The cytolytic animal virus equine herpesvirus type 1 (EHV-1) was evaluated for its oncolytic potential against five human glioblastoma cell lines. EHV-1 productively infected four of these cell lines, and the degree of infection was positively correlated with glioma cell death. No human major histocompatibility complex class I (MHC-I) was detected in the resistant glioma line, while infection of the susceptible glioma cell lines, which expressed human MHC-I, were blocked with antibody to MHC-I, indicating that human MHC-I acts as an EHV-1 entry receptor on glioma cells.

Glioblastoma multiforme (GBM) brain tumors are extremely resistant to all currently approved therapies, including surgical resection, radiotherapy, and chemotherapy. Each year in the United States there are approximately 25,000 newly diagnosed cases of GBM and 13,000 deaths (2). The median survival rate for GBM patients is 12 to 18 months with aggressive therapy (9, 24, 26), and fewer than 5% of patients survive out to 5 years (13, 14).

In recent years, virus therapy as a means of treating cancer has become a promising prospect. In cases where traditional approaches are not feasible or are unlikely to succeed, virus therapy may be an effective means of treating malignancies. To date, a wide variety of viruses have been evaluated for their oncolytic potential, including DNA viruses such as herpesviruses (15, 16, 19), adenoviruses (3, 7), and vaccinia.
virus and RNA viruses such as reoviruses (4, 5, 27) and poliovirus (8).

Equine herpesvirus type 1 (EHV-1) is a member of the alphaherpesvirus family, which includes the human viruses herpes simplex virus 1 (HSV-1) and HSV-2 (17). EHV-1 is an enveloped, double-stranded DNA virus that causes upper respiratory infection in horses and in rare cases causes paralysis and abortigenic disease (1, 20). While EHV-1 does not infect humans, cells obtained from a wide array of species, including humans, are readily infected in tissue culture (25). Recently, we showed that equine major histocompatibility complex class I (MHC-I) is a cellular entry receptor for EHV-1 (12). This receptor is critically important for host defense against invading pathogens, and segments of MHC-I are highly conserved across species (11). In the present study, we investigated the ability of EHV-1 to infect, replicate, and kill a series of human glioblastoma cells and also examined the role of MHC-I in this process.

In order to assess the ability of EHV-1 to infect a panel of human glioma cell lines, the recombinant EHV-1 reporter virus, L11ΔgIΔgE, which contains a lacZ reporter cassette, was used (6). Five human glioma cell lines, A-172, Hs 683, LN-18, SNB19, and U251, were mock infected or infected with L11ΔgIΔgE at multiplicities of infection (MOIs) of 1, 3, and 10 for 6 h. The extent of infection was assessed by measuring β-galactosidase expression (Fig. 1A and B) and viral immediate early (IE) gene transcripts (Fig. 1C). As shown in Fig. 1, four of the five glioma lines (A-172, LN-18, SNB19, and U251) exhibited a dose-dependent increase in infection. The remaining glioma cell line, Hs 683, and the negative-control line, B78H1 (murine melanoma cells that do not express an EHV-1 entry receptor [12]), were resistant to EHV-1 even at high MOIs. These results show that the degree of infection on the susceptible glioma cells varies substantially. A-172 and LN-18 glioma cells were shown in each assay to be highly susceptible to EHV-1, SNB19 and U251 cells were moderately infected, and Hs 683 cells were highly resistant to infection.

In order to determine if EHV-1 could successfully replicate (produce progeny virus) and complete its life cycle in glioma cells after entry, one-step growth curves were performed on each glioma line. As shown in Fig. 2, EHV-1 yields increased by more than one log PFU/ml at 24 h postinfection (p.i.) in A-172, LN-18, SNB19, and U251 cells. At 48 h p.i., the virus yield increased in the LN-18 cells, reaching a maximum of $1.2 \times 10^6$ PFU/ml (1.7 log increase). The total virus yields at 48 h in A-172, SNB19, and U251
cells were similar to the yields obtained at the 24-h time point. In contrast to the increase in virus yield observed on A-172, LN-18, SNB19, and U251 cells, virus yields were significantly lower in the Hs 683 cell line. The ability of EHV-1 to replicate and produce the most progeny virus in A-172 and LN-18 cells and the least progeny virus in Hs 683 cells correlates well with the infectibility of these cells as measured in the infectivity assays. Overall, these data show that EHV-1 can replicate in human glioma cells, but this replication is variable between the different lines.

