Factors That Limit the Effectiveness of Herpes Simplex Virus Type 1 for Treatment of Oral Cancer in Mice

Edward J. Shillitoe and Christopher Pellenz

Abstract

Although the growth of experimental oral cancers can be inhibited by infection with the herpes simplex virus type 1 (HSV-1), the effect is incomplete. To define factors that might limit the effectiveness of the virus, we examined the roles of the innate immune system and the replication status of the tumor cells. AT-84 tumors were induced in strains of mice that had specific immune defects and were treated with the virus. Explanted tumors and tumor cells in culture were also infected. No differences in viral replication or in the effect of virus on the tumor were seen between mice with a lack of T or B lymphocytes, natural killer cells, phagocytic spleen cells, or complement. The virus did not replicate significantly more in tumors that were maintained as explants. Immediately after recovery of cells from a tumor the proportion of cells in the S phase was around 18%, and replication of virus in those cells was very limited. After 3 weeks in culture, the proportion in S had increased to 50% and both the recovery of virus from the cells and the toxic effect of the virus on the cells had increased significantly. The innate immune system thus seemed to have a minimal effect on replication of HSV-1 when used as an oncolytic virus for oral cancers in mice. Instead, the fraction of cells in the S phase was important. Because human oral cancers, like mouse tumors, have low fraction of cells in the S phase, it is likely that the in vivo use of HSV-1 as cancer therapy will be limited by the replication of the virus.

Several recent studies have evaluated the potential of replication-competent strains of herpes simplex virus type 1 (HSV-1) for therapy of cancer. Because the virus is able to multiply within tumor cells, it could be expected to proliferate throughout a tumor, thus eliminating it (1). One tumor type that might benefit from this is squamous cell cancer of the mouth, which arises from the mucous membrane that is the natural host for HSV-1. However, despite the attractiveness of oncolytic viral therapy for oral cancer, the results have yet to match the expectations. In a previous study with the AT-84 mouse model, we found that wild-type HSV-1 was rapidly eliminated from the tumors, and although the growth of the tumor was reduced while the virus was present, it resumed after the virus was lost (2). The injection of a second dose of virus gave greater inhibition of the tumor, which is consistent with the idea that the concentration of virus in the tumor is an important determinant of its antitumor effect. We thus concluded that replication of the virus within the tumor was essential for the optimal antitumor effect and that the limited results to date can be attributed to failure of the virus to multiply effectively.

One possible explanation for failure of HSV-1 to replicate in tumors would be the effects of the immune response, either innate or acquired. In a rodent model of glioblastoma multiforme, it has been shown that replication of HSV-1 is inhibited by the animals’ immunity. Complement and natural antibody act together to reduce the replication of the virus in this system and thus, when the immune response is suppressed, the virus replicates more efficiently and the antitumor effect is enhanced (3, 4). Despite that, the opposite effect has been reported in a rodent model of malignant melanoma. In that case, the virus was effective against tumors in immunocompetent mice but not in tumors of mice that were immunosuppressed, either congenitally or by cyclophosphamide (5). Yet another consideration in evaluating the role of HSV-1 and immunity in cancer therapy is the possibility that the infection might act as an adjuvant, stimulating systemic immunity against the tumor. Evidence for this type of effect has been found in mouse models of colorectal carcinoma and malignant melanoma (6).

Despite work in these systems, the role of immunity in treatment of squamous carcinomas by HSV-1 is relatively unexplored. Significant antitumor effects have been reported in immunocompetent animals (7, 8) and limited data suggest that the effects are neither improved nor decreased by immunosuppression with hydrocortisone or cyclophosphamide (2). There are, however, indications that another important influence in squamous carcinomas is that rapidly dividing and non-differentiated squamous cells are more susceptible to toxicity by the virus than are slowly-dividing or highly-differentiated cells (8, 9). The importance of this to tumor therapy has not been elucidated. We have therefore undertaken the present study to clarify the areas in which advances will be most necessary for the successful development of HSV-1 as treatment for oral cancer.
Materials and Methods

