Tumor Immunity within the Central Nervous System Stimulated by Recombinant *Listeria monocytogenes* Vaccination

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ABSTRACT

Tumors arising within the central nervous system (CNS) present the immune system with a challenging target, given the heterogeneous nature of these neoplasms and their location within an “immunologically privileged” site. We used the lymphocytic choriomeningitis virus nucleoprotein (*LCMV-NP*) as a pseudotumor antigen to investigate recombinant *Listeria monocytogenes* as a tumor vaccine against s.c. and intracerebral challenges with a NP-expressing glioma, 9L-NP. Using Fischer 344 rats, we demonstrate that vaccination with recombinant *L. monocytogenes*-NP stimulates protection against s.c., but not intracerebral, 9L-NP tumor challenge in an antigen-specific, CD8⁺ T-cell-dependent manner. After s.c. tumor rejection, enhanced antitumor immunity is achieved via epitope spreading that permits complete resistance against lethal intracerebral challenge with 9L-NP and with the untransfected parental 9L tumor. Unlike the CD8⁺-dependent immune responses against s.c. 9L-NP tumors, this expanded intracerebral immunity against endogenous tumor-associated antigens is dependent on both CD4⁺ and CD8⁺ T cells. Taken together, these results demonstrate that the mechanisms of tumor immunity within the brain are different from those elicited against non-CNS tumors. Furthermore, vaccination approaches exploiting the concept of epitope spreading may enhance the efficacy of antitumor immune responses within the immunologically privileged CNS, potentially mediating tumor cell killing through both CD4⁺- and CD8⁺-dependent effector pathways.

INTRODUCTION

Among new cancer treatments currently being investigated, none is as theoretically appealing as immunotherapy, because it offers the potential for highly specific antitumor cytotoxicity. However, despite some successful trials of immunotherapy against certain peripheral tumors (1–3), studies of immune responses against i.c. neoplasms have yielded inconsistent results (4, 5). One explanation for this is that the CNS is “immunologically privileged” and devoid of lymphoid reactivity and normal immune surveillance (6). Although this may be true in healthy brain tissue, many studies have documented that T-lymphocytes and MHC antigens are easily detectable in the CNS during illness and disease (i.e., multiple sclerosis, encephalitis, and tumor; Ref. 7). The exact mechanisms by which this change in immunoreactivity occurs are yet unknown. However, the possibility that the immune system can mediate interactions with the CNS during disease, such as in the development of brain tumors, validates further investigation of immunological modes of therapy for these neoplasms.

It is now well accepted that tumor cells express unique antigens that can trigger immune responses and serve as targets for antitumor immunity (8–10). For malignant gliomas, the process of malignant transformation appears to result from a series of defined and characteristic DNA mutations of cancer-related genes (e.g., p53, p16, EGF-R, PTEN, etc.; Refs. 11–13). Genetic instability within malignant cells gives rise to aberrant proteins, which are the products of mutated or rearranged oncogenes, tumor suppressor genes, tumor differentiation genes, or embryonic antigens. Recent advances in molecular biology and cellular immunology have shown that these mutated gene products have the potential to be recognized by the host immune system and, thus, contribute to the elimination of neoplastic cells (9).

To our knowledge, no uniformly expressed tumor-specific antigen has yet been identified for primary brain tumors. Because of this, we have used the LCMV-NP as a pseudotumor antigen to induce antitumor immunity. LCMV infection of mice is one of the best characterized models for studying the interaction of a virus with the immune system of its natural host. This model antigen is well characterized with respect to both immunogenicity and T-cell memory (14, 15). It has been used extensively to study CD8⁺ T-cell-mediated immune responses in various other systems, as it has been well documented that the clearance of LCMV infection is mediated by CD8⁺ CTLs (16, 17).

Lm is a facultative, Gram-positive intracellular bacterium that is able to enter host cells, escape from the endocytic vesicle, multiply within the cytoplasm, and spread directly from cell to cell without encountering the extracellular milieu (18, 19). Thus, it has the unique ability to target its own antigens to both the MHC class I and class II pathways of infected cells for antigen presentation, leading to the induction of CD8⁺ CTLs (20–23). The ability of rLm to induce protective antiviral cell-mediated immunity against a heterologous pathogen was demonstrated previously with LCMV by immunization of animals with rLm strains expressing the LCMV-NP antigen (16, 17). Lm vaccines have also been tested successfully for their ability to elicit T-cell immunity against influenza virus, HIV, and tumor inoculation (25, 26).

