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Subcutaneous vaccination with irradiated, cytokine-producing tumor cells stimulates CD8$^+$ cell-mediated immunity against tumors located in the "immunologically privileged" central nervous system

(central nervous system neoplasms/immunotherapy/melanoma)

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ABSTRACT **Vaccination with cytokine-producing tumor cells generates potent immune responses against tumors outside the central nervous system (CNS). The CNS, however, is a barrier to allograft and xenograft rejection, and established tumors within the CNS have failed to respond to other forms of systemic immunotherapy. To determine what barriers the "immunologically privileged" CNS would pose to cytokine-assisted tumor vaccines and what cytokines would be most efficacious against tumors within the CNS, we irradiated B16 murine melanoma cells producing murine interleukin 2 (IL-2), IL-3, IL-4, IL-6, γ-interferon, or granulocyte-macrophage colony stimulating factor (GM-CSF) and used these cells as subcutaneous vaccines against tumors within the brain. Under conditions where untransfected B16 cells had no effect, cells producing IL-3, IL-6, or GM-CSF increased the survival of mice challenged with viable B16 cells in the brain. Vaccination with B16 cells producing IL-4 or γ-interferon had no effect, and vaccination with B16 cells producing IL-2 decreased survival time. GM-CSF-producing vaccines were also able to increase survival in mice with pre-established tumors. The response elicited by GM-CSF-producing vaccines was found to be specific to tumor type and to be abrogated by depletion of CD8$^+$ cells. Unlike the immunity generated against subcutaneous tumors by GM-CSF, however, the effector responses generated against tumors in the CNS were not dependent on CD4$^+$ cells. These data suggest that cytokine-producing tumor cells are very potent stimulators of immunity against tumors within the CNS, but effector responses in the CNS may be different from those obtained against subcutaneous tumors.**

Tumor cells that have been genetically modified to secrete various cytokines, including interleukin 2 (IL-2) (1–8), IL-4 (9, 10), IL-6 (6, 9, 11, 12), γ-interferon (γ-IFN) (1, 6–8, 13–15), and granulocyte-macrophage colony stimulating factor (GM-CSF) (7–9) have been shown to stimulate an efficacious immune response against tumors outside the central nervous system (CNS). The CNS, however, has been shown to be a barrier to allograft and xenograft rejection and has been considered an "immunologically privileged" site both historically (16, 17) and more recently (18). Indeed, the concept that immunologic reactions against tumors within the CNS may be impaired has been supported by other studies of experimental (19) and clinical (20–22) immunotherapies where systemic responses failed to prevent tumor growth in the CNS. The CNS, then, may pose a significant barrier to the use of cytokine-assisted tumor vaccines.

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We have undertaken a series of experiments, using the well-characterized B16 murine melanoma model, to determine what barrier the CNS might pose to cytokine-assisted tumor vaccines, to determine which cytokines would be most efficacious against tumors within the CNS in this model, and to elucidate the details of the mechanisms of rejection stimulated by these vaccines against tumors within the CNS. Our studies demonstrate that B16-F10 murine melanoma (B16) cells genetically engineered to produce GM-CSF, IL-3, or IL-6, when used as s.c. vaccinations in syngeneic C57BL/6J mice, stimulate a potent and persistent antitumor immune response against B16 tumors located in the brain and increase the survival of tumor-bearing mice. We also show that the antitumor immune response induced by GM-CSF-producing vaccines is the most potent of six cytokines tested. In our brain tumor model, the response induced by GM-CSF-producing vaccines is specific to tumor type and abrogated by depletion of CD8$^+$ cells. Unlike the antitumor responses generated by GM-CSF against s.c. tumors, the efficacious responses observed in the brain were not dependent on CD4$^+$ cells. These data suggest that, although the CNS does not pose an absolute barrier to cytokine-assisted, cell-mediated immunotherapy, the immune responses generated by these vaccines against tumors within the CNS may be significantly different from those generated against tumors outside the CNS.

MATERIALS AND METHODS

Tumor Cell Lines and Animal Models. The B16 cell line (23), provided by I. Fidler (M. D. Anderson Cancer Center, Houston, TX), was grown in zinc option medium (Life Technologies, Grand Island, NY) containing 5% (vol/vol) fetal calf serum. The murine Lewis lung carcinoma (LLC1) cell line (24), obtained from the American Type Culture Collection, was grown in DMEM (Life Technologies) containing 10% fetal calf serum. All cell lines were grown in antibiotic-free medium and were shown to be free from *Mycoplasma* contamination (25). Both the B16 and LLC1 cell lines are syngeneic in the C57BL/6 mouse, and all experiments used 6- to 12-week-old female C57BL/6J mice which were maintained in a virus-free environment in accordance with the Laboratory Animal Resources Commission standards. After an animal's death, the tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. All animal experiments were performed at least twice to confirm results.

