Role of the Immune Response during Neuro-attenuated Herpes Simplex Virus-mediated Tumor Destruction in a Murine Intracranial Melanoma Model

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ABSTRACT

Neuro-attenuated herpes simplex virus-1 (HSV-1) γ34.5 mutants can slow progression of preformed tumors and lead to complete regression of some tumors. However, the role of the immune response in this process is poorly understood. Syngeneic DBA/2 tumor-bearing mice treated with HSV-1 1716 fourteen days after tumor implantation had significant prolongation in survival when compared with mice treated with viral growth sera (mock; 38.9 ± 2.3 versus 24.9 ± 0.6, respectively; P < 0.0001). Additionally, 60% of the animals treated on day 7 had complete regression of the tumors. However, no difference was observed in the mean survival rates of viral- or mock-treated tumor-bearing SCID mice (15 ± 1.7 versus 14.8 ± 2.2, respectively). When DBA/2 mice syngenic for the tumor were depleted of leukocytes by cyclophosphamide administration (before and during viral administration), there was again no significant difference observed in the survival times (19.0 ± 1.9 versus 19.5 ± 2.7, respectively). These data demonstrate that the immune response contributes to the viral-mediated tumor destruction and the increase in survival. Immune cell infiltration was up-regulated, specifically CD4+ T cells and macrophages (which are found early after viral administration). Prior immunity to HSV-1 increased survival times of treated mice over those of naive mice, an important consideration because 50–95% of the adult human population is sero-positive for HSV-1. Our results imply that the timing of viral administration and the immune status of the animals will be an important consideration in determining the effectiveness of viral therapies.

INTRODUCTION

Previous reports have demonstrated the ability of a replication competent neuro-attenuated HSV-11 to slow the progression of preformed tumors in experimental animals and, in some cases, to lead to complete regression of tumors (1–3). One concern regarding the use of neuro-attenuated HSV treatment for tumors is the effect of prior immunity on viral-mediated tumor destruction because 50–90% of the adult human population is sero-positive for HSV-1 (4). If the immune response contributes to tumor destruction, prior immunity to HSV may decrease the effectiveness of the treatment by eliminating the virus before it is able to spread throughout the tumor and destroy it.

The immune response to HSV has been characterized in the context of both the peripheral nervous system and the CNS (5–12). Both arms of the immune response, humoral and cellular, have been shown to be important in limiting the severity of acute HSV infections. The humoral response limits the manifestation of CNS disease, although antibody alone cannot protect mice from reactivation (13). CD4+ T cells and CD8+ T cells clear the virus and prevent it from spreading to the CNS (11, 14, 15). T cells secrete antiviral cytokines in the CNS and thus limit HSV infection by primarily noncytolytic mechanisms within the CNS (16). The primary cytokines secreted are IFN-γ and tumor necrosis factor α, with interleukin 6 also having a proposed role (17). Additionally, microglia cells are activated, and macrophages are recruited during HSV infection of the nervous system (18).

The immune response to tumors has been studied in detail, and much is dependent on the model and therapeutic modality used in the study. It is generally accepted that many tumors are recognized by the immune system but that tumor suppression factors, such as interleukin 10, are released and down-regulate MHC class I expression through various mechanisms, allowing the tumor to escape immune monitoring by CTLs (19–21). A useful property for cancer therapeutics is to not only destroy local treated tumor masses but also to develop new tumor-specific immune responses to destroy distal tumor metastases. Neuro-attenuated HSV-1 may have the potential to do this. Whereas specific lytic infection of tumor cells will destroy these cells at the site of injection, this lysis may also expose new tumor cell antigens to immune cells infiltrating the tumor mass due to viral infection, potentially including CD4+ and CD8+ T cells. However, the immune response could also interfere with the viral therapy by preventing the spread of the virus throughout the tumor mass, thus reducing the ability of the virus to destroy the tumor.

We have determined that the immune response can contribute to tumor lysis and that the response is characterized by an early influx of mainly CD4+ T cells, NK cells, and macrophages (although most types of immune cells are found in the tumor mass after viral therapy). Interestingly, prior immunity to HSV seems to aid in tumor lysis. This novel result underscores the importance of patient selection in clinical trials for viral therapies of tumors because it predicts that an immune-compromised patient with a large tumor mass will have inferior results compared with a non-immune-compromised patient who has a smaller tumor mass.

