maintained in RPMI-1640 media, supplemented with 10% FCS (RPMI-10%). The YAC-1 cell line was maintained as a suspension culture in T-25 cm^2 tissue culture flasks (Corning, Corning, NY, U.S.A.) and passed three times weekly. The C_6 cell line was maintained as an adherent monolayer in T-150 cm^2 tissue culture flasks (Corning) and passed biweekly. The C_6 cells were removed from the monolayers by scraping with a cell scraper and employed as target cells in in vitro and in vivo experiments. All cell lines were incubated at 37°C in an atmosphere of 5% CO_2, 95% air.

Effector Cells

A single spleen from either a male Wistar or Wistar-Munich rat (8–16 weeks old, 200–220 g) was obtained aseptically, mechanically dissociated using scalpel blades, and suspended in 35 ml of RPMI-10%. The cell suspension was then layered onto 15 ml of sterile 1.077 Histopaque (Sigma) and centrifuged at 350 × G for 25 min. After centrifugation, the mononuclear cell interphase layer was collected and washed twice by alternate centrifugation and resuspension in 10 ml of MAK cell media. These cells were then transferred to T-75 cm^2 culture flasks and brought to a final concentration of 4×10^6 cells/ml with MAK cell media. Mononuclear cells were then stimulated with 10 μg/ml Concanavalin A (Con A; Sigma, catalogue no. C-5275) and 10^3 U/ml rIL-2, and incubated at 37°C in an atmosphere of 5% CO_2 and 95% air for the first 48 h. Total number of viable cells was determined each day by 0.01% eosin Y staining. Every 48 h, MAK cells were collected and washed by alternate cen-

trifugation and resuspension in MAK media. MAK cells were recultured in fresh MAK media and 10^3 U/ml of rIL-2, at a concentration of 5×10^5 cells/ml.

In Vitro Cytolytic Assays

Splenic mononuclear cells (SMC) and 2-day MAK cells were tested simultaneously for cytolytic activity. MAK cells from the same cultures were tested 3 (5-day cells) or 7 (9-day cells) days later. All cytotoxic assays included C_6 and YAC-1 cell lines.

Nonadherent Cells

Radiolabeling of target cells and $Na_2{}^{51}CrO_4$ (^{51}Cr)-release, cytolytic assays were performed using the technique described by Yamamoto et al. (17). Assays for nonadherent YAC-1 cells were performed in 96-well, round-bottomed microcytotoxicity plates. Radiolabeling of nonadherent target cells was accomplished by addition of 150 μCi ^{51}Cr to 2×10^6 cells, suspended in 1 ml of RPMI-10%. This suspension was incubated for 24 h at 37°C. At the end of the incubation period, cells were washed three times by alternate centrifugation (300 *g*, for 5 min) and resuspension in RPMI-10%, and dispensed to give a final concentration of 1×10^4 labeled target cells per microtiter well. Effector cells were also washed three times by alternate sedimentation and resuspension in RPMI-10%; they were added to the target cells at various effector-to-target cell (E:T) ratios in a final volume of 120 μl/microtiter well.

Adherent Cells

One hundred μCi ^{51}Cr was added to 1×10^6 C_6 cells in 10 ml of RPMI-10%, and 1×10^4 cells in 100 μl were then seeded into each well of a 96-well flat-bottomed microtiter plate. After incubation for 24 h at 37°C, labeled adherent C_6 cells were washed repeatedly by aspiration and addition of 100 μl cold RPMI-10%, to remove free isotope. At the end of the final aspiration, 100 μl RPMI-10% was added to each well. Effector cells were then added to target cells at various E:T ratios in a volume of 100 μl, to give a final total volume of 200 μl/well.