Retroviral Infection and Cytokine Expression. CRIP viral packaging cell lines were used (9, 26). B16 cells were infected

Abbreviations: γ-IFN, γ-interferon; B16, B16-F10 murine melanoma; CNS, central nervous system; DPBS, Dulbecco's phosphate-buffered saline; GM-CSF, granulocyte-macrophage colony stimulating factor; IL-2, interleukin 2; LLC1, Lewis lung carcinoma; NK, natural killer.

by exposure to viral supernatants from these cells in the presence of (8 μg/ml) polybrene (Sigma). Cytokine production from 1×10^6 B16 cells exposed to virus and grown for 48 h in 10 ml of growth medium was determined by ELISA (Endogen, Cambridge, MA) and confirmed by standard bioassays (27). B16 cells were stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (Sigma) after fixation with 0.5% glutaraldehyde to demonstrate the presence of the LacZ gene.

Subcutaneous Vaccinations. B16 cells were harvested with 0.125% trypsin and 0.02% ethylenediaminetetracetic acid, and washed once in serum-containing medium and twice in Dulbecco's phosphate-buffered saline (DPBS). Cell pellets were resuspended in DPBS, exposed to 3500 cGy from a ^{137}Cs source, and injected s.c. near the right groin in a volume of 500 μl.

Injections in the Brain. B16 cells or LLC1 cells were harvested, mixed with an equal volume of 10% methylcellulose in zinc option medium, and loaded into a 250-μl syringe (Hamilton) with an attached 25-gauge needle. The needle was positioned at bregma, 2 mm to the right of the cranial midline suture and 4 mm below the surface of the skull using a stereotactic frame (Kopf Instruments, Tujunga, CA). Cells were then implanted into the brain in a volume of 5 μl.

***In Vitro* Cytotoxicity Assay.** Cytolysis of B16 cells and YAC cells (28) by splenocytes obtained from tumor-bearing mice 2 weeks after vaccination was determined using standard assays (27). The YAC cell line was obtained from the American Type Culture Collection.

Lymphocyte Depletions. Antibodies were used to deplete $CD4^+$, $CD8^+$, and natural killer (NK) cell subsets *in vivo* using standard techniques (27). Hybridomas GK1.5 (29) and 2.43 (30) were obtained from the American Type Culture Collection and grown i.p. in athymic mice as a source of anti-CD4 and anti-CD8 antibodies, respectively. Polyclonal rabbit anti-asialo GM1 antibody (78 μg/ml) against murine NK cells was obtained commercially (Wako Chemicals, Richmond, VA). Vaccinated mice were injected i.v. once 4 days before tumor challenge and injected i.p. every 3 days thereafter for 12 days after tumor challenge with pretitrated amounts of one of these antibodies. In a cohort of mice that paralleled the experimental groups, flow cytometric analysis of splenocytes, using fluorescein isothiocyanate-labeled anti-CD3 (145–2C11), anti-CD4 (GK1.5), and anti-CD8 (53–6.72) antibodies (PharMingen), confirmed a >97% depletion of the targeted subset and a normal level of the other subsets both at the time of tumor challenge and again before the final antibody injection. NK cell depletion was confirmed by immunohistochemical staining of spleens from depleted mice as described below. Depletion of all subsets was also confirmed by immunohistochemical analysis of tumor-containing tissue as described below.

Immunohistochemistry. Brains from tumor-bearing animals were snap frozen in Tissue-Tek O.C.T. (Miles), sectioned at 4 μm onto gelatin-coated slides, and fixed in cold acetone. Immunoperoxidase staining was performed using the avidin-biotin-peroxidase Vectastain Elite ABC kit (Vector Laboratories). Primary antibodies were GK 1.5 (CD4), 53–6.72 (CD8) (31), F4/80 (macrophages) (32), and the polyclonal rabbit anti-asialo GM1 antibody (NK cells). Secondary antibodies were biotinylated goat anti-rat immunoglobulin (Vector Laboratories) and goat anti-rabbit immunoglobulin (Southern Biotechnology Associates). Sections were developed with 3,3′-diaminobenzidine tetrahydrochloride (Sigma), counterstained with 1% hematoxylin, and permanently mounted. Results were analyzed by a blinded observer.

