Active Specific Immunotherapy against Occult Brain Metastasis

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ABSTRACT

We determined whether lyophilized High Five (H5) insect cells engineered to produce IFN-β (HSVBFVIFN-β) could induce systemic immunity against occult brain metastases. C3H/HeN mice were infected s.c. with syngeneic UV-2237M fibrosarcoma or K-1735M2 melanoma cells. Intracranial injection of 2 × 10⁶ lyophilized HSVBFVIFN-β cells produced complete regression of the s.c. tumors. Six weeks later, UV-2237M fibrosarcoma cells or K-1735M2 melanoma cells were injected into the internal carotid artery of naive or treated mice. UV-2237M brain metastases developed in naive mice or mice cured of K-1735M2 tumors but not in mice cured of UV-2237M tumors. Similarly, K-1735M2 brain metastases developed in naive mice or mice cured of UV-2237M fibrosarcomas but not in mice cured of K-1735M2 melanoma. In the next set of studies, mice were injected s.c. with UV-2237M fibrosarcoma cells. On day 7, UV-2237M fibrosarcoma cells or K-1735M2 cells were implanted into the internal carotid artery, and on day 10, the s.c. tumors were injected with lyophilized HSVBFVIFN-β. Both the s.c. tumors and the occult brain metastases produced from carotid injections were eradicated in a tumor-specific manner. The regression of the brain metastases was abrogated by depletion of CD4⁺ or CD8⁺ cells from immunized mice. These results demonstrate that specific systemic immunity can be induced by lyophilized HSVBFVIFN-β and that the resultant immune response can eliminate established brain metastasis.

INTRODUCTION

In the United States, >170,000 patients develop brain metastasis annually (1, 2). Despite recent advances in the diagnosis and treatment of brain metastases, the median survival of these patients is <1 year (3–5). Clearly, new approaches for treating this fatal aspect of cancer are urgently needed.

Immunotherapy is an attractive and promising strategy for treatment of cancer (6, 7). The goal of active, specific immunotherapy is to activate tumor-specific T cells and tumor-infiltrating macrophages (7, 8) to destroy cancer cells in both primary tumors and metastatic lesions (9, 10). Although the central nervous system has been considered to be an immunologically privileged site (11–14), recent studies indicate that tumors in the central nervous system can be partially or completely suppressed by active immunotherapy (15–19).

We established recently a novel active immunotherapeutic system consisting of a recombinant BV³ expression vector encoding IFN-β (HSVBFVIFN-β; Refs. 20–22). In our previous study, we injected a preparation of lyophilized HSVBFVIFN-β into s.c. murine UV-2237M fibrosarcomas and K-1735M2 melanomas. A potent systemic immune response was induced, leading to immunologically specific eradication of both injected primary tumors and un.injected lung metastases. In contrast, intratrual injection of a preparation containing the same amount of H5 insect cells, control vector-transduced H5 cells, or IFN-β did not significantly alter the growth of the tumors (22). In the present study, we determined whether this active-specific immunotherapy would also eradicate occult brain metastasis. We report that injection of HSVBFVIFN-β into UV-2237M fibrosarcoma or K-1735M2 melanoma growing s.c. eradicated the s.c. lesions and pre-existing occult brain metastases in an immunologically specific and T cell-dependent manner.

MATERIALS AND METHODS

Mice. Specific pathogen-free female C3H/HeN mice were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD). The animals were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care, and in accordance with current regulations and standards of the United States Department of Agriculture, Department of Health and Human Services, and NIH. The mice were used in accordance with institutional guidelines when they were 6–8 weeks of age, except where otherwise indicated.

BV, Insect Cells, and Culture Conditions. Grace’s medium, wild-type BV, pBlueBacHis2A BV transfer vector, liposome-mediated transfection kit, and S9 and H5 insect cells were purchased from Invitrogen Corporation (Carlsbad, CA). Fetal bovine serum was purchased from M. A. Bioproducts (Walkersville, MD), and EXCELL-400 medium from JRH Biosciences (Denver, CO). The S9 cells and the H5 cells were maintained as monolayer cultures in complete TNM-FH medium (Grace’s medium supplemented with 10% fetal bovine serum and Grace’s medium supplements) and serum-free medium EXCELL 400, respectively, at 27°C in an unhumidified chamber. The insect cells and preparations containing H5 cells, BV, and/or IFN-β were free of endotoxins as determined by the Limulus amebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA).