We tested the hypothesis that rLm expressing LCMV-NP (rLm-NP) could induce antitumor immunity against s.c. and i.c. tumor challenges of 9L gliosarcoma cells engineered to express the same NP (9L-NP): (a) we show that rLm-NP immunization of rats protects against 9L-NP tumors when the tumor cells are given s.c. but not when they are given i.c.; this s.c. immunity is antigen specific, requiring the expression of the NP gene by the tumor target, and is completely abrogated by depletion of CD8⁺ T cells; and (b) our results demonstrate that such s.c. elimination of 9L-NP cells not only allows resistance against subsequent i.c. challenges with a lethal dose of 9L tumor cells expressing the NP antigen, but it also protects the animals against s.c. and i.c. challenges with parental 9L cells that do not express NP. We show that this enhanced immunity against paren-

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4 The abbreviations used are: i.e., intracranial; LCMV, lymphocytic choriomeningitis virus; Lm, *Listeria monocytogenes*; cfu, colony-forming unit; NP, nucleoprotein; rLm, recombinant *Listeria monocytogenes*; IL, interleukin; CNS, central nervous system; CMV, cytomegalovirus; TAA, tumor-associated antigen.

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tal 9L cells is because of expanded CTL activity against endogenous 9L TAA3s invoked by elimination of s.c. 9L cells expressing the NP pseudotumor antigen. This cross-over of antitumor immunity is reminiscent of the phenomenon of epitope spreading (27–29) and occurs in a CD4+ and CD8+ T-cell-dependent manner. Here we show that systemic induction of antitumor immunity via epitope spreading can mediate complete protection against a lethal i.c. tumor challenge within the immunologically privileged CNS.

MATERIALS AND METHODS

Cell Lines. 9L gliosarcoma cells and Rat2 fibroblasts (American Type Culture Collection, Manassas, VA) were grown in DMEM with 4.5 g/liter glucose and 1 mM sodium pyruvate (Life Technologies, Inc., Carlsbad, CA), supplemented with 10% fetal bovine serum (Irvine Scientific, Santa Ana, CA), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO2. The LCMV-NP gene was amplified by PCR and cloned into the eukaryotic expression vector pBK-CMV (Invitrogen, Carlsbad, CA). 9L cells were transfected with pBK-CMV encoding the LCMV-NP gene or empty pBK-CMV vector using N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid (Boehringer-Mannheim, Indianapolis, IN) and selected in medium containing 250 μg/ml neomycin (G418; Life Technologies, Inc.). Stable transfectants were selected by growing LCMV-9L clones (9L-NP) or vector alone (9L-V) in vitro in the presence of G418. Reverse transcription-PCR and Western blot analysis confirmed expression of LCMV-NP by 9L-NP clones (data not shown). The morphology of 9L-NP and 9L-V was similar to that of the parental 9L line. 9L-NP and 9L-V tumor cells grew at the same rate as 9L tumors in vitro and when injected in vivo.

rLm Strains. The rLm strain, rLm-NP, was constructed as described previously using the wild-type strain, 10403S, and expresses the entire LCMV-NP gene. After culture under conditions with appropriate selection and growth temperatures, desired recombinant strains that have integrated the antigen expression cassette into their genome were identified and confirmed by Southern blot analysis (16). Expression and secretion of LCMV-NP by rLm-NP was confirmed by Western blot analysis (data not shown). The morphology of 9L-NP and 9L-V was similar to that of the parental 9L line. 9L-NP and 9L-V tumor cells grew at the same rate as 9L tumors in vitro and when injected in vivo.

Animals and rLm Immunizations. All animal experiments used 8–10-week-old syngeneic female Fischer 344 rats (Charles River Laboratories, Wilmington, MA) weighing 150–175 grams and were approved by the UCLA Animal Research Committee. Animals were treated according to the Interdisciplinary Principles and Guidelines for Use of Animals in Research. The approximate i.v. LD50 of rLm-NP in female Fischer 344 rats was determined to be 4.36 × 107 cfu. Groups of anesthetized rats were immunized i.v. with 0.5 ml of PBS containing 1 × 107 cfu of either rLm-NP (recombinant Lm) or 10403S (wild-type Lm) and when injected in vivo.