MATERIALS AND METHODS

Animals. Female DBA/2 mice (4–6 weeks old; body weight, approximately 20 grams) were obtained from Taconic (Germantown, NY). Immunodeficient female SCID (CB-17 scid/scid) mice, originally obtained from M. Bosma (Fox Chase Cancer Center, Philadelphia, PA), were bred and maintained in a pathogen-free environment at the Wistar Institute animal facility. Serum IgM titers of the mice were routinely tested by a direct ELISA when the mice were 6–7 weeks old, and only non-IgM producing mice were used in our studies. All animal work was approved by the University of Pennsylvania’s and the Wistar Institute’s Institutional Animal Care and Use Committee (IACUC).

Tumor Cells. S91 Cloudman M3 melanoma cells (22) were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown using DMEM containing penicillin, streptomycin, and 15% fetal bovine serum. When originally obtained, cells were first implanted s.c. in the flanks of syngeneic DBA/2 mice. Tumors that arose in these mice were removed asexually, grown, and then frozen in 95% culture media/5% DMSO so that all experiments could be initiated with cells of a similar passage number. On the day of i.c. injection, cells in subconfluent monolayer culture were passaged with 0.25% trypsin solution in EDTA, washed once in cell culture medium,
counted using trypan blue, resuspended at the appropriate concentration in medium without serum, and kept on ice.

**i.c. Tumor Production.** Mice were anesthetized with ketamine/xylazine (87 mg/kg ketamine/13 mg/kg xylazine; i.p.). The head was cleansed with 70% ethanol. A small midline incision was made in the scalp, exposing the skull. Stereotactic injection of tumor cell suspensions was performed using a small animal stereotactic apparatus (Kopf Instruments, Tujunga, CA). Injections were done with a Hamilton syringe through a 30-gauge needle. The needle was positioned at a point 2 mm caudal of the bregma and 1 mm left of midline. Using a separate 27-gauge needle, the skull was punctured at these coordinates. The injection needle was advanced through the hole in the skull to a depth of 2 mm from the skull surface and then extracted 0.5 mm to create a potential space.

Cells (5 × 10^4, DBA/2; 5 × 10^5, SCID, SIV) in a total volume of 10 μl were injected over 2 min. After the injection, the needle was left in place for 2 min and then slowly withdrawn. The skin was sutured closed.

**Virus.** To produce virus stocks, subconfluent monolayers of baby hamster kidney 21 clone 13 cells were infected with HSV-1 strain 1716 (stock titer, 1 × 10^9 pfu/ml). The creation of HSV-1 strain 1716 has been described previously (23). Briefly, HSV-1 1716 has a 759-bp deletion that deletes part of the genes encoding ICP34.5, mAT, and ORF (23). Virus was concentrated from the culture and titrated by plaque assay as described previously (24). All viral stocks were stored frozen in viral culture medium (serum-free DMEM containing penicillin and streptomycin) at −70°C and thawed rapidly just before use. Serum-free medium was used for control (mock) inoculation studies as a negative control.

**i.c. Viral Inoculation.** Mice were anesthetized i.p. with ketamine/xylazine (as described above), and the head was cleansed with 70% ethanol. Using a Hamilton syringe with a 30-gauge needle, the appropriate amount of virus was injected in a total volume of 10 μl through a midline incision at the same stereotactic coordinates used for tumor cell injection. The needle was passed through the hole punctured previously for tumor implantation. The injection was performed over a 2-min period, and after the injection, the needle was left in place for 2 min and then slowly withdrawn. The amount of HSV-1 1716 used in all experiments was 5 × 10^4 pfu/mouse, the amount determined to give the best survival at the lowest dosage.

**Intercocular Viral Inoculation.** For latent mice, DBA/2 mice were anesthetized i.p. with ketamine/xylazine as described above. Eyes were scarified in a cross pattern 10 times, and HSV 17+ (5 × 10^4 pfu) was pipetted onto the eyes in 10 μl of viral culture media. Mice were monitored daily for signs of inflammation, and paracriparine hydrochloride was administered ocularly as needed. Virus infection was allowed to become latent for 28 days before tumor injection were done as described above.