Expression of IFN-β in H5 Insect Cells. Vectors were constructed and expression of IFN-β induced using a kit from Invitrogen following the manufacturer’s instructions as detailed in our previous study (22). Briefly, the full coding sequence of murine IFN-β cDNA was subcloned into the BV transfer vector pBlueBacHis2A to derive the recombinant vector pHis2AIFN-β. BV-IFN-β gene was produced by cotransfecting S9 cells with pHis2AIFN-β and linearized Bac-N-Blue BV DNA by using a liposome-based transfection kit. The recombinant virus was propagated in S9 cells to achieve 5 × 10⁸ plaque-forming units/mL. To prepare HSVBFVIFN-β, we infected H5 cells with three multiplicities of infection of recombinant BV encoding the IFN-β for 48 h, which led to an accumulation of 2 × 10⁴ units of IFN-β/10⁶ H5 cells (determined by Access Biomedical Research Laboratories, Inc., San Diego, CA). One unit of HSVBFVIFN-β contained 2 × 10⁴ units of IFN-β, 1 × 10⁴ units of IFN-β, 2 × 10⁴ units of IFN-β, and 2 × 10⁴ units of IFN-β.

Tumor Models and Immunotherapy. The UV-2237M tumor cell line was derived from a spontaneous lung metastasis produced by parental UV-2237M fibrosarcoma cells originally induced in a C3H/HeN mouse by UVB radiation (23). The K-1735M2 melanoma cell line was derived from spontaneous lung metastases produced by parental K-1735M2 melanoma cells originally induced in a C3H/HeN mouse by UVB radiation followed by croton oil painting (24, 25). UV-2237M or K-1735M2 (2 × 10⁵, unless otherwise indicated) cells were inoculated s.c. into syngeneic C3H/HeN mice. When tumors reached 4–5 mm in diameter, the lesions were injected with PBS or HSVBFVIFN-β. The tumor size in two perpendicular diameters was measured with calipers every 5–7 days. Nonpalpable lesions were considered to have been eradicated.
Experimental Brain Metastasis.Suspensions of UV-2237M or K-1735M2 cells were injected into the internal carotid artery of C3H/HeN mice using the technique described previously (26). mice were killed when they were moribund or up to 180 days after the injection of the tumor cells. The brains were removed and fixed in 10% buffered formalin solution. Each brain was serially sectioned. The tissues were stained with H&E and examined for the presence of metastases.

Induction of Long-Term Tumor-specific Immunity and Intracarotid Challenge. Six weeks after eradication of s.c. UV-2237M or K-1375M2 tumors (by intraluminal injection of H5BVIFN-β), C3H/HeN mice were divided into two groups. The mice were challenged by intracarotid injection with UV-2237M cells or with K-1735M2 cells. Naive C3H/HeN mice injected with either cell line served as controls. Mice were killed when they were moribund, and the brains were harvested for histological examination. C3H/HeN mice were inoculated s.c. with UV-2237M or K-1735M2 cells. Two weeks later, all of the mice developed s.c. tumors averaging 7–8 mm in diameter. The mice were anesthetized with Nembutal, and the s.c. tumors were resected. The mice received intracarotid injections of either UV-2237M cells or K-1735M2 cells. Naive C3H/HeN mice injected with tumor cells in the internal carotid artery served as controls. The mice were killed when they became moribund, and the brains were harvested for histological examination.

Therapy of Occult Brain Metastases. We determined whether the injection of H5BVIFN-β into a s.c. UV-2237M tumor generated an immune rejection of brain metastasis. Mice were implanted s.c. with 2 × 106 UV-2237M cells in the right flank. When the s.c. tumors reached 3–5 mm in diameter (day 7), the mice were divided into two groups to receive an intracarotid artery injection of 2 × 106 UV-2237M cells or 2 × 106 K-1735M2 cells. Two days later, the UV-2237M s.c. tumors were injected with either lyophilized H5BVIFN-β in 100 μl PBS or with 100 μl PBS. The mice were observed daily for tumor growth exceeding 15 mm in diameter were resected. The mice were killed when moribund and autopsied. The brains were fixed in 10% formalin and examined histologically for the presence of brain metastasis.