s.c. and i.c. Tumor Challenges. 9L-NP, 9L-V, or 9L cells were harvested by trypsinization and washed once in serum-free medium and twice in PBS. For s.c. challenge, tumor cell pellets were resuspended in PBS and 50 μl (containing 5 × 106 cells) was injected i.c. into the right flank of Fischer 344 rats. Tumor growth was measured at 3–4 days post tumor injection. For i.c. challenge, 5 μl containing 5 × 105 tumor cells were implanted into the right frontal lobe of the brains of anesthetized Fischer 344 rats by stereotactic injection 1-mm anterior and 2-mm lateral to the junction of the bregma and sagittal sutures at a depth of 4.5 mm. Animals in each treatment group were monitored for development of neurological symptoms and survival. Survival estimates and median survival times were determined using the Kaplan-Meier method.

In Vitro Cytotoxicity Assay. Splenocytes from immune and naive rats were harvested, and single-cell suspensions were isolated. Splenocytes were resuspended in RPMI 1640 containing 25 μM HEPES buffer (Fisher Scientific, Tustin, CA) and supplemented with 10% fetal bovine serum, 2 mM GlutaMAX-1 (Life Technologies, Inc.), 100 units/ml penicillin, 100 μg/ml streptomycin, and 5 × 10−3 M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO). Pooled splenocytes (3 × 106 cells/well) from rats within each group were restimulated in vitro with irradiated (5000 rad) 9L-NP cells (3 × 105 cells/well), supplemented with 100 units/ml recombinant human IL-2 (Phar-Mingen, San Diego, CA) in 24-well, flat-bottomed plates (2 ml of final volume). After 5 days of coculture at 37°C in 5% CO2, the 9L-NP-stimulated splenocytes were harvested, washed, and resuspended to various concentrations. 9L-NP, 9L-V, and Rat2 fibroblast target cells (American Type Culture Collection) were harvested, washed with PBS, and labeled with 150 μCi of Na2CrO2 for 2 hours at room temperature. After extensive washing in PBS, 51Cr-labeled target cells were adjusted to 2 × 106 cells/ml and incubated (100 μl/well) with 9L-NP-stimulated splenocytes (100 μl/well) at various effector:target cell ratios for 6 hours at 37°C in 96-well, round-bottomed plates. After the culture period, the plates were centrifuged at 200 × g for 5 minutes, 150 μl of supernatant samples were harvested, and cpm per sample were determined using a Beckman Gamma 5500 counter. The percentage of specific lysis was determined as ((experimental release – spontaneous release)/maximum release – spontaneous release) × 100. All cytotoxic assays were performed in triplicate wells.

Adaptive Transfer Experiments. Splenocytes from immune or naive rats were harvested 3 weeks post-tumor implantation. Single-cell suspensions of splenocytes were isolated, pooled, resuspended to 1 × 107 cells/ml PBS, and split into three aliquots. One aliquot of whole splenocytes was transferred i.v. into naive rats (2 × 105 cells/rat). The other two aliquots were each incubated with purified rat IgG for 30 minutes at 4°C. For depletion of CD8+ and CD4+ T cells, the aliquots were incubated with purified mouse antirat CD8b monoclonal antibody or CD4 monoclonal antibody (PharMingen), respectively, for 45 minutes at 4°C. The aliquots were then washed in PBS, adjusted to 1 × 107 cells/ml PBS, and incubated with goat antimouse IgG conjugated M-450 Dynabeads (Dynal Biotech, Lake Success, NY) for 1 hour at 4°C. After the incubation, each cell suspension was placed within a strong magnetic field to concentrate positively selected cells while the CD8− or CD4+–depleted splenocyte suspensions were removed. The positively selected cells were washed with PBS, and the washed supernatants were added to their respective CD8− or CD4− splenocyte aliquots. The CD8− and CD4−–depleted splenocyte cells were concentrated, resuspended in PBS, and immediately transferred i.v. into naive rats (1.5 × 106 cells/rat). After the procedures for purification of the depleted populations, splenocyte morphology was assessed microscopically, and viability was determined by trypan blue exclusion. In all cases, the CD8− and CD4−–depleted populations were found to be morphologically similar to the unprocessed whole splenocytes, and viability exceeded 95%. Within 24 hours of the adoptive transfer of whole, CD8−–depleted, or CD4−–depleted splenocytes, recipient rats were challenged s.c. or i.c. with tumor cells as described above. Samples from the CD8− and CD4−–depleted splenocyte aliquots were stained with FITC-conjugated antirat CD8 and antirat CD4 (PharMingen), analyzed with a FACSscan (Becton Dickinson, San Jose, CA) flow cytometer, and determined to be >98% depleted of the respective T-cell subsets (data not shown).