**Immunohistochemistry.** Mice were sacrificed by cervical dislocation on days 0, 1, 3, 5, 7, 10, 14, 15, 17, 19, and 21 after tumor implantation, and the brains were dissected for histological and immunohistochemical analysis. These time points were chosen to provide information involving the immediate immune response after tumor cell and viral injection and to provide information on the specific immune response seen later after tumor cell and viral injection. The methods for tissue processing and light microscopic immunohistochemical analysis were similar to those described elsewhere (25, 26). Antibodies used were as follows: (a) anti-HSV-1, polyclonal rabbit antibody (American Qualex, Santa Clemente, CA; 1:1000); (b) MHC II, rat monoclonal antirat TRIBu (class II polymorphic) and mouse H-21-A (Biosource International, Camarillo, CA; 1:5 dilution); (c) MHC I, biotin-conjugated mouse monoclonal antibody H-2K7/H-2D7 monoclonal antibody (PharMingen, San Diego, CA; 1:1000 dilution); (d) secondary antibody, biotin-conjugated goat antirat immunoglobulin (Biosource International; 1:1000 dilution); (e) CD8a, purified rat antimonoclonal antibody CD8a (Ly-2) monoclonal antibody (PharMingen; 1:1000 dilution); (f) CD4, rat monoclonal antimonoclonal L3/T4 CD4 (Biosource International; 1:1000 dilution); (g) neuropeptides, rat monoclonal antimonoclonal antibody (poly-morphic; Biosource International; 1:1000 dilution); (h) macrophage, rat monoclonal antimonoclonal pan macrophage marker (Biosource International; 1:25 dilution); (i) B cells, purified rat antimonoclonal CD19 monoclonal antibody (PharMingen; 1:1000 dilution); (j) NK cells, purified rat antimonoclonal pan-NK cell monoclonal antibody (PharMingen; 1:1000 dilution); (k) IgG2a k isotype, purified mouse IgG2a monoclonal immunoglobulin isotype standard (PharMingen; 1:1000 dilution); (l) IgG2b k isotype, purified mouse IgG2b monoclonal immunoglobulin isotype standard (PharMingen; 1:1000 dilution); and (m) IgG2c k isotype, purified mouse IgG2c monoclonal immunoglobulin isotype standard (PharMingen; 1:1000 dilution). Cells were detected by an indirect avidin-biotin immunoperoxidase method (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) as specified by the manufacturer, with a slight modification. Briefly, tissue sections were rehydrated, quenched in peroxide (H₂O₂), and blocked in 3.5% goat serum (Sigma Chemical Co., St. Louis, MO). Tissue sections were incubated overnight at 4°C with the primary antibody, used at concentrations stated above. Next, the tissues were incubated at room temperature with biotinylated goat antirabbit IgG or goat antirat IgG, the avidin-biotin horseradish peroxidase complex, and 3,3′-diaminobenzidine as the chromagen. Sections were counterstained with hematoxylin and examined under the light microscope. Sections were washed twice with 0.1 M Tris-HCl (pH 7.9) and then washed twice with 0.01 M Tris-HCl containing 5% goat serum between every step except addition of chromagen, in which sections were washed twice with 0.01 M Tris-HCl only. As an additional control for the specificity of immunostaining, tissues were processed as described above, except that 5% nonimmune goat serum alone was substituted for the primary antibody for the overnight incubation and was then followed with secondary antibody. Quantification of positively stained cells was done using the Phase 3 Imaging program (Phase 3 Imaging, Glen Mills, PA). Briefly, the positively stained cells and tumor cells were counted, and the percentage of positively stained cells per number of total tumor cells was determined. Two fields per tumor were counted for each antibody. Each treatment includes three mice, and two slides were counted for each mouse, for a total of 12 fields per antibody.

**Apopoisis Detection.** We used the Dead End Colorimetric Apoptosis Detection Assay from Promega (Madison, WI) per the manufacturer’s directions. Briefly, slides were deparaffinized and rehydrated with sequential ethanol washes. Slides were then incubated in 0.85% NaCl and washed with PBS, and tissue was fixed with 4% paraformaldehyde. The slides were washed again and then incubated with proteinase K. After an additional wash, slides were covered with equilibration buffer, and the biotinylated nucleotide mixture was made. Terminal deoxynucleotidyltransferase reaction mixture was added to slides, and slides were incubated overnight at 4°C. The reaction was stopped by incubating slides in SSC, and the slides were washed and colorized using 3,3′-diaminobenzidine as described above. Negative controls were prepared by treating a sample with DNase I, and these slides were treated as described above but in separate jars to prevent contamination of sample slides with DNase I. Positive cells were counted as described above.