Depletion of T Cells. One day before, and 1 and 3 days after the intratumoral injection of H5BVIFN-β, mice were injected i.p. with rat mAbs against CD4 (GK1.5 mAb; American Type Culture Collection, 200 μg/mouse), CD8 (116–13.1 mAb; American Type Culture Collection, 200 μg/mouse), or CD4 plus CD8. Control mice received three i.p. injections of rat IgG (200 μg/ mouse). In control experiments, we found that three i.p. injections of anti-CD4 and anti-CD8 mAb produced a 75% and a 90% reduction of CD4+ and CD8+ T cells, respectively, in the spleens as indicated by flow cytometric analyses. The depletion persisted for up to 5 weeks.

Immunohistochemistry. Immunohistochemical analyses of tumor tissues were performed as described previously (27). Briefly, after necropsy, tumor tissues were cut into 5-mm pieces, placed in OCT compound (Miles Laboratories, Elkhart, IN), and snap-frozen in liquid nitrogen. Frozen sections (8 μm) were fixed in cold acetone and treated with 3% hydrogen peroxide in methanol (v/v). The treated slides were blocked in PBS containing 5% normal horse serum/1% normal goat serum and incubated with antibodies to CD4 (American Type Culture Collection) or CD8 (Pharmingen, San Diego, CA) antigen for 18 h at 4°C in a humidified chamber. The sections were rinsed and incubated with peroxidase-conjugated secondary antibodies. A positive reaction was visualized by incubating the slides with stable 3,3′-diaminobenzidine (Research Genetics, Huntsville, AL) and counterstained with Mayer’s hematoxylin (Research Genetics). The slides were dried and mounted with Universal mount (Research Genetics). The images were digitized using a Sony 3CD color video camera (Sony Corporation, Tokyo, Japan) and a personal computer equipped with Optimas image analysis software (Optimas Corporation, Bothell, WA). For immunohistochemical staining using an antibody against proliferating cell nuclear antigen, paraffin sections (3–5 μm) of the tumor samples were placed on ProbeOn slides (Fischer Scientific) and stained as described for the frozen sections after deparaffinization and rehydration.

Statistical Analysis. Survival estimates and median survivals were determined using the method of Kaplan and Meier (28). The survival data were tested for significance using a log-rank test. The significance of differences in tumor incidence and tumor size was analyzed by the χ2 test and ANOVA, respectively.

RESULTS

Eradication of s.c. Tumors by H5BVIFN-β Confers Tumor-specific Immune Protection against Brain Metastasis. C3H/HeN mice were implanted s.c. with either UV-2237M or K-1735M2 cells, and on day 7, the resulting tumors were injected with H5BVIFN-β. Six weeks after the complete regression of the UV-2237-M fibrosarcoma or K-1735M2 melanoma (which was 9–10 weeks after injection), the mice were randomized to receive an intracarotid injection of either UV-2237M or K-1735M2 cells. In naive (control) mice, brain metastases developed in 13 of 14 and 14 of 14 mice, with a median survival of 28 and 24 days, respectively (Table 1). Mice cured of s.c. UV-2237M tumors by intraluminal injection of H5BVIFN-β did not develop UV-2237M brain metastases but did develop K-1735M2 brain metastases. The median survival of these two groups of mice was >180 days and 21 days, respectively (P < 0.01). Similarly, 9 of 12 mice cured of s.c. K-1735M2 tumors did not develop brain metastases of K-1735M2 cells but did develop brain metastases of the UV-2237M fibrosarcoma (10 of 12 mice). The median survival of these mice was >180 days and 31 days, respectively (P < 0.01).

The mere growth of tumors in the subcutis did not confer systemic immunity. Mice whose s.c. tumors were surgically excised (rather than treated with H5BVIFN-β) were challenged with tumor cells injected into the internal carotid artery. Brain metastases of UV-2237M or K-1735M2 cells developed in 12 of 15 and 10 of 10 mice originally implanted s.c. with UV-2237M tumors. Median survival of the mice was 31 and 23 days, respectively (Table 1). Similarly, the surgical removal of s.c. K1735M2 tumors did not significantly alter the development of brain metastasis by UV-2237M or K-1735M2 cells (Table 1). The growth of UV-2237M and K-1735M2 tumors was confirmed by histological analysis. Images of a typical histological staining are shown in Fig. 1, demonstrating that the intracarotid injection of UV-2237M or K-1735M2 cells produced tumors in con-
Eradication of Established s.c. Tumors and Occult Brain Metastasis by H5BVIFN-β Therapy. Next, we determined whether the injection of H5BVIFN-β into s.c. tumors could eradicate pre-existing, occult brain metastases. First, UV-2237M cells were inoculated s.c. into syngeneic C3H/HeN mice. When the tumors reached 3–5 mm in diameter, the mice were injected in the internal carotid artery with UV-2237M or K-1735M2 cells. Two days later, the s.c. tumors were injected with PBS or 2 units of H5BVIFN-β. The data summarized in Fig. 2 show that a single injection of H5BVIFN-β into the s.c. UV-2237M tumors led to complete regression of the s.c. tumors in 60–80% of mice (Fig. 2, A and C) and prolonged the survival of mice with UV-2237M brain metastases (P < 0.05; Fig. 2B) but not the survival of mice with K-1735M2 brain metastases (Fig. 2D). Moreover, we noted that mice (2 of 10; Fig. 2A) in which the H5BVIFN-β treatment failed to completely regress the s.c. tumors developed brain metastases. Histological examination of the brain confirmed that the injection of H5BVIFN-β into the s.c. tumors eradicated UV-2237M but not K-1735M2 tumors (Fig. 2E).