Release of ^{51}Cr from suspension and adherent cells was measured—after 4 h incubation, at 37°C, in a CO_2 incubator—by uptake of cell-free supernatant, using the Titertek supernatant collection system (Skatron, Norway), and was quantitated in an automated biogamma counter (Beckman, Fullerton, CA, U.S.A.). Total ^{51}Cr releasable (90–95% of total counts) was determined by lysing cells with 100 μl 3% (w/v) sodium dodecyl sulfate solution. Spontaneous ^{51}Cr release was 2–3% per h. Percent lysis was determined by the following formula:

$$\frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100 = \%\ \text{specific lysis}$$

Cell Phenotyping–Flow Cytometric Analysis

Cell suspensions were analyzed by FACScan (Becton Dickinson, Mountain View, CA, U.S.A.). The FACscan was calibrated with the Autocomp program and optimized for each sample tested. Forward and side scatter thresholds were set in order to eliminate debris and dead cells. Particles with usually low forward and side scatter were considered nonviable and were not counted. Subthreshold particles were <5% of the total population of MAK cells, which is in agreement with dye exclusion studies of these same cultures. Cells were gated forward vs side scatter to obtain a uniform distribution; green (FL1) or red (FL2) fluorescence was measured by analyzing cells stained with fluorescein isothiocynate or phycoerythrin-labeled antibodies, respectively. Cells stained with isotypic control antibodies were used to determine background fluorescence. Data were expressed as percent positive cells for corresponding surface antigen. Surface phenotypes on rat lymphocytes were analyzed by staining the cells for surface antigens, with the following monoclonal antibodies: a) T cells T-CD5 (clone MRC OX-19), b) helper/inducer T-CD4 (clone W3/25), c) cytotoxic/suppressor T-CD8 (clone MRC OX-8), d) IL-2 receptor (clone MRC OX-39, which recognizes the 55 kD chain), and e) major histocompatibility complex-class II Ia antigen (clone MRC OX-17). Monoclonal antibodies were fluorescein- or phycoerythrin-conjugated and were obtained from Bioproducts for Science, Inc. (Indianapolis, IN, U.S.A.). Cells were incubated with desired monoclonal antibodies for 30 min on ice and washed twice with phosphate buffered saline (PBS, 0.01 *M* phosphate, pH 7.2, 0.15 M NaCl) by alternate centrifugation (5 min at 400 *g*) and resuspension. Cells were fixed in 1.0% paraformaldehyde solution and stored at 4°C until further analysis.

Implantation and Growth of C_6 Glioma in Wistar and Wistar-Munich Rats

Animals were anesthetized by injection of 0.05 mg/100 mg body weight Acepromazine (Ayerst Labs, New York, NY, U.S.A.) and 5 mg/100 mg body weight ketamine hydrochloride (Bristol Labs, Syracuse, NY, U.S.A.). A 3 cm midline scalp incision was made, exposing the cranium. A 1.5 mm diameter hole, located 3 mm caudal to the frontal suture and 3 mm to the right of the sagittal suture, was made through the calvarium, using a hand-held drill. The dura mater was left intact. We found that an inoculum of 5×10^5 C_6 glioma cells, in a volume of 20 μl PBS, consistently generated brain tumors, which were spherical in shape and had a diameter of >0.5 cm at 21 days. All animals with untreated tumors died by 35 days. This dose of tumor cells was routinely employed in all subsequent studies. After injection, the skin was closed with a stainless steel clip.

Gross and Histopathologic Examination

All animals were monitored daily for signs of neurologic dysfunction (poor grooming, lethargy, and ataxia). Animals were killed at 14-day intervals after intracranial injection, and the entire brain removed and fixed in 2% paraformaldehyde. After 72 h, each brain was sectioned, embedded in paraffin, and stained with hematoxylin and eosin Y. Multiple serial sections representing the entire tumor were examined under low power, with an Olympus microscope. The greatest cross-sectional area of tumor was determined with the aid of a 4 mm^2 100 quadrant ocular grid (Olympus), each quadrant of which was 0.04 mm^2. Mean tumor size for each experimental group was determined by averaging the largest cross-sectional area of tumor from each of the animals, on coded slides.

One group of animals was harvested at 6 and 12 h post-injection, and at every 24 h period up to 14 days. Brains were prepared for microscopic examination, as stated above, and subjected to histological inspection.