Tumor cells
The murine oral cancer cell line, AT-84, was obtained from Dr. S. Karp (Medical College of Virginia, Richmond, VA) and was maintained and used to induce oral cancers in mice as described previously (1, 2). The proportion of cells that were in the S phase was measured by staining with 40 μg/mL propidium iodide followed by flow cytometry using a Becton Dickinson BDLSRII Flow Cytometer with fluorescence-activated cell sorting DiVa acquisition software and ModFit LT analysis software (Verity Software House, Inc., Topshem, ME).

Animals
Mice of the C3H strain and the NOD.CB17-Prkdcscid/J strain (scid/ nod) were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice of the strains BALB NU (nu/nu), CB17 SCID (scid/scid), and CB17-SCID BEIGE (scid/beige) were obtained from Taconic Farms (Germantown, NY). All mice were 6-week-old females.

Viruses
The red fluorescent protein (RFP) strain of HSV-1 was kindly provided by Dr. C. Hwang (Upstate Medical University, Syracuse, NY). This was derived from strain KOS by transfer of a gene that encodes RFP from the plasmid pDsRed2 (BD Biosciences Clontech, Palo Alto, CA) into the thymidine kinase region of the viral genome under control of the cytomegalovirus immediate-early promoter. Virus was propagated in Vero cells and harvested at a concentration of 5 × 10^7 plaque-forming units (pfu)/mL.

Assays of immune functions
T-cell function. T-cell function was assessed by measuring the response of spleen cells to a mitogen. Spleen cells were harvested, red cells were lysed, and the cells were dispensed at 2 × 10^6/mL in RPMI with 10% FCS (U.S. Department of Agriculture grade) into round-bottomed 96-well plates. Concanavalin A was added at either 4, 2, 1, 0.5, or 0.25 μg/mL. Cells were labeled with 3H thymidine at 1 μCi/well after 48 hours and were harvested 18 hours later onto filter papers. The uptake of thymidine was assessed by scintillation counting with results expressed as cpm (10).

B-cell function. B-cell function was assessed by the Jerne plaque assay, which counts the number of spleen cells able to make antibody to sheep red cells following sensitization (11). Mice were sensitized to the red cells by i.p. injection of 200 μL of 3% sheep RBC (Rockland Immunochemicals, Gilbertsville, PA). After 5 days, splenocytes were harvested and 20 μL of 25% sheep RBC were added with 20 μL of guinea pig complement and 60 μL of medium and 4 × 10^5 splenocytes in 100 μL. The mixture was incubated in a 50-μL slide chamber for 2 hours and hemolytic plaques were counted under a light microscope. Data were expressed as plaque-forming cells per spleen.

Phagocytic cells. Phagocytic cell activity was tested by the ability of cells to ingest latex beads. Splenocytes were isolated and incubated in plastic dishes; nonadherent cells were washed away and the adherent cells were incubated with latex beads. The proportion of phagocytic cells was derived by counting 100 cells of a representative field under a light microscope and data was recorded as the proportion of cells which had ingested five or more beads (12).

Natural killer cells. Natural killer (NK) cell functions were assessed by a chromium release assay using splenocytes and 51Cr-labeled YAC1 cells (American Type Culture Collection, Rockville, MD), which are sensitive to mouse NK cells (13). Activity was expressed as percent specific release, which was calculated using the spontaneous and maximum release from appropriate control cultures.

Complement. Total hemolytic complement activity in fresh plasma was measured by a standard hemolytic assay. Serial 1:2 dilutions of fresh serum in gelatin veronal buffer were incubated with antibody-sensitized sheep erythrocytes (Sigma, St. Louis, MO) at a concentration of 1 × 10^8/mL at 37°C for 1 hour in 96-well microtiter plates (14). The plate was centrifuged for 3 minutes at 120 × g and the absorbance of the supernatant was determined as A405 nm. The value was corrected using the same serum that had been heat-inactivated and expressed as a percentage of the equivalent value where freshly reconstituted lyophilized guinea pig serum (Sigma) was used.