Eradication of Brain Metastases Is Mediated by Both CD4+ and CD8+ Cells. Because we have demonstrated that the eradication of s.c. tumors by H5BVIFN-β therapy requires both CD4+ and CD8+ cells (22), we determined whether these T-lymphocyte subsets were also involved in the destruction of UV-2237M brain metastases. C3H/HeN mice were injected s.c. with UV-2237M cells. When the resulting tumors reached 5–6 mm in diameter (day 7), the mice were injected in the carotid artery with UV-2237M cells. Two days later (day 9), the mice were injected i.p. with 200 μg/mouse of anti-CD4 and/or anti-CD8 antibodies. The i.p. injections were repeated on days 11 and 13. The s.c. tumors were injected once with the H5BVIFN-β preparation on day 10 (Fig. 3). Control mice whose s.c. tumors were treated with PBS had a median survival of 36 (29–56) days. Mice injected i.p. with PBS and H5BVIFN-β or IgG and H5BVIFN-β had a median survival of 115 (33–180) days and 180 (33–180) days, respectively. Surviving mice were killed on day 180, and the mice (5) treated with PBS plus H5BVIFN-β and 5 of 6 mice with control IgG plus H5BVIFN-β were histologically free of any brain metastases (P < 0.001). In sharp contrast, the median survival of mice injected with anti-CD4 antibody was 37 (31–51) days; with anti-CD8 antibody, 33 (27–61) days; and with anti-CD4 plus anti-CD8, 33 (25–49) days. Additionally, we noted that mice (2 of 10; Fig. 3) in which the H5BVIFN-β treatment failed to completely regress the s.c. tumors developed brain metastases. Histological examination of the brain confirmed that the injection of H5BVIFN-β into the s.c. tumors eradicated UV-2237M but not K-1735M2 tumors (Fig. 3E).
days ($P < 0.001$). These data suggest that both CD4 $^+$ and CD8 $^+$ T cells are involved in H5BVIFN-$\beta$ activity against the UV-2237M tumors in the brain of mice. Immunohistochemical analyses of brain metastases strengthened this suggestion. To determine whether brain metastases were infiltrated by CD4 $^+$ and/or CD8 $^+$ cells, mice were killed on day 17 of the experiment, i.e., 7 days after the injection of H5BVIFN-$\beta$ preparation into s.c. tumors. The brains were frozen and examined histologically (Fig. 4). In control mice, the brain metastases contained numerous CD4 $^+$ and CD8 $^+$ cells. In mice injected with H5BVIFN-$\beta$ and IgG, the brain metastases were densely infiltrated by CD4 $^+$ and CD8 $^+$ cells. These metastases eventually regressed. In mice injected with H5BVIFN-$\beta$ and antibodies against CD4 and/or CD8 antigens, the number of infiltrating CD4 $^+$ or CD8 $^+$ cells was reduced significantly. The median survival of mice given anti-CD4 and/or anti-CD8 antibodies did not exceed that of mice that did not receive H5BVIFN-$\beta$ treatment.