Statistical Analysis. Survival estimates and median survivals were determined using the method of Kaplan and Meier (33), and 95% confidence intervals, shown in parentheses after the median survival, were calculated using the method of Hettmansperger and Sheather (34) for nonparametric data. Survival data were compared using the proportional hazards regression model described by Cox (35). Student's *t* test was used for calculating the significance of other data. Statistical significance was determined at the 0.05 level.

RESULTS

We obtained the following high levels of cytokine production from infected populations of B16 cells: IL-2 (3300 units/ml), IL-3 (65 ng/ml), IL-4 (30 ng/ml), IL-6 (175 ng/ml), γ-IFN (35 ng/ml), and GM-CSF (430 ng/ml). The amount of cytokine produced by each of these unselected B16 cell populations was comparable to that previously reported by others for *in vivo* vaccination studies (9) and exceeded the concentrations generally used for *in vitro* immunologic assays (27). Cytokine production from these transfected cell lines was stable over a period of 14 days *in vitro*. The efficiency of our transfection protocol was estimated to be between 20% and 30% based on 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (Sigma) staining of B16 cells exposed to retroviral supernatants containing the LacZ construct as described. Uninfected B16 cells did not produce detectable levels of any cytokine tested. The retroviral constructs used in this study have previously been shown to be incapable of dissemination when injected into mice (36).

Subcutaneous Vaccination with Irradiated B16 Cells Producing GM-CSF, IL-6, or IL-3 Protects Against B16 Tumor Challenge in the Brain. To determine whether the production of various cytokines could enhance the ability of irradiated tumor cells to protect against a subsequent tumor challenge in the brain, we gave C57BL/6J mice s.c. vaccinations of 5×10^5 untransfected or cytokine-producing B16 cells and challenged them in the brain 1 week later with 200 untransfected B16 cells. All mice vaccinated with untransfected B16 cells succumbed to tumor with a median survival of 25 days (20.5–26.5; $n = 33$) (Fig. 1*A*). Under the same conditions, vaccination with DPBS alone or B16 cells expressing the bacterial LacZ gene produced similar negative results (data not shown).

Vaccination with cytokine-secreting B16 cells produced results that varied according to the cytokine being produced. Survival of mice vaccinated with B16 cells producing IL-4 or γ-IFN was not significantly different from mice treated with untransfected B16 cells. Mice in these groups had median survivals of 22 days (18.8–32.1; $n = 11$; $P = 0.67$) and 22 days (15.0–37.1; $n = 9$; $P = 1.00$), respectively. On the other hand, vaccination with B16 cells producing IL-2 resulted in a significant reduction in median survival to 16.5 days (15.3–20.3; $n = 10$; $P = 0.0088$) (Fig. 1*A*). In contrast, mice vaccinated with 5×10^5 irradiated B16 cells producing GM-CSF, IL-6, or IL-3 had median survival times that were significantly greater than mice vaccinated with untransfected B16 cells. Median survival times in these groups were 49 days (39.9–65.1; $n = 23$; $P < 0.0001$) (Fig. 1*A*), 40 days (30.0–38.0; $n = 10$; $P = 0.0007$) and 29 days (23.4–36.6; $n = 28$; $P = 0.0074$), respectively. In addition, eight of the 23 mice (34.8%) vaccinated with GM-CSF-producing B16 cells survived for >100 days and were rechallenged in the brain with 200 viable B16 cells at that time. One of these mice died from tumor 29 days after the second challenge, but the other seven have survived for >60 additional days without evidence of tumor. Based on these initial studies, we chose to further evaluate the properties of GM-CSF-producing vaccines.