Winn Test

Animals were injected intracerebrally, as described originally by Winn (18) for injection of tumors. Cultured C_6 glioma cells (5×10^5) were mixed with effector cells at different E:T ratios, in a volume of 20 μl PBS. Control animals were injected with either 5×10^5 C_6 cells alone or nonactivated fresh SMC mixed with 5×10^5 C_6 cells at an E:T ratio of 40:1. All animals were killed at 14 days. Brains were paraffin-embedded, sectioned, and stained. Tumor size was determined by microscopic examination of serial sections of brain tissue, as described in the previous section.

Survival Studies

Three groups of six animals each were employed for these studies. The first (control) group received 5×10^5 C_6 glioma cells intracranially. The second group of animals received SMC admixed with C_6 glioma cells at an E:T ratio of 40:1. The third group received 5-day MAK cells and C_6 glioma cells at a ratio of 40:1. Animals in each group were examined daily for indications of neurologic dysfunction, due to tumor growth, and were killed when severe lethargy or ataxia became evident. Growth of tumor in the brains of these animals was evaluated, as described previously. Mean survival time was then calculated for each experimental group.

RESULTS

In Vitro Growth Characteristics of SMC in the Presence of Con A and rIL-2

SMC were stimulated with Con A and rIL-2 for 48 h and then cultured with rIL-2 alone, as described in Materials and Methods. Growth characteristics of the nonadherent cells in these cultures, over a 5-day period, are shown in Fig. 1. Data shown are averages of growth curves from three separate experiments, conducted with cells from Wistar and Wistar-Munich animals. There is a decrease in the number of nonadherent viable cells over the course of the first 1–2 days, then the remaining cells begin to proliferate. Cell growth rate may slow after 5–6 days; however, it can be reinitiated by a second 24 h pulse with 10 μg/ml Con A. Reinitiated cells will continue vigorous in vitro growth for up to 18 days.

Phenotypic Analysis of Nonadherent, Rat Splenic Cultures

Nonadherent cells, collected at various intervals from the same culture over a 9-day period, were

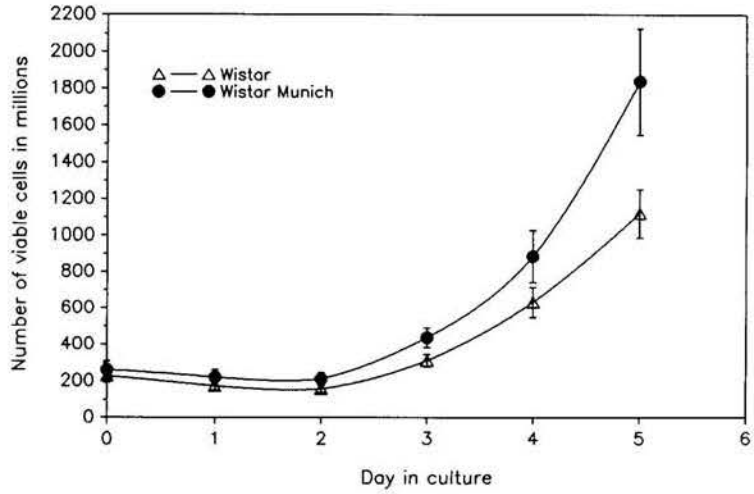

FIG. 1. Growth of rat splenic nonadherent mononuclear cells after stimulation with Con A and subculture in IL-2. SMC were stimulated by coculture with 10 μg/ml Con A and 10^3 U/ml rIL-2 for 48 h in MAK media. They were then washed and incubated with 10^3 U/ml rIL-2 in MAK media.