Histological Analysis. Brains or s.c. tumors were removed at necropsy for histological and/or immunohistochemical examination. Before removing the tissues, rats were perfused with 500 ml of PBS followed by 750 ml of 4% paraformaldehyde. The brains or s.c. tumors were then removed and postfixed in 4% paraformaldehyde overnight at 4°C. The specimens were stored at 4°C in 15% sucrose in PBS before cryosectioning. Sections through tumor or the area of tumor implantation were cut at a thickness of 30 μm by a cryostat, and sections were either mounted on glass slides for routine histological staining with H&E or floated in PBS for immunohistochemistry. For immunohistochemistry, floating sections were blocked with normal goat serum, then incubated with biotinylated mouse anti-rat CD8 or CD4 monoclonal antibodies (Phar-Mingen). The sections were washed twice with PBS and incubated with biotinylated goat anti-mouse IgG (PharMingen). After washing sections with PBS, immunoreaction products were visualized using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) per manufacturer’s protocol, followed by incubation in a solution of 0.02% 3,3’-diaminobenzidine tetrahydrochloride.

Statistical Analysis. Data were analyzed using StatView 4.51 for Macintosh (Abacus Concepts, Inc., Piscataway, NJ) and are represented as the mean ± SE. Survival data were compared using Wilcoxon’s log-rank test. Student’s t test, unpaired and two-sided, was used for calculating the significance of all other data. Statistical significance was determined at the level of P < 0.05.
RESULTS

Vaccination with rLm-NP Protects Against s.c. Tumor Challenge with 9L-NP. We first tested the ability of rLm-NP vaccination to protect rats against s.c. or i.c. challenges with syngeneic 9L glioma cells engineered to express LCMV-NP as a pseudotumor antigen (Fig. 1). Fischer 344 rats were immunized with i.v. inoculations of either wild-type Lm or a well-characterized recombinant Lm vaccine strain (rLm-NP) that secretes LCMV-NP (16). Three weeks after immunization, naïve control animals and vaccinated rats were challenged s.c. (5 × 10⁶ cells/right flank, n = 25) or i.c. (5 × 10⁵ cells/right cerebrum, n = 10) with either empty vector-transfected 9L cells (9L-V) or 9L cells stably transfected with an LCMV NP-containing vector (9L-NP). Naïve controls and wild-type Lm-vaccinated rats demonstrated continuous, uncontrolled growth of s.c. 9L-NP tumors up to 3 cm² and were eventually sacrificed (Fig. 1A). Rats vaccinated with rLm-NP and challenged with s.c. 9L-V were also unable to control their tumor growth. In contrast to these control groups, all rLm-NP-vaccinated rats challenged s.c. with 9L-NP showed initial tumor development at 7 days, control of tumor growth and initiation of tumor regression by 15 days, and complete tumor eradication by 40 days postimplantation (P < 0.001).

rLm-NP vaccinated rats were also challenged i.c. with 9L-V or 9L-NP and observed for the development of neurological symptoms and survival (Fig. 1B). In contrast to the complete protection seen against s.c. tumors, all rLm-NP-vaccinated rats challenged i.c. with either 9L-V or 9L-NP developed neurological symptoms and eventually succumbed to their brain tumors within 30 days (P > 0.05). These results suggested that rLm-NP vaccination stimulated a NP-specific immune response that was sufficient to protect against s.c. 9L-NP tumors but was ineffective against primary i.c. tumor challenge within the CNS.

s.c. Anti-9L-NP Immunity Is Dependent on CD8⁺ T Cells. We next investigated the importance of T-cell subsets for s.c. tumor protection induced by rLm-NP vaccination. Splenocytes from rLm-NP-vaccinated rats were harvested, and aliquots of whole splenocytes were depleted of CD4⁺ or CD8⁺ T cells. Whole or depleted splenocyte preparations were injected i.v. into naïve rats, which were subsequently challenged s.c. with 9L-NP tumors. Data represent the mean tumor area ±SE of five naïve control rats that received no splenocytes (●) and 20 adoptive transfer recipient rats that received whole splenocytes (●, n = 7), CD4⁺-depleted splenocytes (●, n = 7), or CD8⁺-depleted splenocytes (●, n = 6) from rats immunized previously. Of the rats that received CD4⁺-depleted splenocytes, 2 of 7 were not able to control their tumor growth, whereas 5 of 7 controlled and eliminated their tumors. Data were combined from three independent experiments.