Treatment with Irradiated B16 Cells Producing GM-CSF Prolongs Survival in Mice with Established Tumors in the Brain. Although the protective effect of cytokine production by B16 cells was demonstrated by the above studies, a question that may be more relevant is the potential for such therapies to eradicate established tumors. Therefore, 200 B16 cells were implanted in the brains of unvaccinated mice, and these mice were allowed to recover for 3 days before being treated. Mice killed at the time of treatment verified the presence of tumor within the brain, and treatment consisted of a single s.c. injection

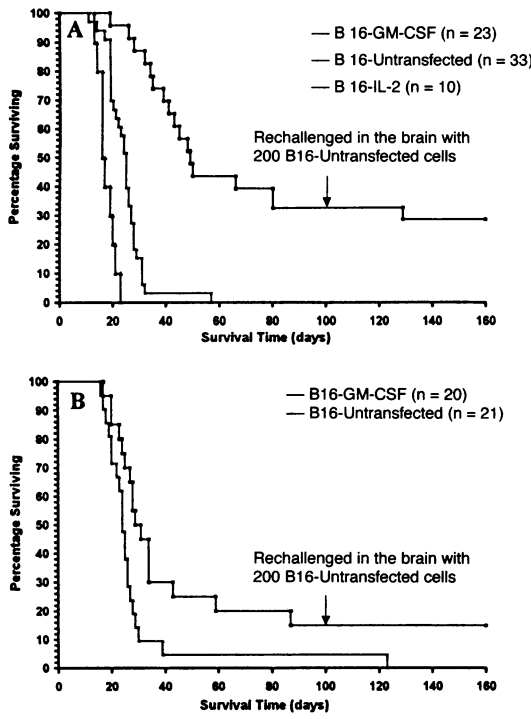

FIG. 1. Survival data for C57BL/6J mice vaccinated s.c. with irradiated tumor cells. (*A*) Mice vaccinated with 5×10^5 untransfected or cytokine-producing B16 cells and challenged in the brain 1 week later with 200 viable untransfected B16 cells. Mice surviving more than 100 days were rechallenged in the brain with another 200 untransfected B16 cells (arrow). (*B*) Survival data for mice treated with a single s.c. vaccination of 1×10^7 untransfected or GM-CSF-producing B16 cells, 3 days after intracerebral B16 tumors were established in the brain by injection of 200 viable untransfected B16 cells.

of 1×10^7 irradiated, untransfected B16 or GM-CSF-producing B16 cells.

In mice with established tumors, treatment with GM-CSF-producing B16 cells again enhanced survival. Mice treated with untransfected B16 cells had a median survival of 24 days (21.4–26.3; $n = 21$), whereas mice treated with GM-CSF-producing cells had a significantly longer median survival of 30 days (25.5–40.9; $n = 20$; $P = 0.014$) (Fig. 1*B*). In addition, three of the 20 mice (15.0%) vaccinated with GM-CSF-producing B16 cells survived for >100 days. These mice were rechallenged as described above and have survived for >60 additional days without evidence of tumor. One mouse in the group treated with untransfected B16 cells also survived for >100 days after the initial tumor challenge but succumbed to rechallenge with B16 cells in 23 days. Although treatment with the same number of either IL-3- or IL-6-producing B16 cells also resulted in a slight increase in median survival, this did not reach statistical significance, and there were no long-term survivors in either of these groups.

Splenocytes from Mice Vaccinated with B16 Cells Producing GM-CSF Have Enhanced Antitumor Cytotoxicity *in Vitro*. To determine whether the increases in survival produced by these vaccines could be a consequence of enhanced cell-mediated immunity, we studied the ability of splenocytes from vaccinated mice to lyse B16 cells and YAC cells *in vitro*. Splenocytes from mice vaccinated with either DPBS or irradiated, untransfected B16 cells demonstrated minimal ability to lyse B16 cells or YAC cells at all effector/target ratios tested (Fig. 2). However, splenocytes from mice vaccinated with B16 cells producing GM-CSF demonstrated a significant increase in their ability to lyse both B16 cells and YAC cells at most effector/target ratios tested ($P < 0.05$). This enhanced lysis of B16 cells could be abrogated by co-incubation with the anti-CD8 antibody, 2.43, at the lower effector/target ratios tested (data not shown).

Antitumor Response Produced by B16 Cells Producing GM-CSF Is Specific to B16 Tumors *in Vivo*. The demonstration that splenocytes from mice vaccinated with B16 cells producing GM-CSF possessed an enhanced ability to lyse YAC cells suggested that the antitumor response generated by this vaccination protocol might be nonspecific. Therefore, to determine if the antitumor response *in vivo* was specific to tumor type, mice vaccinated with untransfected or GM-CSF-producing B16 cells were assessed for their ability to reject syngeneic LLC1 tumors. Mice were vaccinated as described above and challenged in the brain 1 week later with either 200 B16 cells or 200 LLC1 cells. The production of GM-CSF by the B16 cell vaccines protected mice challenged with B16 cell tumors as reported above. The median survival time of mice with LLC1 tumors that had been vaccinated with untransfected B16 cells was 29 days (25.9–31.2; $n = 11$), which did not differ from the median survival of 29 days (26.0–32.0; $n = 15$; $P > 0.31$) in mice vaccinated with GM-CSF-producing B16 cells.