subjected to FACS analysis, as described in Materials and Methods. Phenotyping data are expressed as percent positive cells for corresponding surface antigen. Percentages were determined by Consort 30 software supplied by Becton Dickinson Immunotherapy Systems; histograms were subjected to analysis by the Consort 30 software. Peak separation was obtained in a lag fluorescence scale. Positive and negative gates were set using unlabeled cells such that 97–98% of 10^5 events fell within the gated parameter. Next, monoclonal treated samples were run through the FACscan, which had been gated to calculate the percentage of the region of the peak with the highest fluorescent intensity. Results of these studies are shown in Table 1. Percentages shown in Table 1 for CD5, CD4, IL-2R, and Ia are from clearly-defined peaks; however, CD8 is calculated from a broad peak. These studies were repeated a total of three times on cultures of Wistar and Wistar-Munich spleen cells, with essentially identical results. Results of the analysis of these cultures are shown in Table 1. By day 2, the percentage of cells expressing the T lymphocyte markers $CD5^+$, $CD8^+$, and cells expressing IL-2 receptors and Ia (class II) proteins had increased. By day 5, cells expressing $CD5^+$ and $CD8^+$ markers continued to increase, and $CD5^+$ T cells became the predominant cells in these cultures. While all the data are not shown, $CD5^+$ T cells continued to predominant these cultures (85–95%) at day 9 (Table 1) and up to day 18. Additional studies (not shown) revealed that IL-2 receptor expression dropped, after day 5, in cultures whose growth rate slowed. Exposure of these cells, at day 6, to a 24 h pulse of 10 μg/ml Con A in the presence of rIL-2, and the subsequent culture of these cells in rIL-2 alone resulted in increased expression of IL-2 receptors and an increase in growth rate by day 9.

TABLE 1. *Percent of cells expressing selected phenotypic markers in SMC, and nonadherent 2, 5, and 9-day MAK cells in vitro*

	Wistar			Wistar-Munich	
Phenotype	SMC	2-day MAK	5-day MAK	5-day MAK	9-day MAK
CD5	37 ± 3	50 ± 5	81 ± 9	85 ± 5	92 ± 4
CD4	27 ± 4	35 ± 6	36 ± 5	35 ± 7	33 ± 6
CD8	13 ± 6	35 ± 16	73 ± 9	61 ± 10	81 ± 6
Ratio (CD4/CD8)	2.2 ± 0.4	1.3 ± 0.7	0.5 ± 0.1	0.6 ± 0.2	0.90 ± 0.1
IL-2-R	4 ± 1	79 ± 2	87 ± 9	82 ± 7	97 ± 1
IA	16 ± 16	61 ± 19	22 ± 3	27 ± 1	45 ± 36

Splenic mononuclear cells (SMC) were stimulated for 48 h with Con A and rIL-2; they were then cultured with 10^3 units/ml rIL-2. The 9-day culture was restimulated at day 6 for 24 h with 10 μg/ml Con A and cocultured with 10^3 U/ml rIL-2. Cells in these cultures were exposed to a panel of monoclonal antibodies, directed at defined cellular antigens and analyzed by FACS. Data shown are mean percents of cells expressing each marker from three separate experiments.

Cytolytic Activity of Cultured Rat SMC Tested on YAC-1 Lymphoma and C_6 Glioma Cells In Vitro

Cells of cultures derived from Wistar and Wistar-Munich spleens were next tested for their capacity to induce cytolysis of C_6 glioma cells and NK-sensitive YAC-1 cells at various E:T ratios in the 4 h ^{51}Cr release assay, as described in Materials and Methods. Data presented in Table 2 are summaries of data obtained in ten separate experiments. For clarity, only the mean value for ^{51}Cr release at each E:T ratio tested is shown. Standard deviations ranged from ±3–15%. Wistar SMC were incapable of lysing C_6 glioma cells lines at any E:T ratio tested. However, these cells possessed NK activity, since they could induce cytolysis of NK-sensitive YAC-1 cells. Based on these results, C_6 glioma cells appear to be NK-resistant target cells. While not shown, SMC from Wistar-Munich animals were also without effect on these target cells. Effector cells from 2-day cultures had increased cytolytic activity against YAC-1 cells and C_6 glioma cells, although it was minimal. In contrast, cells from 5-day cultures expressed the ability to cause cytolysis of YAC-1 and C_6 glioma cells. Cytolytic activity continued to be expressed by 9-day effector cells (Table 2) derived from Wistar-Munich animals.