**Cyclophosphamide Administration.** Mice for cyclophosphamide experiments were implanted with tumors as described above. On days 6 and 8 after tumor implantation, 75 mg/kg cyclophosphamide (Sigma Chemical Co.) was injected i.p., followed every 2 days by 50 mg/kg cyclophosphamide. Mice were bled to insure leukocyte depletion, which was determined to be >97% in sampled mice throughout the study period. Viral or mock therapy was administered as described above on day 10, and mice were followed for signs of morbidity or neurological symptoms, at which point they were sacrificed.

**Statistics.** Data analysis, including calculations of means, SDs, repeated measures ANOVA, Fisher’s PLSD for survival, and unpaired t test, was performed using StatView statistical software (Abacus Concepts, Berkeley, CA) on an Apple Macintosh computer ( Cupertino, CA).

**RESULTS**

**HSV-1 1716 Prolongs Survival of i.c. Tumor-bearing Immuno-competent Mice.** We have previously shown that neuro-attenuated HSV-1 1716 is able to significantly prolong survival of i.c. Harding-Passey tumor-bearing C57Bl6 mice when the virus is administered at the midpoint of survival (1). However, the Harding-Passey tumor line is not a syngenic tumor line because it is derived from an outbred mouse strain. Because we are interested in the role of the immune response in viral-mediated tumor destruction, we have used the M3 Cloudman S91 melanoma (S91) model in syngenic DBA/2 mice. Here we demonstrate that HSV-1 1716 is also able to significantly prolong survival of i.c. S91 tumor-bearing immunocompetent DBA/2 mice.

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4 B. Randazzo, personal communication.
Specifically, 6-week-old DBA/2 mice were injected i.c. with S91 cells as described in “Materials and Methods.” Fourteen days after this implantation, the animals were split into two groups, with half of the animals receiving HSV-1 1716 treatment, and the other half of the animals receiving mock treatment. Animals were followed for signs of severe illness and sacrificed according to IACUC guidelines. A second group of mice was treated similarly to the above-mentioned group, but virus or mock treatment was administered 7 days after tumor cell implantation.

Tumor-bearing mice treated with HSV-1 1716 fourteen days after tumor implantation had a mean survival of 38.9 ± 2.3 days, whereas tumor-bearing mice mock-treated with viral growth media had a mean survival of 24.9 ± 0.6 days (P < 0.0001; Fig. 1A). To further characterize this model, we determined whether the previously seen increase in survival with earlier (day 7) administration of virus (1) was also seen. When HSV-1 1716 or mock treatment was administered on day 7 after tumor implantation, 60% of the HSV-1 1716-treated mice showed complete regression of tumors. There was also a significant prolongation of mean survival in the remaining mice (54 ± 9.1 days), whereas mock-treated mice had a mean survival of 27.8 ± 1.1 days (P < 0.0003; Fig. 1B). Although a more significant prolongation in survival of mice after viral treatment than mock treatment is seen when virus is administered on day 7 after tumor implantation, the size of the tumor was small at this time, and it was difficult to insure that virus was administered within the tumor mass. Therefore, the results were not as consistent as those of mice with treatment administered at day 14, and we have used this model for further studies. These data demonstrate the feasibility of using this model to study the role of the immune response in neuro-attenuated HSV-1 therapy of murine i.c. melanoma.

**HSV-1 1716 Does Not Prolong Survival of i.c. Tumor-bearing Immunodeficient Mice.** We have previously shown that HSV-1 1716 is able to decrease human tumor cell growth in SCID and nude mice (27–29). The ability of HSV-1 1716 to destroy tumors in immunodeficient mice implies that the immune system does not contribute substantially to viral-mediated tumor destruction. However, nonspecific immune responses to the xenographic tumor cells may be triggered by viral administration, and these responses, along with unchecked viral replication throughout the tumor mass, may be sufficient to mediate tumor destruction. Additionally, it has been shown that HSV-1 replicates more efficiently in human tissue than in rodent tissue (30). This fact may make the viral-mediated destruction of tumors more effective in the human tumor/mouse host models than in mouse tumor/mouse host models.

