Brief Definitive Report

Bone Marrow–generated Dendritic Cells Pulsed with Tumor Extracts or Tumor RNA Induce Antitumor Immunity against Central Nervous System Tumors

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Summary

Recent studies have shown that the brain is not a barrier to successful active immunotherapy that uses gene-modified autologous tumor cell vaccines. In this study, we compared the efficacy of two types of vaccines for the treatment of tumors within the central nervous system (CNS): dendritic cell (DC)-based vaccines pulsed with either tumor extract or tumor RNA, and cytokine gene–modified tumor vaccines. Using the B16/F10 murine melanoma (B16) as a model for CNS tumor, we show that vaccination with bone marrow–generated DCs, pulsed with either B16 cell extract or B16 total RNA, can induce specific cytotoxic T lymphocytes against B16 tumor cells. Both types of DC vaccines were able to protect animals from tumors located in the CNS. DC-based vaccines also led to prolonged survival in mice with tumors placed before the initiation of vaccine therapy. The DC-based vaccines were at least as effective, if not more so, as vaccines containing B16 tumor cells in which the granulocytic macrophage colony-stimulating factor gene had been modified. These data support the use of DC-based vaccines for the treatment of patients with CNS tumors.

The concept that the brain is an immunologically privileged site has been supported clinically by the failure of central nervous system (CNS) tumors to respond to immunotherapy protocols that were successful systemically (1, 2). Recently, several groups including ours have described active immunotherapy protocols using intradermal vaccines of genetically modified tumor cells as being effective in rodent brain tumor models (3, 4). However, animal studies have shown that immunization with CNS-derived tumor material can induce fatal experimental allergic encephalitis (5). Due to the limited ability of reliably obtaining and growing a high percentage of tumor specimens without contamination by normal nervous tissue, the application of similar approaches to human patients with brain tumors may therefore carry the risk of causing such autoimmune complications (6, 7).

The dendritic cell (DC) network is a specialized system for presenting antigen to naive or quiescent T cells, and it plays a central role in the induction of T and B cell immunity in vivo. Immunizations using DCs loaded with tumor antigens may, therefore, represent a powerful method of inducing antitumor immunity. Recent studies have shown that immunizing mice with DCs pulsed with specific antigens can prime a CTL response that is tumor-specific and engenders protective tumor immunity in the treated mice (8–10). Immunization using defined tumor antigens is, however, limited at present to a handful of human tumor types in which candidates for tumor rejection antigens have been identified (11). More recently, effective tumor immunity in mice was induced using DCs pulsed with unfractionated tumor-derived antigens in the form of peptides (12, 13), cell sonicates (14), or messenger RNA (mRNA; 15). The advantages of vaccinating with total tumor-derived material are that the identity of the tumor antigen(s) need not be known and that the presence of multiple tumor antigens reduces the risk of antigen-negative escape mutants. The potential benefit of using total tumor antigens in the form of mRNA is that it can be amplified from a small number of tumor cells. Hence, DC vaccine treatment may be extended to patients with brain tumors from which only a small, possibly microscopic, biopsy can be taken for diagnosis. Furthermore, isolating bona fide tumor cells from patient specimens by ex vivo purification methods and combing this with the use of RNA subtractive hybridization techniques may reduce the concentration of self, potentially autoreactive, antigens in the vaccine preparation. This would be of crucial importance for vaccinations with CNS tumor-derived antigens, as it may diminish the risk of severe autoimmune complications.

The studies presented here evaluated and compared the efficacy of DC-based tumor vaccines pulsed with either tumor extract or tumor-derived total RNA, with that of tu-

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mor vaccines in which the gene for GM-CSF had been modified. The vaccines were studied in a model of active immunotherapy for CNS tumors.

Materials and Methods

Tumor Cell Lines and Animal Models. The B16/F10 murine melanoma cell line (B16) derived from a spontaneous melanoma in a C57BL/6 mouse (H-2^b) was provided by I. Fidler (M.D. Anderson Cancer Center, Houston, TX; reference 16). The SMA 560 cell line was derived from an intracerebral transplant of a spontaneous astrocytoma from a VM/Dk mouse (H-2^b) (17). The SMA 560 cell line was chosen as a control for the B16, since both cell lines are derived from neural crest. EL-4 (H-2^b) murine thymoma cells were obtained from American Type Tissue Culture Collection (Rockville, MD). Cell lines were grown in zinc option medium (GIBCO BRL, Gaithersburg, MD) containing 5% (vol/vol) FCS. All cell lines were shown to be free from *Mycoplasma* contamination as previously described (18). All experiments used 6–12-wk-old female C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME), which were maintained in a virus-free environment in accordance with the Laboratory Animal Resources Commission standards.