Rejection of B16 Tumors Within the Brain by Mice Vaccinated with B16 Cells Producing GM-CSF Requires $CD8^+$ Cells but Not $CD4^+$ or NK Cells. It has been previously demonstrated that induction of an antitumor immune response by GM-CSF-producing tumor vaccines is dependent on both $CD4^+$ and $CD8^+$ cells but not NK cells (9). To further clarify the role of NK cells in the effector arm of the antitumor response against a tumor within the CNS and to define the lymphocyte subsets necessary to produce such a response, vaccinated mice were depleted of NK, $CD8^+$, and $CD4^+$ cell subsets *in vivo*.

These lymphocyte depletion studies demonstrated that only $CD8^+$ cells were essential effector cells. Mice vaccinated with 5×10^5 irradiated, untransfected B16 cells had a median survival of 19 days (15.1–25.5; $n = 8$), and those vaccinated with B16 cells producing GM-CSF had a median survival of 42 days (30.0–63.0; $n = 10$). In addition, three mice (30%) in the group vaccinated with GM-CSF-producing B16 cells survived for >100 days without evidence of tumor. NK cells were shown not to be vital to the effector mechanism, as mice vaccinated with B16 cells producing GM-CSF and depleted of NK cells had a median survival of 35 days (25.1–37.6; $n = 6$), which was not significantly different from that in undepleted mice vaccinated with GM-CSF-producing B16 cells ($P = 0.23$). Conversely, mice vaccinated with B16 cells producing GM-CSF and then depleted of $CD8^+$ cells completely lost the survival advantage usually conferred by these vaccinations and had a median survival of only 22 days (17.0–25.0; $n = 5$), which was significantly less than that in undepleted mice ($P = 0.012$). Unexpectedly, $CD4^+$ cells, which have been shown to be essential to the effector arm of the antitumor response against s.c. B16 tumors (9), were not found to be essential in the effector arm of the response against B16 tumors in the brain. Mice vaccinated with GM-CSF-producing B16 cells and then depleted of $CD4^+$ cells had a median survival of 33.5 days (22.4–60.9), which was not significantly different from that in undepleted mice vaccinated with GM-CSF-producing B16 cells ($P = 0.55$). In addition, one mouse in the group depleted of $CD4^+$ cells (16.7%) survived for >100 days without evidence of tumor. This mouse was rechallenged as described above and has survived for >60 additional days without evidence of tumor.

Prolonged Survival Is Associated with Infiltration of Tumor with $CD8^+$ Cells in Mice Vaccinated with B16 Cells that Produce GM-CSF. To further define the importance of NK, $CD8^+$, and $CD4^+$ cells at the tumor site, we performed immunohistochemical analysis of the tumor inoculation site in the brain. Tumors examined from mice vaccinated with DPBS and challenged in the brain 1 week later with 200 untransfected B16 or LLC1 cells demonstrated infiltration with small num-

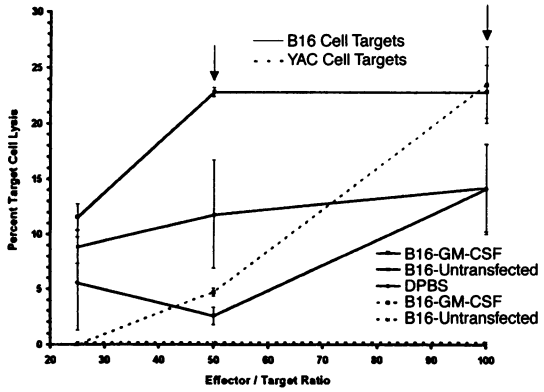

FIG. 2. Lysis of B16 and YAC cells *in vitro* by splenocytes from vaccinated mice. Splenocytes were harvested from C57BL/6J mice that had been vaccinated s.c. 2 weeks earlier with DPBS or 5×10^5 irradiated, untransfected B16 or GM-CSF-producing B16 cells and challenged in the brain 1 week earlier with 200 viable B16 cells. Splenocytes were stimulated *in vitro* for 5 days with untransfected B16 cells. Data for B16 cell lysis is shown with solid line, and data for YAC cell lysis is shown with dotted line. Arrows indicate effector/target ratios where splenocytes harvested from animals vaccinated with GM-CSF-producing B16 cells produced significantly greater lysis of B16 and YAC cells ($P < 0.05$). Error bars indicate one standard deviation from the mean.