Growth of C_6 Glioma After Implantation in the Brain of the Wistar Rat

Various dosages of glioblastoma cells were injected into the right cerebral hemisphere of male Wistar rats; animals were killed at different intervals and growth of the tumors assessed by histopathologic methods, as described in Materials and Methods. Growth of the C_6 glioma, after implantation of 5×10^5 cells into the brain of the Wistar rat, is presented in Fig. 2. While these data are from a single experiment, in which three animals were killed at each time point, it is characteristic of all subsequent studies. At this dosage, tumor growth was consistent and progressive in all animals; by day 10, the tumor had developed into an organized vascular mass, which was easily seen macroscopically in cut sections of brain. By day 30, all animals showed signs of significant neurologic impairment, followed by death within 3–4 days.

Histologic studies revealed progressive primary and satellite tumor growth into surrounding normal tissue. After 14 days, the tumor was well-vascularized, visible grossly, and had a spherical shape. In some tumors, cells were proliferating within the needle tract and, in a few cases, the tumor had spread onto the surface of the brain, presumably by migration up the needle tract. By day 21, the tumor

TABLE 2. *Percent in vitro lysis of target cells, measured in a 4 h ^{51}Cr release assay, induced by fresh or cultured rat splenic mononuclear cells (SMC)*

Target cell employed	Effector: target cell ratio	Percent ^{51}Cr release induced by effector cells				
		Wistar fresh SMC[a]	Wistar 2-day MAK[b]	Wistar 5-day MAK[c]	Wistar-Munich 5-day MAK[d]	Wistar-Munich 9-day MAK[e]
C_6 glioma	100:1	5.1	14.4	47.9	—	—
	40:1	1.8	8.8	35.2	41.3	64.0
	20:1	1.7	5.8	31.5	—	—
	10:1	3.5	3.7	17.1	12.2	17.4
	5:1	2.4	2.3	11.0	—	—
YAC-1	100:1	35.7	53.6	79.9	—	—
	40:1	26.8	28.0	86.6	79.8	62.1
	20:1	15.0	18.4	73.0	—	—
	10:1	10.3	14.5	56.3	50.1	56.0
	5:1	7.5	9.7	40.4	—	—

Different effector cell populations were mixed with ^{51}Cr labeled target cells and isotope release was measured after 4 h, as described in Materials and Methods. Data shown are mean values of ten separate experiments. Variation was from 3–15%.

[a] Unstimulated Wistar SMC.

[b] Wistar MAK cells cultured for 2 days with lectin and rIL-2.

[c] Wistar MAK cells cultured for 2 days with lectin and 5 days with rIL-2.

[d] Wistar-Munich MAK cells cultured for 2 days with lectin and 5 days with rIL-2.

[e] Wistar-Munich MAK cells cultured for 2 days with lectin and 9 days with rIL-2. These 9-day cells were restimulated at day 6 for 24 h with 10 μg/ml Con A in the presence of rIL-2.