Natural antibody. Serum levels of naturally occurring antiviral antibody were measured by a virus neutralization assay. Serum was collected from mice and heat-inactivated at 56°C for 30 minutes. Serum was diluted 1:2, 1:4, and 1:8 and 50 μL were mixed with 100 pfu of virus in 50 μL of buffer with 2 μL of reconstituted lyophilized guinea pig serum. The mixture was incubated at 37°C for 1 hour and plated onto Vero cells. After 48 hours, the number of virus plaques was counted. As a positive control, two sera were included from C3H mice that had been sensitized to HSV-1 by three i.p. injections of the virus over a period of 4 weeks.

All assays of immune function were done on two or three different occasions with two or three replicates for each measurement on each occasion.

Tumors
Tumors were induced in mice by injection of 1 × 10^6 AT-84 cells to the floor of the mouth, and tumors were allowed to develop over 2 to 3 weeks. Undiluted HSV-1 was injected into tumors in a volume of 25 μL. To find the concentration of virus in a tumor the tumor was removed, minced, and incubated in type 1 collagenase (Invitrogen Co., Carlsbad, CA) at 0.9 mg/mL at 37°C. Strained through a 70-μm sieve, spun, the supernatant replaced with medium, frozen and thawed, and plated onto Vero cells. Plaques were counted after 2 days.

For studies of explanted tumors, tumors were removed under sterile conditions, cut into 4-mm cubes, and maintained in culture medium with 15% FCS at 37°C for up to 5 days. Some were infected by injection of 25 μL of virus as was done for tumors in vivo. Representative tumors were fixed, embedded in paraffin, sectioned, stained with H&E, and examined microscopically.

To obtain cells from tumors, the tumor was disaggregated as above but cells were placed in culture for up to 3 weeks.

Inhibition of growth of tumors by herpes simplex virus type 1
To determine the effects of virus treatments on the growth of tumors, tumor volumes were measured thrice per week for 2 weeks with graduated calipers. Experiments were terminated early if a tumor exceeded 1 cm in any direction or if an animal showed signs of distress.

Replication of herpes simplex virus type 1 in cells in vitro
Cells were seeded into 96-well plates at 20,000 cells per well. The next day, when the monolayers were around 80% confluent, they were infected with 50 μL of HSV-1 at 10^4 pfu/mL. After 48 hours, cells were scraped into the medium and were disrupted by freezing and thawing twice. Supernatants were plated onto monolayers of Vero cells and plaques were counted after 48 hours. Assays for each sample were measured twice, in duplicate, and numbers of plaques were averaged.

Replication of cells in culture
Cells were plated in 96-well plates at a starting number that would provide equal cell densities the next day. This was 6.25 × 10^4 for AT-84 cells or tumor cells and was 3.12 × 10^5 for Vero cells. The next day (day 0) cultures were infected with 50 μL of virus at 10^5 pfu/mL. The number of cells in each well was evaluated on days 0, 1, 2, and 5 by addition of (3-4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide and using the A560-690 nm value as a measure of cell number. The doubling time was estimated by examining the rate of growth in the first two days and calculating the time in which the A560-690 nm value would increase from 0.1 to 0.2.
Statistical analyses

Numbers of cells in cultures were compared with a one-tailed unpaired t test, and P values < 0.05 were taken as significant. Data were analyzed with assistance of the computer program GraphPad Prism, v4.0 (GraphPad Software, Inc., San Diego, CA).

Results

Immune functions of each strain of mouse. C3H mice had a mean of $6.1 \times 10^7$ splenocytes per spleen in each mouse. The nu/nu mice had around one tenth as many, with $4.8 \times 10^6$ in scid/scid mice, $5.7 \times 10^6$ in scid/nod mice, and $4.3 \times 10^5$ in scid/beige mice.