DC Generation from Bone Marrow Cultures. The procedure used in these studies was the same as previously described (8, 19). In brief, bone marrow was flushed from the long bones of the limbs and depleted of red cells with ammonium chloride. Bone marrow cells were depleted of lymphocytes, granulocytes, and Ia^+ cells using a mixture of mAbs and complement. The mAbs used were 2.43 or 53-6.72 (CD8), GK1.5 (CD4), RA3-3A1/6.1 (CD45R), B21-2 anti-Ia (Tumor Immunology Bank 210, 105, 207, 146, and 229, respectively; American Type Tissue Culture Collection), and RB6-8C5 anti–Gr-1 (provided by DNAX, Palo Alto, CA). Cells were plated in 6-well culture plates (10^6 cells/ml, 3 ml/well) in RPMI 1640 medium supplemented with 5% heat-inactivated FCS, 50 mM 2-mercaptoethanol, 10 mM Hepes (pH 7.4), 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 3.3 ng/ml GM-CSF (provided by Amgen, Inc., Thousand Oaks, CA). On day 3 of culture, floating cells were gently removed, and fresh medium was added. On day 7 of culture, nonadherent cells and loosely adherent proliferating DC aggregates were collected and replated in 100-mm Petri dishes (10^6 cells/ml, 10 ml/dish). At 10 d of culture, nonadherent cells (DCs) were removed for analysis and immunizations.

The quality of DC preparation was characterized by cell surface marker analysis, morphological analysis, and the ability of the preparation to induce OVA-specific CTLs in immunized mice as previously described (data not shown; references 19, 20).

Vaccination with DCs Pulsed with Tumor Extracts. DCs were washed twice in Opti-MEM medium (GIBCO BRL) and then resuspended at 5–10 $\times$ 10^6 cells/ml in 50 ml polypropylene tubes (Falcon, Lincoln Park, NJ). The cationic lipid, DOTAP, (Boehringer Mannheim Biochemicals, Indianapolis, IN) was used to deliver tumor extracts into cells. Tumor extracts were obtained by sonicating tumor cells in Opti-MEM (10^7 cells/500 μl) using a Special Ultrasonic Cleaner (Laboratory Supplies Company, Hicksville, NY) and were used without any further manipulation. Tumor extracts (500 μl) and DOTAP (125 μg in 500 μl Opti-MEM medium) were mixed in 12 $\times$ 75–mm polystyrene tubes at room temperature for 20 min. The complex was added to the DCs and incubated at 37°C in a water bath with occasional agitation for 25 min. The cells were washed and resuspended in PBS (10^5 extract-pulsed DCs in 500 μl PBS/mouse) for intraperitoneal immunizations.

Vaccination Using RNA-pulsed DCs. Total RNA was isolated from actively growing tissue culture cells by standard methods as previously described (15). Pulsing DCs with RNA was performed in serum-free Opti-MEM medium (GIBCO BRL) as described for tumor extracts with the following modifications. RNA (25 μg in 250 μl Opti-MEM medium) and DOTAP (50 μg in 250 μl Opti-MEM medium) were mixed in 12 $\times$ 75 mm polystyrene tubes at room temperature for 20 min. The complex was added to the DCs (2–5 $\times$ 10^6 cells/ml) and incubated at 37°C in a water bath with occasional agitation for 25 min. The cells were washed twice and resuspended in PBS (10^5 RNA-pulsed DCs in 500 μl PBS/mouse) for intraperitoneal immunizations.

PBS, B16 extract from 10^5 cells in PBS, or DCs prepared as described above were injected intraperitoneally in a volume of 500 μl.

Vaccination with GM-CSF–secreting Tumor Cells. Crip cells genetically engineered to produce replication-incompetent recombinant retrovirus with an amphotropic host range and with the ability to encode the cDNA for the murine GM-CSF gene were used as previously described (4, 21). B16 cells were infected by exposure to viral supernatants from these cells in the presence of polybrene (Sigma Chemical Co., St. Louis, MO). GM-CSF production from B16 cells exposed to virus was confirmed by ELISA (Endogen, Cambridge, MA) and standard bioassays (21).