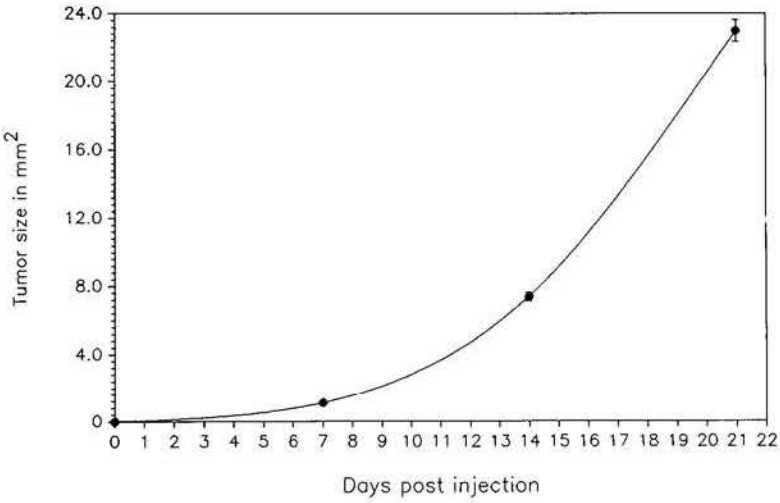

FIG. 2. Growth of C_6 glioblastoma in the brain of the Wistar rat. 5×10^5 cultured C_6 glioblastoma cells were injected into the forebrain of male Wistar rats. At intervals, animals were killed and tumor size established by histopathologic techniques. One tumor unit = 0.04 mm^2.

had displaced structures of the entire right cerebral hemisphere. In general, the host inflammatory response was very minor. However, at 14 days, a small amount of scattered perivascular infiltrates of small mononuclear cells, which did not invade the tumor mass, was noted. Few, if any, polymorphonuclear cells were seen in these tissue sections at any time. By day 21, this inflammatory response had largely subsided.

Effect of Cultured SMC on Growth of C_6 Glioma in Brains of Wistar Rats

Effector cells, admixed with fresh C_6 cells at various E:T ratios and injected into the brain of Wistar rats, as described in Materials and Methods, were harvested from these cultures at different days. Animals were killed after 14 days and tumor size established by histopathologic methods. Experiments were conducted over a 12-month period. Results of one of three experiments are presented in Fig. 3, where data from each bar represent means of five animals. Animals that received SMC at an E:T ratio of 40:1 developed tumors whose size was not statistically distinguishable ($p = 0.56$) from that of untreated, control tumors. In contrast, both 2- and 5-day effector cells had similar effects on tumor size. At an E:T ratio of as little as 5:1, the 2- and 5-day effector cells caused a significant decrease in tumor size, when compared to controls. This effect

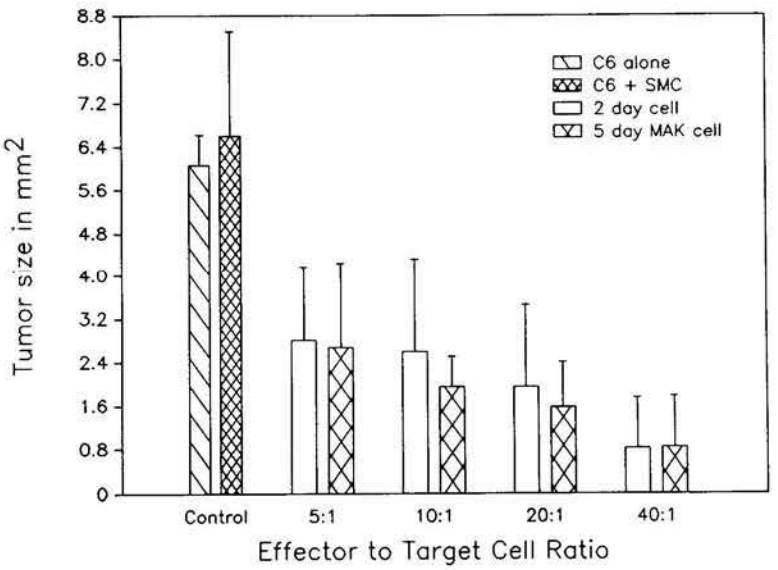

FIG. 3. Effect of SMC, and 2- and 5-day MAK cells on growth of C_6 glioma in the brain of Wistar rats, when injected in a Winn assay. Effector cells were mixed with 5×10^5 C_6 glioma cells at various E:T ratios and injected into the brain of Wistar rats. Animals were killed after 14 days and tumor size established by histopathologic methods. One tumor unit = 0.04 mm^2.

increased as the ratio of effector to tumor cells was increased. Two out of five animals in the 40:1 E:T group of 2- and 5-day effector cells showed no microscopic evidence of tumor growth.