**DISCUSSION**

The regression of s.c. tumors after intralesional injection of a lyophilized preparation of H5 insect cells and murine IFN-$\beta$ (H5BVIFN-$\beta$) generated active-specific T cells (CD4 $^+$ and CD8 $^+$) that crossed the blood-brain barrier, and infiltrated and destroyed brain metastases. Systemic administration of antibodies against CD4 and/or CD8 antigens abrogated the active-specific therapeutic effects of H5BVIFN-$\beta$ in both s.c. tumors (22) and brain metastases (this study). The efficiency of rejecting the intracranial tumor challenge depended on the elimination of s.c. tumors with H5BVIFN-$\beta$ therapy.
Traditionally, the brain has been considered to be an immune-privileged site (11–14); however, several recent studies dealing with brain tumors suggest that the blood-brain barrier is not an absolute barrier for lymphocytes and macrophages (15, 18). In fact, activated T cells in the systemic circulation have been shown to freely traverse the barrier (29). Furthermore, s.c. injection with IFN-γ, IL-7, or B7-1 gene-transfected rat glioma cells has been shown to lead to the regression of occult intracerebral glioma isografts (19). Similarly, s.c. immunization with GM-CSF gene-engineered tumor cells have been shown to induce immune responses that protect mice from a second challenge by tumor cells implanted in the periphery and the brain (15). Interestingly, the subsets of T cells involved in host response induced by GM-CSF gene-engineered tumor cells in the periphery and brain differed (15). Both CD4+ and CD8+ T cells were required for immune protection against s.c. tumors, but immune protection in the brain required only CD8+ T cells (15). Our data show that, in mice whose s.c. tumors regressed after injection with H5BVIFN-β, both tumor-specific CD4+ and CD8+ T cells infiltrated the brain metastases. These data are consistent with those from studies on the regression of lung metastases (22) and suggest that the subsets of T cells required to eradicate tumors in the brain may vary with the cytokine used to initiate the therapy (15) and the type of tumor growing in the brain. On the other hand, whereas the lymphocyte depletion experiments in this study revealed critical roles for both CD4+ and CD8+ cells, their relative importance for immune priming versus effector function remains unclear. Because the administration of antibodies at day 10 resulted in the growth of s.c. tumors, the failure to control an intracranial disease might reflect the requirement of T cells during the priming phase. In future studies, we will additionally investigate the possible role of T cells in the effector phase by depleting CD4+ and/or CD8+ T cells in mice cured of primary tumors before intracranial injection of tumor cells. A therapy using lyophilized preparation of H5BVIFN-β, but not its individual component (H5 cells or IFN-β), was necessary for inducing the immune protection against the intracranial challenge. We base this conclusion on the observation that the induction of the immune protection depends on the elimination of s.c. tumors, and only treatment with H5BVIFN-β, but not H5 cells nor IFN-β, can eradicate s.c. tumors (22). However, the exact components in the preparation of H5BVIFN-β that augmented the immune stimulatory effects of IFN-β remain unknown. Recent studies demonstrate that the innate immune response against pathogens is dependent on pattern recognition receptors on antigen-presenting cells (30–33). These receptors recognize common patterns shared by bacteria or viruses that are not present on normal host cells. The triggering of pattern recognition receptors can lead to expression of high levels of costimulatory molecules, such as CD80 and CD86, that prime and activate antigen-specific T cells, and to the secretion of proinflammatory cytokines, e.g., IL-1, IL-6, IL-12, tumor necrosis factor α, GM-CSF, and type I IFN (30–33). Several recent studies show that the unmethylated CpG motifs in the insect cell DNA, by inducing type I IFN production, can augment T-cell responses to specific antigens (34–36). However, in our study, the intratumoral injection of H5 cells, or in other studies, H5 cells transduced with a baculoviral vector-expressing GM-CSF (data not shown), had minimal therapeutic effects on UV-2237M tumors. These data suggest that other components in the H5 cells serve as an adjuvant to augment the specific immune response against tumor cells. The present data do not exclude the possibility that other inflammatory stimuli, such as whole bacteria, endotoxins, and unmethylated DNA, combined with IFN-β could be as effective as insect cells in eradicating tumors. Furthermore, in the present study, we only investigated the role of insect cells plus IFN-β in eradicating tumors. Because IFN-β and IFN-α share type I IFN receptors, it is possible that IFN-α could substitute for IFN-β.

In summary, we show that the injection of lyophilized H5BVIFN-β into established s.c. tumors can eradicate the skin tumors and occult brain metastases. Unlike therapies using genetically modified tumor cells, the success of the H5BVIFN-β therapy does not require the transfection of tumor cells or the use of tumor antigens. The eradication of the brain metastases by the H5BVIFN-β therapy was not associated with any detectable behavioral changes in the treated tumor-bearing mice. Even 10 consecutive weekly s.c. injections of 20 units of H5BVIFN-β did not lead to demonstrable toxicity. For all of these reasons, H5BVIFN-β could be a potent adjuvant for active-specific immunotherapy against lung (22) and brain metastases.

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