B16 parent cells with the modified GM-CSF gene and the untransfected cells were harvested, washed once in serum-containing medium, and washed twice in PBS. Cell pellets were resuspended in PBS at 10^5/500 μl, irradiated (3,500 centiGray), and injected subcutaneously. The subcutaneous route of administration was chosen because we previously demonstrated that this is the preferred method for administering tumor vaccines (data not shown).

In Vitro Cytotoxicity Assay. In vitro cell–mediated cytotoxicity assays were performed using standard procedures as we previously described (22). In this study, splenocytes obtained from immunized animals and controls were restimulated in vitro for 5 d on monolayers of irradiated and mitomycin C–treated B16 cells or SMA 560 cells. Target cells included SMA 560 and B16 cells.

Implantation of Brain Tumors. B16 cells were harvested by trypsinization, washed twice in Dulbecco's PBS, and mixed with an equal volume of 10% methylcellulose in zinc option medium. The cells (500 cells in a volume of 5 μl) were then implanted into the right caudate nucleus of the brain of C57BL/6 mice by stereotactic injection as previously described (3, 4).

Statistical Analysis. Survival estimates and median survivals were determined using the method of Kaplan and Meier (23). Survival data was compared using Wilcoxon's log-rank test. Student's *t* test was used for calculating the significance of other data. Statistical significance was determined at the <0.05 level.

Results

Vaccination with Bone Marrow-derived DCs Pulsed with Tumor Extract or Tumor RNA Induces Tumor-specific CTLs. To test whether bone marrow–derived DCs pulsed with tumor extracts or tumor RNA are capable of inducing tumor-specific CTLs, we first immunized C57BL/6 mice with three intraperitoneal injections of DCs spaced 1 wk apart. Standard cytotoxicity assays were performed using

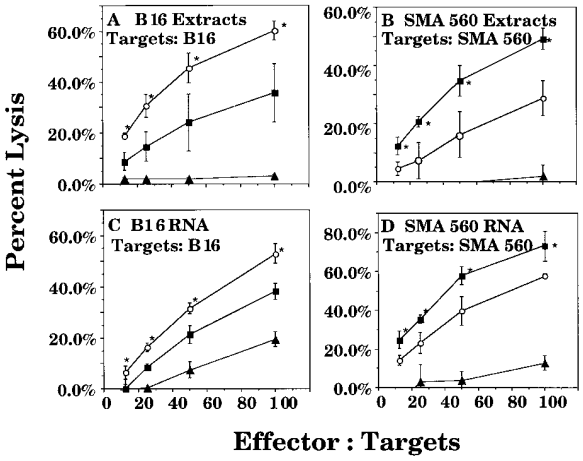

Figure 1. Induction of specific lytic activity against tumor cells by immunization with DC pulsed with tumor extracts or with tumor-derived total RNA. Triplicate C57BL/6 mice were immunized three times with either DC pulsed with (*open circles*) B16 tumor extract or (*filled squares*) SMA 560 tumor extract (*A* and *B*, respectively), or with DC pulsed with (*open circles*) B16 tumor RNA or (*filled squares*) SMA 560 RNA (*C* and *D*, respectively) or with PBS (*filled triangle*). 7 d later, splenocytes were isolated and restimulated for 5 d with irradiated B16 cells (*A* and *C*) or SMA 560 cells (*B* and *D*). Cytotoxic activity was then measured by chromium release assay using the targets indicated in each panel. *($P < 0.05$) indicate points of significant differences in lysis when B16-immunized animals are compared to SMA 560–immunized animals. Error bars indicate 1 SD of the mean.