The oncolytic potential of EHV-1 was measured using an MTS cell viability assay. Each glioma cell line was mock infected or infected in triplicate with L11ΔgIΔgE at MOIs of 1, 3, and 10 for 48 h, and then cell death was measured (Fig. 3). Both the B78H1 and the Hs 683 cell lines showed close to 100% cell viability at all of the MOIs, indicating that even at a high MOI these cells were highly resistant to lysis by EHV-1 infection. The other four glioma lines showed increasing amounts of cell death at increasing MOIs. At an MOI of 10, 66% of the A-172 cells and 69% of the LN-18 cells (the two glioma lines most susceptible to infection) were lysed. This level of cytotoxicity was similar to the cell death observed on the positive-control cell line RK13, in which the virus is normally cultured, at MOIs of 3 and 10. These data show that EHV-1 infection leads to glioma cell death by 48 h p.i. and the degree of cell death correlates positively with the amount of cells that are initially infected.

Previously, we showed that Equus caballus MHC-I is an entry receptor for EHV-1 (12). MHC-I receptors are expressed on most nucleated cells, and regions of this receptor are highly conserved within the animal kingdom (11). To investigate whether the five glioma lines used in the current study express MHC-I and if there is a difference in expression, reverse transcriptase (RT)-PCR assays were performed on each line to assess MHC-I transcript lev-
els. As shown in Fig. 4, human MHC-I was detected in all of the glioma lines except the Hs 683 cell line. The B78H1 cell line, which is highly resistant to EHV-1 infection, also does not express MHC-I (12). To directly assess whether EHV-1 uses MHC-I as an entry receptor on the four susceptible glioma lines, an antibody blocking assay was performed with anti-human MHC-I. As shown in Fig. 5, EHV-1 infection on each of the four susceptible glioma cell lines was inhibited in a dose-dependent manner by the anti-human MHC-I antibody W6/32. This blocking of EHV-1 was specific, as the antibody had no effect on HSV-2 (gC2BBΔIEP::lacZ), which uses nectin-1 as a cellular entry receptor (18). These data indicate that EHV-1 uses MHC-I on these glioma cells to initiate infection.

The current study is the first to demonstrate that the animal virus EHV-1 can infect and lyse human glioma brain cancer cells. As GBM is one of the deadliest of all human cancers and current conventional therapies have had only minimal success in prolonging survival times, new treatment strategies based on alternative or novel approaches are increasingly sought after. One of the nontraditional approaches for GBM therapy that has been attempted is the use of viruses as oncolytic vectors either alone or in conjunction with other modes of therapy (reviewed in references 10 and 29).

EHV-1 has many favorable properties that make it a potential new member in the arsenal of anticancer agents. The virus has a short, well-defined growth cycle that results in the death of infected cells, the genome has been sequenced, and the virus can be grown to high titers. Another attractive feature of an EHV-1 vector is the lack of preexisting immunity against the virus. This would allow for the introduction of EHV-1 into human tumors and consequent replication and spread of the virus that would not be hindered by preexisting antibodies or memory cytotoxic T lymphocytes. Finally, EHV-1 is a large virus and thus has an extensive packaging capacity. This feature allows for the relatively facile insertion of therapeutic genes that may act to increase the oncolytic or selective properties of an engineered EHV-1 oncolytic vector.

Equus caballus MHC-I has been shown to be an entry receptor for EHV-1 (12). In the current study, we investigated whether the glioma cell lines expressed human MHC-I and whether this expression is correlated with infectivity. Many cancer cells, including gliomas, have been shown to downregulate or not express MHC-I (21, 22, 28). This downregulation is an important immune evasion mechanism employed by these malignant cells allowing for escape from immunosurveillance and CD8+ cytotoxic T-cell killing. Our results clearly show that each glioma line, with the exception of the Hs 683 line, expresses MHC-I. The lack of expression on Hs 683 cells was particularly noteworthy, as this cell line is highly refractory to EHV-1 infection. Previous work by Trapp et al. showed that EHV-1 could infect a broad array of cell lines from multiple species (25). The ability of this virus to exhibit such a broad tropism could be attributed to its use of the MHC-I receptor, which is expressed on most cell types across species. Interestingly, equine and human MHC-I share significant sequence homology. In particular, an alanine at residue 173 of equine MHC-I that was previously shown by Sasaki et al. to be critical for EHV-1 entry is also found in the human MHC-I receptor (23). As MHC-I is found on normal healthy cells, in addition to tumor cells, it will be necessary to engineer second-generation EHV-1 oncolytic vectors that are specifically targeted solely to tumor cells. This can be achieved in various ways, such as re-directing the tropism of the virus via insertion of ligands that will specifically recognize receptors that are unique or overexpressed on glioma cells or by placing the vector under the control of a tumor-specific promoter.

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