Regression of s.c. 9L-NP Tumors Expands CTL Activity against Shared 9L TAAs within the CNS. Murine studies have provided evidence of expanded antitumor immune responses, which recognize additional TAAs expressed on genetically modified tumor that are “shared” with the parental untransfected tumor. The possibility of such an expanded antitumor CTL response was investigated by harvesting splenocytes from naïve rats, tumor-bearing rats, and rLm-NP-vaccinated rats. Splenocytes were stimulated in vitro for 5 days with irradiated 9L-NP cells and IL-2. After the in vitro stimulation, effector cell populations were examined for cytotoxic activity against 9L-NP, 9L-V, 9L tumor cells, and syngeneic Rat2 fibroblasts in standard CTL assays (Fig. 3). Effector cells from naïve rats and tumor-bearing rats did not exhibit any CTL activity against any of the target cell populations (Fig. 3A). In marked contrast, effector cells from rLm-NP-vaccinated rats that had eliminated s.c. 9L-NP tumors demonstrated specific lysis of all 9L cell targets (parental 9L, empty vector-trans-
Enhanced Antitumor Immunity via Epitope Spreading Completely Protects Against i.c. Challenge with 9L-NP, 9L-V, and Parental 9L. With in vitro evidence of enhanced antitumor cytotoxicity, we tested the hypothesis that after the epitope-spreading event, immune rats would have improved resistance against subsequent s.c. and i.c. challenges with 9L tumors that do not express NP. rLm-NP-vaccinated rats that had eliminated s.c. 9L-NP tumors and naïve animals were challenged either s.c. (5 × 10^6 cells) or i.c. (5 × 10^5 cells) with 9L-NP, 9L-V, or parental 9L tumor cells (Fig. 4). Naive control rats demonstrated continuous, uncontrolled growth of s.c. tumors over 30 days and rapidly succumbed to i.c. tumors within 35 days. In contrast to naive controls, all immune rats that previously eliminated s.c. 9L-NP tumors were completely resistant to s.c. 9L-V and 9L challenges, showing no evidence of s.c. tumor development (Fig. 4A). These immune rats were also able to survive i.c. 9L-NP, 9L-V, and 9L challenges (Fig. 4B), demonstrating no neurological symptoms during 100 days of observation and no evidence of i.c. tumors at necropsy.

Because the 9L tumor cells were propagated in vitro, it is possible that they underwent antigenic shift or picked up tissue culture-associated antigens over extended passages. To test the inherent immunogenicity of the parental 9L tumor cell line in our animal model, 10 Fischer 344 rats were immunized with unmodified 9L tumor cells s.c. (5 × 10^5) and then challenged with 9L tumor cells i.c. (5 × 10^5) 1 week later. Two of 10 rats (20%) immunized with unmodified 9L cells were alive at 60 days (data not shown), although 0 of 10 (0%) survived past 100 days after i.c. 9L challenge (data not shown). This prolongation of survival of rats immunized with nontransformed 9L cells suggests that the 9L tumor cell line is immunogenic in itself. Nevertheless, treatment with rLm-NP followed by 9L-NP resulted in a significantly higher number of animals surviving i.c. 9L tumor challenge (100% tumor protection, Fig. 4), compared with immunizations with unmodified 9L cells alone (0% survival at 100 days, data not shown). Therefore, although the 9L tumor model is partially immunogenic; the results of our rLm-NP vaccination strategy are still of considerable interest.

Our results showed that the rLm-NP-vaccinated rats were protected from all subsequent 9L challenges, whether the 9L cells expressed the NP epitope or not. This suggests that antitumor immune responses targeted against the NP antigen, which initially only permitted elimination of s.c. 9L-NP tumors, were now expanded against endogenous TAAs on parental 9L cells and allowed complete protection against i.c. 9L tumor challenges within a traditionally "immune privileged" site.