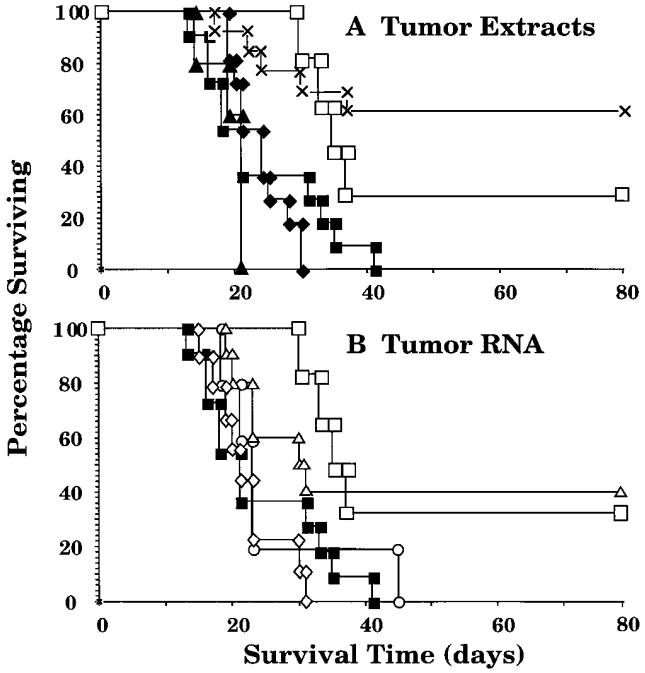

Figure 2. Vaccination with DCs pulsed with tumor extract or tumor RNA protects against CNS challenge with B16 tumor and is equipotent to vaccination with GM-CSF–producing B16 cells. Vaccination of C57BL/6 mice was performed a total of three times before intracranial tumor challenge with B16 cells as described in Materials and Methods. Mice were evaluated daily until death. Data are representative of two experiments performed with similar results. The results are divided in two panels for clarity. The results for the PBS-immunized group (*closed squares*) and the GM-CSF modified B16 cell immunized group (*open squares*) are represented in both *A* and *B* for ease of comparison. Median days of survival, range, number of animals, and significance compared to PBS-immunized animals based on log-rank analysis for each group are as follows: *closed squares*, PBS: 21, 13–41, $n = 10$; *X*, DC pulsed with B16 tumor extract: >80, 17–>80, $n = 13$, $P = 0.0006$; *closed diamonds*, DCs pulsed with SMA 560 extract; 24, 19–30, $n = 11$, $P = 0.50$; *closed triangles*, B16 extract: 21, 14–21, $n = 5$, $P = 0.45$; *open squares*, GM-CSF–modified B16 cells: 36, 30–>80, $n = 6$, $P = 0.022$; *open triangles*, DC pulsed with B16 RNA: 31, 19–>80, $n = 10$, $P = 0.0001$; *open diamonds*, DC pulsed with SMA 560 RNA: 21, 15–31, $n = 9$, $P = 0.40$; *open circles*, unmodified B16 cells: 23, 18–45, $n = 5$, $P = 0.39$.

splenocytes harvested from immunized animals 7 d after the third immunization and restimulated for 5 d in vitro with irradiated B16 cells. Cytotoxic activity was tested against B16 cells. We previously demonstrated that the B16 is a poorly immunogenic tumor cell line such that no induction of CTLs occurs after immunization with unmodified parent cells, and such immunization affords no antitumor immunity in vivo (4).

As shown in Fig. 1, immunization using DCs pulsed with either B16 tumor extract or B16 RNA induced B16-specific CTL responses that were statistically significant compared with animals immunized using DCs pulsed with either SMA 560 tumor extract or SMA 560 RNA, or with animals injected using PBS (Fig. 1, *A* and *C*). Conversely, splenocytes harvested from groups of animals immunized with SMA 560–pulsed (extract or RNA) DCs specifically lysed SMA 560 targets (Fig. 1, *B* and *D*), further demonstrating the tumor-specific nature of these responses. As seen in Fig. 1, high levels of nonspecific lysis are observed when animals are injected with bone marrow–derived DCs pulsed with control antigen, but not those injected with PBS. High levels of nonspecific lysis associated with the use of bone marrow–derived DCs have been previously described and were shown to be dependent upon the presence of sygeneic MHC class II molecules on the immunizing DCs (8) and can be partially overcome by the adherence depletion of antigen-presenting cells before the restimulation of effectors (19).

B16 Tumor Challenge in CNS After Immunization Using DCs Pulsed with Tumor Extracts or RNA. Next, experiments were performed to determine whether vaccination using B16-pulsed DCs generated specific and protective immunity against B16 tumors within the CNS. Groups of C57BL/6 mice received three intraperitoneal vaccinations spaced 1 wk apart and composed of PBS, B16 extract alone, or DCs pulsed with either B16– or SMA 560–derived extracts or RNA. Mice were then challenged in the brain 1 wk later with 500 viable B16 cells.