We have evaluated whether a specific immune response contributes to viral-mediated tumor cell destruction using an allogenic immunodeficient model. Six-week-old SCID mice were implanted with S91 cells as described in “Materials and Methods.” Due to the accelerated growth of S91 melanoma cells in SCID mice as compared with syngeneic DBA/2 mice, we injected 2-fold less tumor cells and administered the virus on day 7. Fig. 2A shows the mean survival for HSV-1 1716-treated and mock-treated S91 i.c. tumor-bearing SCID mice treated on day 7. No significant difference in survival was seen between mock- and HSV-1 1716-treated mice (14.8 ± 2.2 and 15.0 ± 1.7 days, respectively; P = 0.95). This may be due to the accelerated growth kinetics of the S91 melanoma cells in SCID mice because the tumor may have grown too large for the virus to kill a significant portion of the tumor and thus have an effect on survival. We have shown previously that administration of the virus at a later period during tumor growth decreases its effectiveness in tumor destruction and decreases survival times (30).

To readdress whether a specific immune response is important to viral-mediated tumor cell destruction, we used a second murine tumor model using i.p. cyclophosphamide injections to deplete leukocytes in syngenic DBA/2 mice (31–34). After implantation of 5 × 10^4 S91 melanoma cells, cyclophosphamide administration was initiated on day 6 and continued until the mice were moribund, at which point they were sacrificed. On day 10 after tumor implantation, viral therapy was administered. After HSV-1 1716 or mock treatment, leukocyte-depleted mice showed no significant difference in mean survival (19.0 ± 1.9 and 19.5 ± 2.7 days, respectively; P = 0.6702; Fig. 2B). Mice were bled before viral treatment and throughout the posttreatment monitoring period to determine the degree of leukocyte depletion (>97%; data not shown). Because there was some variance in average mean survival times between experiments and to assess the effect of cyclophosphamide treatment on tumor growth, a third group of mice was implanted with tumor cells concurrently with the other groups but was not treated with cyclophosphamide or given the virus. These mice had a mean survival of 25 ± 0.8 days. Therefore, the leukocyte depletion due to the cyclophosphamide therapy may contribute to a slightly accelerated tumor growth rate. Furthermore, we saw no difference in the growth rates of s.c. S91 flank tumors between cyclophosphamide-treated, HSV-1-treated, and mock-treated mice (data not shown). Taken together, these data suggest that an immune
response develops after viral administration that aids in tumor cell destruction.

**Immune Cells Infiltrate the Tumor but not the Surrounding Brain Mass.** The S91 melanoma cell line used in these studies is immunogenic (35). Thus we investigated the immune response to the i.c. implantation of tumor cells in DBA/2 mice to better understand the response to the tumor and virus administration. The major class of cells infiltrating into tumors after implantation was CD4+ T lymphocytes (10.6%; Fig. 4A). Significant numbers of CD8+ T lymphocytes, NK cells, and microglia cells (7.2%, 9.1%, and 6.8% respectively) were also seen (Fig. 4A). The immune cells were often located in edge regions of the tumor mass, although positively stained cells were found throughout the tumor masses. This response could be due to the breach of the BBB during tumor implantation, although the recruitment of leukocytes into the CNS is now considered common in response to virus, bacteria, and so forth. However, no significant immune cell infiltration was seen when mock-implanted mice were examined (Fig. 3E). Therefore, breaching the BBB is not enough to cause recruitment of immune cells into the CNS; stimulation is required. The infiltration into tumor-bearing mouse tissue disappeared after several days; however, on day 12, a slight increase in infiltrating cells was again seen for a short period of time. Specifically, CD4+ and CD8+ T cells (4.1% and 3.7%, respectively), NK cells (3.5%), and B cells (3.0%) were detected (Fig. 4A). At the time of virus administration, little or no immune cells were present (data not shown).