i.e. Antitumor Immunity Is Dependent on CD4+ and CD8+ T Cells. We next determined the relative importance of T-cell subsets during protection against i.c. 9L tumor challenge. Splenocytes from rLm-NP-vaccinated rats that had eliminated s.c. 9L-NP tumors and naïve rats were harvested and fractionated into three aliquots, two of which were depleted of CD8+ or CD4+ T cells, respectively. Naïve recipient rats received i.v. adoptive transfers of either whole splenocytes from naïve rats (n = 5), whole splenocytes from immune rats (n = 8), CD8+ -depleted splenocytes from immune rats (n = 8), or CD4+ -depleted splenocytes from immune rats (n = 8). These rats were then challenged i.c. with 5 × 10^5 9L glioma cells within 24 h. Age-matched naïve control rats (n = 8) were also implanted i.c. with 9L cells. All rats that received naïve splenocytes or no adoptive transfer died of their i.c. tumors within 21 days postimplantation. In contrast, 100% of animals (8 of 8) that received immune whole
splenocytes survived >100 days after lethal i.c. 9L tumor challenge (Fig. 5). Neither CD4- nor CD8-depletion completely abrogated the protection conferred by the immune splenocytes. However, adoptive transfer of each of these depleted T-cell subsets conferred reduced protection compared with the transfer of whole immune splenocytes, with only 63% (5 of 8) of the CD8⁺-depleted group and 25% (2 of 8) of the CD4⁺-depleted rats surviving at 100 days. These results indicate that i.c. immunity within the CNS mediated by the enhanced anti-9L immune response is dependent on both CD4⁺ and CD8⁺ effector T cells, with CD4⁺ T cells playing a dominant role in this model.

**Histological Characterization of CNS Tumor Implantation Sites.** Brains from i.c. tumor challenge survivors were examined for the presence of tumor cells and T-cell infiltrates. Brains from naive control rats, immune animals, and adoptive transfer recipient rats challenged i.c. with 9L-NP, 9L-V, or 9L were harvested at necropsy, sectioned with a cryostat, and stained with H&E or antibodies for immunohistochemical analysis (anti-CD4 or anti-CD8). H&E-stained sections cut through brains obtained from all immune rats that survived their i.c. tumor challenge had no microscopic evidence of remaining tumor cells (Fig. 6A), whereas brains from all naïve rats that succumbed to i.c. tumor challenge had large tumor masses within the right frontal lobes (Fig. 6B). Immunohistochemical analysis of immune brain sections stained with antirat CD4 or CD8 antibodies demonstrated marked infiltration of CD8⁺ T cells around the tumor implantation site, with occasionally observed CD4⁺ cells (Fig. 6, C and D). Neither CD8⁺ nor CD4⁺ cellular infiltrates into the CNS were detectable in the brains of untreated tumor-bearing rats or sham-operated animals injected i.c. with PBS (Fig. 6, E and F). These results suggest that i.c. tumor survival was associated with complete elimination of tumor cells and evidence of remaining CD8⁺ and CD4⁺ T-cell infiltrates at the i.c. tumor implantation site.
DISCUSSION

In this study, we have shown that an antigen-specific immune response, stimulated by vaccination with recombinant Listeria secretory LCMV-NP (rLm-NP), resulted in 100% tumor rejection after s.c. implantation of 9L tumor cells expressing the NP antigen (9L-NP). Previous studies have demonstrated that rLm can invoke a specific antitumor response by functioning as an antigen-delivery vehicle (25, 33–35). In addition, Lm is known to induce strong T-helper responses biased toward the Th1-type CD4+ T-cell subset as a consequence of IL-12 induction, which results in the release of IFN-γ and subsequent up-regulation of MHC class I molecules on tumor cells (36, 37). Therefore, in our syngeneic rat model, we postulate two separate but cooperative mechanisms of antitumor immunity: (a) rLm functions as an adjuvant promoting the appropriate cytokine milieu and T-cell help for the development of strong cell-mediated immunity; and (b) the immunogenic NP antigen expressed on rLm-NP elicits an effective antigen-specific, CD8+ T-cell cytotoxic response against NP-transduced tumor cells. Although this CD8+-mediated antitumor immunity was sufficiently strong to eliminate s.c. NP-transfected tumors that may have otherwise escaped immune attack, it could not protect against tumors growing in the CNS.