bers of $CD4^+$, $CD8^+$, and NK cells (Table 1). In mice with B16 tumors, this infiltrate was not substantially different in mice vaccinated with 5×10^5 irradiated, untransfected B16 cells, and no tumors could be identified in mice vaccinated with GM-CSF-producing vaccines. However, vaccination with 5×10^5 irradiated, untransfected or GM-CSF-producing B16 cells did increase the infiltrate into LLC1 tumors of $CD4^+$ and NK cells. However, upon examination of mice having established B16 brain tumors that were treated with 1×10^7 irradiated, untransfected or GM-CSF-producing B16 cells (Table 1; Fig 3), mice vaccinated with GM-CSF-producing vaccines demonstrated a marked increase in the infiltration of both $CD8^+$ and NK cells.

Immunohistochemical examination of tissues from mice that were vaccinated with B16 cells which produced GM-CSF and were depleted of specific lymphocyte subsets at the time of tumor challenge demonstrated that mice vaccinated with GM-CSF-producing B16 cells that were then depleted of $CD4^+$ cells still maintained a significant infiltrate of $CD8^+$ cells into the tumor (Table 1). Mice depleted of $CD8^+$ lymphocytes, however, not only failed to develop a peritumoral infiltrate of $CD8^+$ cells but also completely failed to develop any infiltrate of $CD4^+$ cells despite normal levels of $CD4^+$ cells in the spleen.

DISCUSSION

It has been convincingly demonstrated by others that vaccination with tumor cells genetically engineered to secrete various cytokines stimulates a potent immune response against tumors outside the CNS. The most significant finding reported here is that such vaccines were capable of inducing a similar response against tumors within the brain, suggesting that the immunologically privileged CNS may not be an absolute barrier to this form of active, specific immunotherapy. Although a limited number of previous experimental studies have been able to produce systemic sensitization against tumors within the CNS, these studies have generally employed chemically induced tumors that have been shown to be poor models of human tumors because of their high inherent immunogenicity (37). Other studies have also used vaccinations consisting of viable cells that are undesirable clinically or have compared the survival of their vaccinated animals only to completely unimmunized animals (38–45). In so doing, previous investigations have failed to control for the inherent immunogenicity that has been readily demonstrated in rodent models of tumor immunotherapy (9, 37). Results from such studies cannot be taken as evidence that such strategies have therapeutic potential for human tumors. In comparison, our study demonstrates that, under conditions where irradiated tumor cells produce no response, a single vaccination with irradiated GM-CSF-producing tumor cells produces a significant increase in median survival and a 15% incidence of long-term survivors in mice with pre-established tumors. This demonstrated potency of cytokine-producing tumor cells as vaccinations against pre-established tumors in the brain provides evidence that such vaccination strategies may be considerably more effective against CNS tumors in humans than previous strategies. Efficacy against tumors such as melanoma in the CNS is important because of the high incidence of brain metastases in patients with melanoma (46, 47); therefore, failure to treat tumors within the CNS would have a major impact on the efficacy of any immunotherapy approach.

Table 1. Quantitative immunohistochemical analysis of lymphocyte infiltrate into brain tumors of vaccinated mice

Tumor challenge	Vaccination	CD4 cell count*	CD8 cell count*	NK cell count*	Lymphocyte subset depleted
		Protection experiments†			
B16	DPBS	15	10	18	None
B16	B16-untransfected	9	19	10	None
B16	B16-GM-CSF†	—	—	—	None
LLC1	DPBS	7	13	16	None
LLC1	B16-untransfected	34	15	59	None
LLC1	B16-GM-CSF	52	14	48	None
		Treatment experiments‡			
B16	B16-untransfected	36	5	11	None
B16	B16-GM-CSF	43	42	57	None
		Lymphocyte depletion experiments§			
B16	B16-GM-CSF	0	85	58	CD4
B16	B16-GM-CSF	0	0	10	CD8
B16	B16-GM-CSF	17	21	1	NK

*Mean number of positively staining cells per 0.2 mm^2 field.