Histopathology of Rat Brain Sites Injected with 5-Day MAK and C_6 Cells

Histopathologic studies were conducted on brain sites injected with 5-day effector and C_6 cells at an E:T ratio of 40:1. Microscopic examination of tissue sections collected at 6 and 12 h postinjection, and at 24 h intervals thereafter over a period of 14 days, revealed that lymphoid and tumor cells were visible for up to three days at the injection site; thereafter, lymphoid cells were no longer detectable. Effector and tumor cells remained in contact with each other and localized primarily to the injection site. Tumor cell numbers remained unchanged, or decreased, for the first three days in all sites receiving effector and tumor cells; however, it appeared that tumor cells surviving this initial period began to proliferate and developed into tumors. No histopathologic changes in normal brain tissues surrounding the injection site(s) were detected. These studies were conducted with Wistar and Wistar-Munich animals.

Survival of Wistar Rats After Intracranial Injection of C_6 Glioma, SMC, and 5-Day MAK Cells

Male Wistar rats received injection either of 5×10^5 C_6 cells alone or C_6 cells mixed with SMC or 5-day MAK cells, at an E:T ratio of 40:1. All animals were examined every two days for signs of neurologic dysfunction and were killed when serious ataxia and/or lethargy became evident. Results of one study are summarized in Fig. 4. Six control animals, injected with C_6 glioma, survived for 30 ± 4.6 days, whereas six animals, which received C_6 glioma plus SMC, survived for 27.5 ± 7.6 days. Survival times of these two groups were not significantly different. In contrast, the six animals treated with C_6 and 5-day MAK had greatly-increased survival times. One animal died after 40 days; however, 5 of the 6 animals were killed after 120 days, and had no histopathologic evidence of disease.

DISCUSSION

These procedures provide an in vitro method for the production of a population of highly enriched T cells (85–95%), which have antitumor activity in vitro and in vivo. Treatment of SMC from outbred Wistar and inbred Wistar-Munich rats, with Con A, and coculture with recombinant human IL-2, resulted in stimulation and proliferation of a nonadherent T cell population. Con A, which is a polyclonal T cell mitogen, provides the initial stimulation, which renders T cells to be more responsive to the rIL-2, and activating T cells to become nongenetically restricted killers. However, lectin alone cannot maintain cell proliferation, viability, or cytolytic activity beyond a few days in vitro (19,20). Percentage of cells expressing T cell $CD5^+$ and $CD8^+$ phenotypes increased and predominated the cultures by 5 days. Percentage of $CD5^+$ and $CD8^+$ cells continued to increase up to 9 days, and remained high for the duration of the culture.

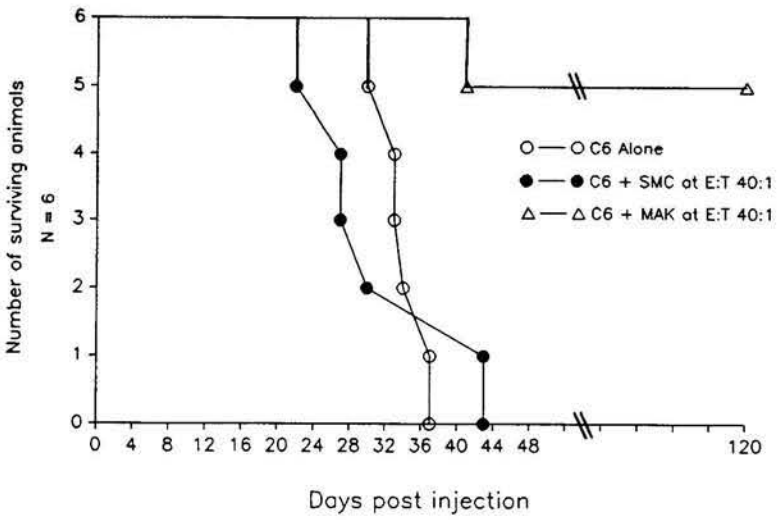

FIG. 4. Survival of Wistar rats after intracranial injection of a 40:1 mixture of effector cells and C_6 glioma cells. SMC or 5-day MAK cells were mixed with 5×10^5 C_6 glioma cells and injected into each brain of two groups of six Wistar rats. Six control animals received C_6 cells alone. Each group of animals was examined every two days. Survivors were killed after 120 days and their brains examined by histopathologic techniques.