However, once the host’s immune response rejected NP-transfected 9L cells injected s.c., rats developed systemic antitumor immunity and were completely resistant to subsequent challenges with high doses of 9L-NP cells both within the CNS and peripherally. Furthermore, these animals were protected from s.c. and i.c. rechallenges with 9L-V and parental 9L cells, suggesting that elimination of 9L-NP tumors may prime immune responses to recognize shared endogenous 9L antigens. In these rechallenge experiments, there was no initial tumor formation before tumor rejection, which suggested that systemic 9L immunity had already been well established and that immunological memory was sufficient to prevent tumor development.

Systemic immunity within the CNS was mediated by synergistic CD4+ and CD8+ T-cell responses. Adoptive transfer of immune spleen cells could confer anti-9L immunity to naïve recipients, and this passive transfer was eliminated partially by the depletion of either CD8+ or CD4+ T cells, indicating that both T-cell subsets play a role in the control of i.c. tumor growth in vivo. Specific CTL activity against glioma cells (9L-NP, 9L-V, and parental 9L), but not against syngeneic Rat2 fibroblasts, was easily detectable after in vitro stimulation. Histological analysis of the brains of the rats that survived i.c. tumor challenge revealed infiltration of the tumor site by both CD8+ and CD4+ T cells, again suggesting possible effector roles for these T-cell subsets in the i.c. antitumor response.

Our results and those of several other investigators have established the ability of effector immune cells to be activated against CNS tumors (5, 38–42). Previous preclinical studies using genetically modified tumor cells that express immunostimulatory cytokines, such as granulocyte macrophage colony-stimulating factor, IL-4, or tumor necrosis factor-α; Refs. 40 and 43–47) or that abrogate immunosuppressive cytokines (e.g., transforming growth factor-β2; Refs. 48 and 49) have been reported to effectively stimulate the immune response against tumors located in the immunologically privileged CNS. In comparison to these previously reported systems of tumor immunotherapy, the paradigm presented here differs in several distinct ways. First, rather than using biologically active cytokine molecules, we used glioma cells genetically engineered to express a foreign viral antigen (LCMV-NP) with no known biological activity or specific receptors in its host. We speculate that the effect provided by LCMV-NP is a result of the viral protein serving as an effective target antigen, which activates antigen-specific CD8+ cytotoxic T cells against tumor cell targets in which LCMV-NP is expressed. After CD8+ T cell-mediated lysis of tumor cells, inflammatory antigen-presenting cells, such as macrophages and dendritic cells, presumably phagocytize the tumor debris and subsequently present processed tumor antigens (both LCMV-NP and endogenous tumor peptides) to naïve CD4+ and CD8+ T cells. The use of an inert viral antigen expressed by tumor cells may have certain advantages over current cytokine-based tumor vaccines, which have been associated with severe brain edema because of cytokine-induced overstimulation of immune reactions within the CNS (43, 50, 51). The second distinction of our strategy lies in the use of Lm as an adjuvant promoting the appropriate Th2- and Th1-type immune responses for the process of antigen recognition and presentation to result in effective cell-mediated immunity against tumor epitopes (23, 36). Recent reports have established the safety and efficacy of rLm antitumor vaccines in several animal models without CNS disease (25, 33–35, 52). Our data shown here provide evidence that immunization with this attenuated bacterium is relatively safe in rats with i.c. tumors, as no brain edema or other neurological toxicities were noted in the treated animals.

Perhaps the most fundamental insights provided by our results are the findings of epitope spreading causing tumor-specific CTL responses in the CNS and the implications of this phenomenon for brain tumor immunotherapy. Epitope spreading is a process whereby epitopes distinct from and noncross-reactive with an inducing epitope become major targets of an ongoing immune response (27–30). We have demonstrated that specific, systemic antitumor immunity can be elicited within an immune-privileged site by vaccination with rLm and tumor cells genetically engineered to express a pseudotumor antigen. This immunotherapy approach not only eradicates tumors transfected with the foreign antigen but also elicits potent immune responses against parental tumors inoculated s.c. and i.c. Our finding of epitope spreading is in agreement with recent observations by other investigators, which suggest that CTL-mediated tumor cell destruction in vivo may involve cross-presentation of additional epitopes with the consequent activation of additional tumor-reactive lymphocytes (28–30). This process is obviously interesting in the clinical context of designing tumor vaccines, because it bypasses the need to define natural tumor-specific antigens to which cellular immune responses are directed. Because true tumor-specific antigens have not yet been identified for human brain cancer, targeting an extrinsic nontumor antigen to elicit tumor-specific immunity to natural tumor peptides may be an intriguing approach to cancer immunotherapy for CNS neoplasms.

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