†Mice were vaccinated with DPBS, or 5×10^5 irradiated, untransfected or GM-CSF-producing B16 cells and challenged in the brain 1 week later with 200 untransfected B16 or LLC cells.

‡Mice had 200 untransfected B16 cells implanted in the brain and were treated 3 days later with 1×10^7 irradiated, untransfected or GM-CSF-producing B16 cells.

§Mice were vaccinated as for protection experiments and depleted of specific lymphocyte subsets as described. Mice were killed 7 days after vaccination and the tumor site within the brain examined immunohistochemically.

Although GM-CSF was found to be the most potent cytokine under the conditions evaluated, the production of IL-6, a cytokine found by Dranoff and coworkers (9) to stimulate a response against s.c. tumors was also found to be active against B16 tumors in the brain. In addition, the production of IL-3, a cytokine that has been shown to generate lymphocytes *in vivo* that have cytotoxic activity *in vitro* (48) and to have antitumor activity against s.c. tumors (G.D. and R. C. Mulligan, unpublished data) was also active in this model. Conversely, we were not able to show any efficacy of IL-4-producing s.c. vaccinations against tumors within the CNS despite their established efficacy against s.c. tumors (9, 10). Unexpectedly, IL-2-producing vaccinations were found to significantly reduce the survival of mice with tumors in the brain. This is interesting in light of a recent study demonstrating that local production of IL-2 by tumors in the brain generates a lethal edematous response (18). These authors attributed the edema to a direct effect of IL-2 on the blood–brain barrier. The results reported here, however, demonstrate that extracranial vaccination with IL-2-producing cells can also reduce the survival of tumor-bearing animals. These studies raise the possibility that IL-2 may initiate a type of immune response that is deleterious in the brain.

GM-CSF has been hypothesized to act by stimulating the uptake and processing of tumor material by specialized antigen presentation cells, such as dendritic cells, leading to very effective antigen presentation *in vivo* (49). The studies reported here demonstrate that the GM-CSF-producing B16 cell vaccines also cause an increase in NK cell-mediated cytolysis of B16 cells *in vitro* and stimulate nonspecific invasion of NK cells into different types of tumors. Despite these findings, the studies reported here demonstrate that NK cells probably play a minor role, if any, in the antitumor immune response. This has important implications regarding the monitoring of immune responses induced by such vaccines. Clearly, routine *in vitro* cytotoxicity assays alone, which do not differentiate tumor-specific cytolysis, cannot be relied upon to predict an efficacious response *in vivo*. Our studies demonstrate that infiltration of tumor by $CD8^+$ cells *in vivo* may be more predictive of efficacious responses when GM-CSF-producing vaccines are used.

The most unexpected finding reported here, however, is the demonstration that, for GM-CSF-producing tumor cell vaccines, $CD4^+$ cells play a limited role in the effector arm of immune response against tumors within the brain. In contrast to other studies in which both $CD8^+$ and $CD4^+$ cells were shown to be necessary for effector responses against s.c. tumors (9), these studies found that efficacious immune responses against tumors within the CNS were observed even after apparent depletion of $CD4^+$ cells. It is unlikely that the number of $CD4^+$ cells appeared artificially low during flow cytometric analysis of splenic lymphocytes because $CD4^+$ cells were also not detected during immunohistochemical analysis of tumor tissue. In addition, the number of cells detected by the panlymphocyte marker, CD3+, on flow cytometric analysis was found to closely match the number of $CD8^+$ cells in mice depleted of $CD4^+$ cells.

The demonstration that the migration of $CD4^+$ cells into the CNS was also completely blocked in the absence of an activated $CD8^+$ lymphocyte subset is also an unexpected finding. The data reported here suggest that the CNS may pose a barrier that preferentially discriminates against a certain $CD4^+$ T-cell subset in the absence of activated $CD8^+$ cells. Alternatively, since the C57BL/6J mouse strain is poorly susceptible to the induction of certain autoimmune diseases of the brain (50) that are primarily mediated by $CD4^+$ T cells (51), these findings may be strain- or species-specific rather than univer-