We next characterized the immune cell infiltration into the tumor after viral administration. The main infiltrating cells early after viral treatment were CD4+ T cells (11.7%) and macrophages (8.2%), but PMN (7%), CD8+ T cells (2.1%), B cells (3.2%), NK cells (4.4%), and microglia cells (3.6%) were also present (Fig. 4B). This immune cell infiltration was sustained until day 21, when tumor size in most sampled mice was negligible. These mice would have survived until the maximum time (i.e., day 50 after tumor implantation) because a small number of tumor cells escaped destruction and would have resumed tumor growth. No infiltration or signs of inflammation occurred in the surrounding normal brain tissue on any day (data not shown). Infiltration into the tumor proceeded from small, localized sites on days 1 and 3 to more widespread sites in the tumors on days 7 and 12 (Fig. 3, F–N). Significant NK (16.3%) and polymorphonuclear leukocytes (16.5%) infiltration was seen on day 7, with significant CD4+ T cells (14.5%) again present on day 12. Staining for HSV antigen showed a growth curve throughout sampling, with the highest staining on day 7 (15.3%; Fig. 4). HSV-1 staining was found throughout the tumor mass (Fig. 5, A–H). No staining was seen in mock-treated tumor-bearing mice (Fig. 5I). MHC class I expression was down-regulated 3 days after viral therapy in treated mice when compared with mock-treated mice. This is in accordance with reports on the ability of HSV-1 to down-regulate MHC class I expression through ICP47 (36–38). The down-regulation of MHC class I expression also corresponds with the concurrent shift from CD4+ and CD8+ T cells to NK cell and PMN infiltration. This correlates with the proposed escape from CTL recognition of tumors and the importance of NK cells in tumor clearance (39). This corresponds the findings of Lewandowski et al. (40), who state that HSV-1 KOS is able to block the surface expression of MHC class II after infection, but that strain F is not able to do so. Because HSV strains KOS and 17+ are both more pathogenic than strain F, it is interesting to suggest that more pathogenic strains are able to down-regulate MHC class II expression through some as yet unknown mechanism.

**Apoptosis Increases within the Tumor Mass after Viral Administration.** An important factor in understanding the mechanism of tumor destruction is whether tumor cells are being destroyed via necrosis and/or apoptosis. Necrosis leads to potentially undesirable inflammatory responses within the CNS. However, necrosis also increases the potential development of a tumor-specific immune response because infiltrating immune responses clean up necrotic cell debris and present tumor cell and viral antigens to lymphocytes. Death through apoptosis, on the other hand, would limit the inflammation at the site of tumor destruction but may block the development of a tumor-specific immune response that would be useful in potentially eliminating distant metastases. To determine whether tumor destruction occurs by apoptosis, we used the Dead-End Colorimetric Apoptosis Detection System (Promega) to localize apoptotic cells within the tumor mass of viral-treated and mock-treated mice. Detection of apoptotic cells increased throughout the monitoring period in viral-treated mice, beginning at 5% on day 0 and increasing to >30% by day 15 (Fig. 6). The apoptotic cells were found throughout the viral-treated tumor mass (Fig. 5, I–N). In mock-treated mice, baseline apoptotic cell detection was seen early in monitoring period (days 1–6) but was not detected after day 7. These results suggest that although apoptotic cell death is induced after viral administration, necrotic cell death may also be occurring because some cells in viral-treated tumor masses appear necrotic and do not stain for apoptosis (data not shown).
Fig. 3. Detection of infiltrating inflammatory cells after i.c. implantation of S91 melanoma cells into DBA/2 mice. All pictures were taken within 1 h of tumor cell implantation. A, CD4+ T cells; B, CD8+ T cells; C, NK cells; D, microglia cells; E, negative control. Note the lack of detection of infiltrating inflammatory cells after mock tumor implantation. F–N, detection of infiltrating inflammatory cells after i.c. implantation of S91 melanoma cells into DBA/2 mice. Fourteen days later, they were inoculated with $5 \times 10^4$ pfu HSV-1 1716. F, CD4+ T cells, day 1; G, macrophages, day 1; H, PMN, day 7; I, CD8+ T cells, day 3; J, B cells, day 7; K, NK cells, day 7; L, microglia cells, day 12; M, MHC class I, day 3; N, MHC class II, day 1. Magnification = original magnification $\times 20 \times$ objective.
Prior Immunity to HSV Increases Survival Time of Viral-treated Mice. Because the immune response to viral administration plays an important role in viral-mediated tumor cell destruction, we wanted to determine the effect of prior immunity to HSV on tumor cell destruction. For immune mice, animals were infected ocularly with HSV 171 and allowed to become latent for 28 days before tumor implantation. Control mice were mock-infected at the same time. Tumors were implanted, and 14 days later, HSV-1 1716 was administered. Immune (latent) viral-treated tumor-bearing mice had a significant increase in survival when compared with naïve viral-treated tumor-bearing mice (Fig. 7). Specifically, immune mice mean survival was 35.5 days, whereas naïve mice mean survival was 28.8 days (P, 0.0001). To insure that previous immunity to HSV-1 had no effect on tumor growth, immune tumor-bearing mice were mock-treated as described before. These mice had a mean survival of 25 days (P = 0.0001 to naïve and immune-treated survival). Immunity had no effect on mortality in nontreated groups. These results not only demonstrate that prior immunity is not detrimental to viral therapy but also reiterate the importance of the immune response in terms of cell destruction. If virus administration accelerates the immune response to the tumor, in the presence of prior immunity, the immune response will be up-regulated and will aid in better tumoricidal activity.