As shown in Fig. 2, immunizations with PBS, B16 extract alone, or DCs pulsed with SMA 560 extract or RNA did not protect against CNS challenge with B16 cells. All animals in these groups succumbed to tumor; median survival was between 21 and 24 d (Fig. 2, *A* and *B*). In contrast, the median survival of animals undergoing vaccinations of DCs pulsed with B16 extract was significantly prolonged to >80 d ($P = 0.0006$), with 8 of 13 animals surviving when the experiment was stopped at 80 d (Fig. 2

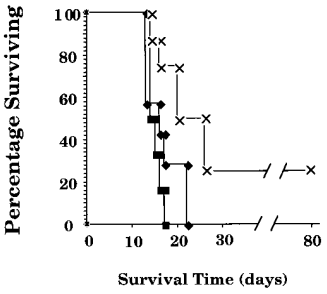

Figure 3. Treatment of established CNS B16 tumors with DCs pulsed with tumor extract prolongs survival. Intracranial tumor challenge with B16 cells was performed first and then, starting 4 d later, C57BL/6 mice were treated with a total of three immunizations as described in Materials and Methods. Mice were evaluated daily until death. Median days of survival, range, number of animals, and significance compared to PBS-immunized animals based on log-rank analysis for each group are as follows: *filled squares*, PBS: 16, 15–18, $n = 6$; *X*, DC pulsed with B16 tumor extract: 26, 14–>80, $n = 7$, $P = 0.037$; (*filled diamonds*), DC pulsed with SMA 560 extract: 16, 15–26, $n = 7$, $P = 0.57$.

A). Likewise, animals receiving vaccinations of DCs pulsed with B16 RNA experienced a significant improvement in median survival to 31 d ($P = 0.0001$), with 4 of 10 animals surviving when the experiment was stopped at 80 d. No statistical significance was demonstrated in the difference between groups immunized using DCs pulsed with either B16 extract or RNA ($P = 0.29$).

It has been convincingly demonstrated that vaccination with tumor cells genetically engineered to secrete various cytokines stimulates a potent immune response against tumors outside the CNS (21, 24, 25). We recently showed that subcutaneous vaccination with B16 cells that are genetically engineered to produce GM-CSF stimulates a potent antitumor immune response against B16 tumors located in the brain and increases the survival of tumor-bearing C57BL/6 mice. It was, therefore, of interest to compare the efficacy of DC-based immunotherapy with immunization using tumor cells having the modified GM-CSF gene. As shown in Fig. 2, *A* and *B*, mice vaccinated with GM-CSF gene-modified B16 cells had a median survival of 36 d with two of six mice surviving beyond 80 d, whereas mice vaccinated with B16 cells alone did not exhibit a survival advantage. Vaccination using DCs pulsed with cell extracts (Fig. 2 *A*) or tumor RNA (Fig. 2 *B*) was as at least as effective as vaccination using the B16 cells containing modified GM-CSF. Although the median survival of the group vaccinated using DCs pulsed with B16 tumor extract was higher than the group vaccinated with GM-CSF–modified B16 cells, the level of protection achieved with the DC-based vaccines was not statistically greater than that obtained with vaccines using GM-CSF–modified B16 cells ($P = 0.069$).

Prolonged Survival of Mice Bearing CNS B16 Tumors and Treated Using DCs Pulsed with B16 Tumor Extract. In considering the clinical application of a tumor vaccination strategy, it is more realistic to treat animals with tumor present at the time of vaccination. Thus, in the next experiment, 500 B16 cells were implanted in the brain of naive mice, and these mice were treated starting 4 d later, at which point vascularized tumor can be demonstrated histologically (4). Animals received treatments with three intraperitoneal vaccinations spaced 1 wk apart with PBS, DCs pulsed with SMA 560 extract, or DCs pulsed with B16 extract (Fig. 3). Mice in the first two groups had median survivals of 16 d. Mice treated with the DCs pulsed with B16 extract had a significantly longer median survival of 26 d ($P = 0.037$), with two of seven animals surviving at 80 d when the experiment was stopped.