(i) T cells of C3H mice showed thymidine incorporation of 396 cpm into unstimulated cells compared with 21,853 cpm into mitogen-stimulated cells; a difference of 21,457 with a stimulation ratio of 157. In contrast, the immunosuppressed mouse cells showed stimulation that varied from 2.6% of this in the case of the nu/nu mice to 6.1% in the case of the scid/nod mice.

(ii) B-cell assays showed no hemolytic plaques from mice to 6.1% in the case of the scid/nod mice.

(iii) Phagocytic cells were present at an average number of 17.7 per well in the C3H mice and 19.0 per well in the scid/beige mice. The other strains of mice showed fewer phagocytic splenocytes per well with 10 from the nu/nu mice, 7.6 from scid/scid mice, and 4 from the scid/nod mice.

(iv) NK cell assays showed a mean spontaneous release of $^{51}$Cr by YAC1 cells of 1,628 cpm and a mean maximum release of 14,292 cpm. In the presence of splenocytes of C3H mice, the mean specific release was 16.2%. Each of the other strains of mice showed lower specific release, with no NK activity being detected in splenocytes of the scid/nod mice.

The above assays were all done on equal numbers of splenocytes per assay. Because the nu/nu, scid/scid, scid/beige, and scid/nod mice all had fewer splenocytes than the C3H mice, the activity of each immune function would seem lower than reported above if results were expressed on a per spleen basis.

(v) Complement assays showed that guinea pig complement produced a level of lysis of sensitized red cells that was similar to that of water at all dilutions up to 1:64. This was reduced by 80% to 90% by heating of the serum to 56°C. Evaluations of mouse sera were then done at a dilution of 1:4. Hemolysis of <50% of that from guinea pig serum was observed in C3H, nu/nu, and scid/nod mice.

(vi) Natural antibody assays showed that sera from all strains of mice failed to cause 50% reduction of viral plaques at any of the dilutions tested. The positive control sera from C3H mice that had been sensitized to HSV-1 caused a >50% reduction in the number of viral plaques in all dilutions up to 1:32.

The immune functions of each strain of mouse are summarized in Fig. 1.

Effect of immune functions on replication of virus in tumors.

To find if the virus replicated more effectively in the tumors of mice with immune defects, infected tumors were harvested at regular intervals and the amount of virus that they contained was measured. As shown in Fig. 2, the recoverable virus from each strain of mouse fell rapidly after injection. Although there was a small and brief increase in the amount of virus present on day 5 in the nu/nu, scid/scid, and scid/beige mice, the recoverable virus never exceeded the input quantity, and all mice had lost all virus by day 7. Explant cultures did not show any replication of virus.

Effect of immune functions on antitumor effects of virus.

AT-84 oral cancers were induced in each strain of mice by injection of cells to the floor of the mouth, and incipient tumors were then injected with HSV-1 after 4 days. On day 10, the tumor volumes of the treated mice were significantly smaller in the nu/nu and scid/scid mice (t = 2.89, P = 0.0081 and t = 2.96, P = 0.0042, respectively) but were not significantly different in the scid/beige or scid/nod mice (Fig. 3), and at later times all tumors continued to grow.

Effect of cell cycle status of cells on replication of virus and growth inhibition by virus.

To find the association between the status of the tumor cells and the replication of the virus, AT-84 cells and cells derived from AT-84 tumors were infected with HSV-1. Both the release of virus and the expression of RFP were compared with the fraction of cells in the S phase. For comparison, the same measurements were done on Vero cells and three human oral cancer cell lines. As shown in Fig. 4, AT-84 cells that were recovered from tumors had 18.4% of cells in the S phase and released $1.1 \times 10^3$ pfu/mL after infection, whereas AT-84 cells that had been in culture indefinitely showed 46.7% in the S phase and released $6.0 \times 10^3$ pfu/mL. Vero cells and the human oral cancer cells showed intermediate proportions of cells in the S phase and released more virus (Fig. 4A). When examined by fluorescent microscopy, AT-84 cells showed expression of RFP in under 2% of cells, whether they were recently derived from a tumor or not, whereas Vero and human oral cancer cells showed between 70% and 100% that expressed the protein (Fig. 4B).