DISCUSSION

Although the brain is historically considered an immune privileged site, recent studies in cases of infection or damage have shed light on the ability of the immune response to infiltrate into the CNS. The concept of immune privilege of the CNS is based on the low levels of resident lymphocytes, low levels of MHC expression on resident microglia, and the presence of the BBB, which blocks immunoglobulin and complement access to the CNS (41–43). Many recent studies have shown that lymphocytes are able to migrate into the CNS and that many cytokines are expressed within the CNS in response to inflammatory or injury markers (41). Therefore, it is important to determine the importance of the immune response to viral (HSV) replication during tumor destruction and the contribution of the immune response to tumor cell destruction.

Neuro-attenuated HSV-1 strains are able to destroy human xenograft tumors in immunodeficient mice (27–29). However, it has been shown that HSV-1 replicates with better efficiency in human tissue than in rodent tissues (30). In the absence of an immune response to eliminate replicating virus, it was shown that neuro-attenuated HSV-1 is able to spread through xenograft tumors and destroy them. Because HSV-1 is currently being studied in clinical trials as a treatment for human tumors in patients (who have probably been immunocompromised due to prior treatment) with this virus, it is important to determine the ability of neuro-attenuated HSV-1 to destroy syngeneic murine tumors in immunodeficient mice.

Although many reports of the tumoricidal activity of neuro-attenuated HSV have been published (1, 2, 3, 29), few studies have investigated the effect of the immune response on replication-competent viral treatment of tumors (44). M3 Cloudman S91 melanoma cell tumor-bearing mice treated with HSV-1 1716 virus have a significant
Fig. 5. A–H, detection of positive staining for HSV-1 antigen in the tumor mass of DBA/2 mice after i.c. implantation of S91 melanoma cells followed 14 days later by $5 \times 10^4$ pfu HSV-1 1716. A, day 0; B, day 1; C, day 3; D, day 5; E, day 7; F, day 12; G, day 15; notice the lack of staining in mock-treated tumor (H, day 3). Magnification = original magnification $\times$ 4 $\times$ objective. No staining was seen in any areas of the surrounding normal brain tissue. I–N, detection of positively stained apoptotic tumor cells within tumor mass after viral administration of DBA/2 tumor-bearing mice. Mice were injected with $5 \times 10^4$ S91 melanoma cells followed 14 days later with $5 \times 10^4$ pfu HSV-1 1716. I, day 0; J, day 3; K, day 7; magnification = original magnification $\times$ 4 $\times$ objective. Higher magnification of the above-mentioned pictures detailing apoptotic cells. L, day 0; M, day 3; N, day 7; magnification = original magnification $\times$ 20 $\times$ objective.
prolongation in mean survival over mock-treated mice (Fig. 1A). Examination of the contribution of the immune response and the cells that mediate this response indicates that it is important for tumor destruction. The depletion of leukocytes by cyclophosphamide administration demonstrated that a cellular immune response played a role. Furthermore, the immune cell infiltration, mainly CD4+ T cells and macrophages, seen early after viral administration demonstrated that immune cells are present within the tumor. Finally, previous immunity to HSV-1 was found to prolong the survival of viral-treated tumor-bearing DBA/2 mice (Fig. 7).