In extensive studies, Hiserodt and colleagues (21–25) have shown that LAK cells can be generated from Fischer 344 rat spleen cells cultured for 4–6 days with IL-2 alone in vitro. However, they found that these LAK cells were adherent and expressed NK phenotypes (ASIALO/GM-1$^+$, Lamanin$^+$, CD5$^-$). Adherent LAK cells were termed ALAK. In addition, they also demonstrated that nonadherent cells were only weakly cytolytic. Thus, the ALAK and MAK cell, generated by the above in vitro techniques, are quite distinct cell populations. In vitro testing indicated that nonadherent MAK cells in these cultures became increasingly cytolytically active on C_6 glioma and YAC-1 target cells in vitro. Thus, these cells are nongenetically restricted killers. SMC had little or no cytotoxic activity against C_6 glioma cells at all E:T ratios tested; however, they were effective against NK-sensitive YAC-1 cells. These data support the concept that the C_6 glioma is relatively insensitive to NK-induced cell lysis. It has also been shown that human gliomas are also resistant to cytolysis induced by autologous and allogeneic NK effector cells in vitro (11). Two-day MAK cells possessed increased cytolytic activity against YAC-1 and some activity against C_6. However, 5-day MAK cells were highly effective against YAC-1 and C_6 glioma cells. This was even more obvious in 9-day MAK cells. Cytolytically-active T cells can be generated from the rat spleen, but they require stimulation and a period of differentiation before being able to express high levels of cytolytic activity. Thus, 5-day MAK cells appear similar to nongenetically-restricted, lymphokine-activated killer T-cells, as reported by many different laboratories (26–28).

Intracranial injection of 5×10^5 cultured C_6 glioma cells into Wistar or Wistar-Munich rats led to consistent development of brain tumors, which were lethal by 35 days postinjection. Histopathologic studies revealed minimal host inflammatory response to surgery, intracranial implantation, or growth of the C_6 glioma. Two and 5-day MAK cells had inhibitory effects on the size of C_6 gliomas, as determined by the brain Winn assay. Even at an E:T ratio of only 5:1, tumor size, at 2 weeks, was half that of untreated control tumors ($p < 0.008$ and 0.005, respectively). Increasing the E:T ratio resulted in greater cytolytic activity in vitro and inhibition of tumor size. At a ratio of 40:1, tumors were minute or not detectable. In contrast, SMC did not significantly alter the in vivo growth rate of the C_6 glioma, as compared to untreated controls. Thus, in vitro cytolytic activity did not correlate to in vivo activity, and NK cells were not capable of affecting growth of the C_6 glioma either in vitro or in vivo.

Histologic studies revealed some important information. First, it appears that MAK cells were only present at the injection site, exerting their antitumor effects for as little as 2–3 days. Second, no evidence of damage to normal brain tissue was apparent in treated outbred Wistar or inbred Wistar-Munich rats. Thus, even allogeneic MAK cells, at the levels we employed in these studies, did not appear to cause injury to normal tissues in vivo.

These studies demonstrate that MAK cells of T cell phenotype can be produced by in vitro techniques from spleen cells derived from outbred Wistar and inbred Wistar-Munich rats. The MAK cell is genetically nonrestricted, can cause cytolysis of C_6 cells in vitro, and can also inhibit growth and destroy C_6 cells in vivo in the brain. While the present studies were conducted under the ideal conditions of the Winn test, further studies on the cells responsible for in vivo antitumor activity, and their effects when stereotactically implanted into established brain tumors, are underway. This animal system could lend itself to studies serving as models for design and improvement of MAK-cell immunotherapy of human brain tumors.

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