When AT-84 tumor cells were maintained in culture for up to 3 weeks, the proportion of cells in the S phase increased from 18.4% to 50.5% (t = 5.28, P = 0.0002; Fig. 5A). At the same time, there was an increase in the quantity of virus that the cells released after infection, from $1.1 \times 10^3$ pfu/mL to $3.3 \times 10^5$ pfu/mL. When the proportion of AT-84 cells in the S phase was reduced experimentally to 27% by reduction of the concentration of serum, there was a reduction in the amount of virus that was released to $6.3 \times 10^3$ pfu/mL (Fig. 5B). The changes in the amount of virus released did not, however, achieve statistical significance at the level of P < 0.05.

To find the association between the replication state of AT-84 cells and their susceptibility to growth inhibition by HSV-1, the virus was used to infect AT-84 cells directly from culture, cells
recovered from disaggregated tumors, cells from tumors that had been cultured for 3 weeks, and Vero cells. As shown in Fig. 6, uninfected Vero cells and uninfected AT-84 cells grew rapidly with a doubling time over the first two days of 10.8 and 11.5 hours, respectively. AT-84 cells from disaggregated tumors grew slower with a doubling time of 16.8 hours, but this reduced over the following 3 weeks to 14.4 hours. These differences were associated with different reductions in the number of surviving cells after 5 days of infection. Cells from freshly disaggregated tumors showed only a 30.2% (P = 0.094) reduction in number due to virus infection for 5 days, whereas after 3 weeks of culture they showed a reduction of 50.7% (P < 0.05). Vero cells and AT-84 cells showed reductions of 84.3% (P < 0.001) and 51.6% (P < 0.01) after 5 days of infection, respectively.

Discussion

The use of oncolytic viruses as therapy for cancers, including oral cancer, has received much attention lately, with both HSV-1 and adenoviruses having been proposed as candidates for clinical use. The use of replicating viruses is based on the assumption that they would infect more cells than nonreplicating viruses, which only transduce a limited number of oral cancer cells (15). Because HSV is naturally toxic to cells, its spread through a tumor might be expected to eliminate the tumor very effectively.

As a test of such hopes, we have developed the use of a realistic animal model of oral cancer (1) but found that HSV-1 produced only a limited inhibition of tumor growth (2). It was noted that the tumor seemed inhibited so long as virus was recoverable; the loss of virus from the tumor happened at
the same time as regrowth of the tumor commenced. This implied that the limiting factor for effective treatment was failure of the virus to replicate, and the present study was undertaken to find the likely causes of that.

A possible reason for failure of viral replication would be the immune system. One group, using brain tumors of rats, has shown that innate immunity can inhibit replication of HSV-1 and that immunosuppression allows increased viral replication and increased antitumor effects (3, 4).

To test if this could be so in the case of oral cancer, we selected strains of immuno-suppressed mice that represented defects in the major categories of immunity. Assays were done to confirm their phenotypes, and the mouse strains did indeed show a range of important defects (Fig. 1). At least one strain of mice showed <50% of the function of T cells, B cells, NK cells, or phagocytic cells of C3H mice. Low levels of complement were seen in two strains of mice and natural antibody was not detected in any strain of mouse (Fig. 1). These findings were consistent with previous reports on each strain (16). Thus, it was expected that if any of these components of the immune system were responsible for the failure of HSV-1 to replicate in oral cancers, this would become evident by an increased replication of the virus in one or more strains, along with improved response of the tumors to viral treatment in those same mice.