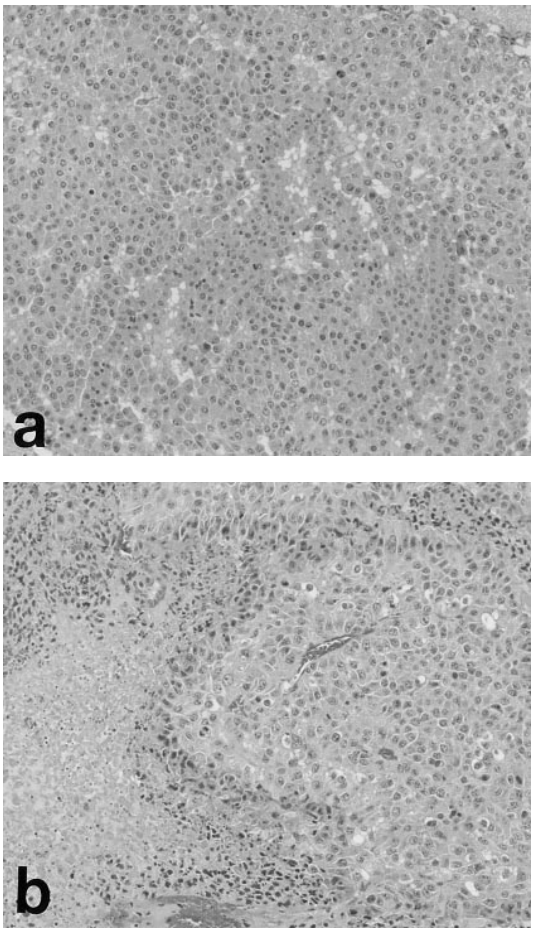

Figure 4. CNS B16 tumors from animals vaccinated with DC pulsed with B16 tumor extract demonstrate large areas of necrosis, hemorrhage, and inflammatory infiltrate (*b*). No such changes are seen in CNS B16 tumors from animals vaccinated with DC pulsed with control tumor extract (*a*) or PBS (not shown).

Histologic Characterization of CNS Tumors in Immunized Animals. CNS tumors from triplicate animals immunized only once with PBS or DCs pulsed with B16 or control EL-4 tumor extract were examined histologically. Tumors of the group vaccinated with DCs pulsed with B16 extract demonstrated large areas of hemorrhage and necrosis with an associated heavy inflammatory infiltrate composed of both mononuclear cells and polymorphonuclear leukocytes. In comparison, no such areas were observed in either control group (Fig. 4). Outside the immediate peritumoral regions, the brain parenchyma appeared histologically normal.

Discussion

In this study, we showed that immunization with DCs pulsed with either unfractionated tumor extracts or with

total tumor RNA elicits potent immunity against CNS tumors in mice. We showed that this therapy can be used for both protection against CNS tumor challenge and in the treatment of established tumors. Furthermore, when compared directly, B16-pulsed DCs had potency at least equivalent to GM-CSF–modified B16 vaccines.

A number of barriers exist, in practice, to the treatment of human brain tumors using genetically modified autologous tumor cell vaccines. These vaccines require the considerable tasks of ex vivo purification, culture, expansion, and transfection of tumor specimens, a difficult undertaking even for tumors outside the CNS (6, 7). DC-based vaccines may overcome some of these problems. First, human DCs can be generated from peripheral blood; therefore, supplies are not limited. Secondly, the ex vivo manipulations required to produce antigen-pulsed DCs are simpler than those required for generating autologous tumor cell vaccines.

It has been shown that immunizing nonhuman primates and guinea pigs with human glioblastoma multiforme tissue can induce allergic encephalomyelitis that is lethal (5). Vaccination with unfractionated tumor–derived antigens, such as those possibly contained in an autologous tumor cell vaccine derived from the CNS and modified genetically, may lead to potentially disastrous consequences such as autoimmune encephalitis. This risk may limit the use of such vaccines to a minority of patients: those suffering with brain tumors from which highly purified tumor specimens can not be guaranteed. One approach that may overcome these drawbacks is to use, as a source of antigen, mRNA from tumor cells. In the case of brain tumors, an important advantage is that RNA may allow the use of subtractive hybridization techniques to reduce the concentration of antigens shared between tumor and normal CNS tissue, lessening the potential for autoimmunity.

By demonstrating that vaccines based on DCs pulsed with tumor extracts or RNA are active against CNS tumors and are equipotent to cytokine gene–modified vaccines, these studies establish a basis for future preclinical studies of human DC-based vaccines for treating brain tumors.

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