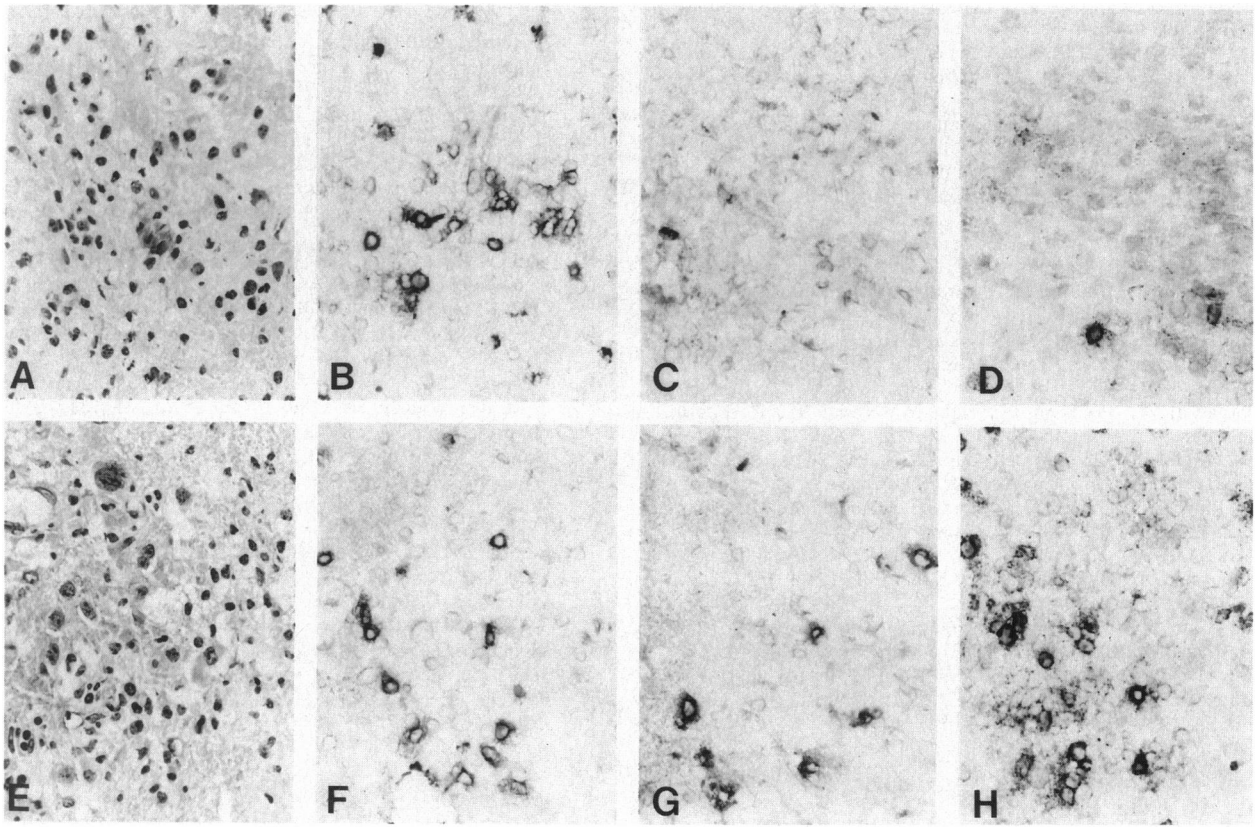

FIG. 3. Enhanced peritumoral infiltrates of $CD8^+$ and NK cells in mice vaccinated with GM-CSF-producing B16 cells. Brains from C57BL/6J mice with established B16 tumors in the brain were analyzed immunohistochemically 7 days after s.c. treatment with irradiated, untransfected (*A–D*) or GM-CSF-producing (*E–H*) B16 cells. Shown are hematoxylin and eosin (*A* and *E*) and immunoperoxidase staining with antibodies against CD4 (GK 1.5) (*B* and *F*), CD8 (53–6.72) (*C* and *G*), and NK (anti-asialo-GM1) (*D* and *H*) cells. (×400.)

sal. If this phenomenon is universal, then some of these findings may have very important implications for the treatment of primary brain tumors with such vaccines. One of the risks of vaccination with primary brain tumor cells is the potential for crossreaction between antigens on the tumor cells and normal brain antigens (52). Such crossreactivities may produce a potentially lethal postvaccinational autoimmune encephalomyelitis (53). Therefore, a carefully timed elimination of $CD4^+$ T cells may become a critically important method of reducing the risk of these vaccines when they are used against primary brain tumors.

Another potential limitation to the therapeutic use of cytokine-assisted tumor vaccines is the production of potent immunosuppressive cytokines, such as transforming growth factor β (TGF-β) (54). Studies that evaluate these possibilities are currently underway in our laboratory.

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