Both humoral and cellular immune responses have been demonstrated to be important in HSV clearance and prevention of viral spread to the CNS. The humoral response is important in protecting the CNS from disease (13). CD4+ T cells reduce primary replication and protect against latent infection (45), whereas CD8+ T cells prevent the immunopathological activity of CD4+ T cells (31). Interestingly, our results closely parallel those seen previously using nonreplicating HSV-1 as a gene therapy vector (43). In these studies, MHC class I and II expression was up-regulated along with the infiltration of macrophages, T cells, and NK cells soon after viral administration (43). However, our response is accelerated and is seen before that of other models (43, 46, 47). During gene therapy, the goal is generally long-term gene expression from a viral vector; therefore, an immune response to the virus or the recombinantly expressed protein is undesirable. However, during cancer therapy, the goal is tumor cell destruction; therefore, an immune response to the virus, which is expressing its genome only in tumor cells, may help tumor destruction by leading to a tumor-specific immune response that may aid in destruction of distal metastases. Additionally, nonreplicating viruses used for gene therapy carry a foreign gene of therapeutic interest, which may alter the immune response to the virus. In the present study, the virus is restricted to infection in dividing tumor cells by its ICP34.5 deletion. The accelerated increase in infiltration of immune cells seen in viral-treated tumors is due in part to an immune response to viral proteins expressed on infected tumor cells but may also be due to the prior immune response elicited by the tumor itself, suggesting that virus injection either up-regulates the expression of tumor antigens or that lytic infection increases tumor antigen expression. Additionally, viral infection may up-regulate the expression of new tumor antigens, helping in the treatment of highly metastatic tumors. Unfortunately, the predominant cells that are seen in our model after HSV-1 1716 therapy are CD4+ T cells, NK cells, and macrophages, of which only CD4+ T cells will exert antitumor effects at metastatic sites.

The decrease in MHC class I expression over time is interesting because it appears that the tumor cells express MHC class I on implantation but are down-regulating it as viral infection proceeds. MHC class I peaks 3 days after viral administration and decreases thereafter. Because HSV encodes a protein, ICP47, that interferes with transporters associated with antigen processing (TAP) and thus down-regulates the antigen presentation through MHC class I (37, 38), this may also be due to ICP47 action. Although ICP47 has been shown to not interact with murine TAP in vitro, a recent report by Goldsmith et al. (48) demonstrates that it is able to interfere with antigen processing in vivo. MHC class II expression is also down-regulated after viral infection. It has been shown previously that after infection with HSV-1 strain KOS, MHC class II cell surface expression is blocked (40). This is not seen with HSV-1 strain F (40), a less pathogenic strain. Because HSV-1 strains KOS and 17+ are both highly pathogenic, there may be a similar mechanism between the viruses, and this may be occurring within the CNS in our model.

Up to 90% of the adult human population is sero-positive for HSV antibodies. Our result that prior immunity aids in viral-mediated tumor cell destruction suggests that HSV-1 therapies will have better effectiveness in humans. The increased response to HSV may either lead to an increased infiltration of immune cells into the tumor mass or suggest a switch for the dominant immune cell type infiltrating into the tumor mass. In contrast to these results, Herrlinger et al. (47) observed no increase in survival in immune animals in their studies and saw a decrease in gene transfer in immune mice, suggesting that the response may be tumor or model specific. Their model uses rat D74 gliomas in which the mutant HSV-1 is unable to replicate sufficiently due to resistance of the rat cell line to HSV infection. These authors did report more inflammatory infiltrates in the tumors of immune mice at early time points compared with those of naïve mice. Therefore, prior immunity may aid in tumor destruction only in the presence of a clearly replicating viral infection of the tumor cells.

In conclusion, the role of the immune response in viral-mediated tumor cell destruction is important when the virus is administered late in tumor progression. This is important when timing virus administration in clinical trials, considering that many current clinical trials are being performed in which patients have their immune response depressed (by chemotherapy and radiation treatment). Earlier administration of virus or administration of virus in the absence of immune compromise may facilitate the usefulness of neuro-attenuated HSV-1 for i.c. tumors.
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Role of the Immune Response during Neuro-attenuated Herpes Simplex Virus-mediated Tumor Destruction in a Murine Intracranial Melanoma Model

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