Unexpectedly, the recovery of virus from infected tumors of each strain of mice did not show important differences from what was seen in C3H mice (2). Virus disappeared rapidly from the infected tumors, and although an increase in the amount of virus in the tumors was seen in three strains of mice, this was small and limited to a single day. By day 7, no virus was found in the tumor of any animal (Fig. 2).
If the immune system was responsible for the failure of HSV-1 to replicate well in tumors, then it might be expected that explanted tumors, which are no longer supplied with mouse serum or immune cells, would be more permissive for viral replication than tumors in vivo. The explants were found to appear viable for several days as judged by histologic signs of viability. Nonetheless, no significant spread of virus through the explanted tissues was seen by immunohistochemistry, and no replication of virus was detected.

The effect of the virus on the growth of the tumors in nu/nu and scid/scid mice was similar to that in the C3H strain, in that moderate reductions in tumor volume were obtained on day 10. After that time, the tumors continued to grow (Fig. 3). No significant therapeutic effect was seen with the scid/beige or scid/nod mice, which implies that some component of the immune response of nu/nu or scid/scid mice contributes to the antitumor effect of the virus. This would be consistent with a previous report (5). The most likely cell type, based on the data of Fig. 1, would be NK cells, and functional studies of lymphoid cells taken from the tumors could be done to test this. However, the inflammatory infiltrate to the tumors is sparse whether they are infected with HSV-1 (2) or are not infected (1). It would be difficult to obtain enough cells to study and for this reason and because the therapeutic effect was small, the question was not pursued further.

As an alternative to the immune system, another explanation for the failure of virus to grow in a tumor when the tumor cells are otherwise susceptible would be that the tumor consists only of a fraction of susceptible cells. Alternatively, it might be that the three-dimensional structure of a tumor or its level of

Fig. 4. Relationship between proportion of different cell types that were in the S phase while growing exponentially, and the release of virus following infection (A) and expression of the RFP marker gene from HSV-1 (B). Data from AT-84 disaggregated tumors cells in (A) are also on the two leftmost columns of Fig. 5A. Measurements were taken on three occasions. Points, means; bars, SE (usually not seen because they are smaller than the symbols).

Fig. 5. Effect of the replication status of AT-84 cells on propagation of HSV-1. AT-84 cells were obtained by disaggregation of tumors and used for propagation of the virus either immediately, or after 7, 14, or 21 days in culture (A). Alternatively, AT-84 cells that had been only been grown in culture were maintained in different concentrations of serum for 24 hours before being used for propagation of the virus (B). Columns, mean of three to seven replicates; bars, SE. Values that differed from those of 21-day cultures (A) or those with 15% serum (B) with *P < 0.05 (asterisks).
differentiation prevents viral replication (9, 17, 18). As a test of these hypotheses, we infected cells from tumors along with AT-84 cells that had been cultured indefinitely as well as Vero cells and three lines of human oral cancer cells (Fig. 4A). The cells that had been derived the previous day from a tumor showed only 18.4% of cells in the S phase and produced very little virus, whereas cells that had been in culture indefinitely showed a much higher proportion in S and released more virus. This suggested that the proportion of cells in the S phase was important in viral replication; however, Vero cells and human oral cancer cells released more virus than AT-84 cells without showing a greater fraction in the S phase. Apparently, other factors than the proportion in the S phase are relevant to viral replication. As part of an effort to count infected cells, they were examined by fluorescent microscopy because the strain of HSV-1 expresses RFP. Although the Vero and human cells expressed RFP in up to 100% of cells, the AT-84 cells did not show >2% of fluorescing cells (Fig. 4B). The reasons for this remain unknown, but the lack of expression did preclude the use of fluorescent microscopy of tumor sections, which had been planned as a method of locating infected cells in tumors.

To confirm the importance of the cell cycle status in the infection by HSV-1, we cultured cells from AT-84 tumors for up to 3 weeks. Initially, they grew slowly but later they grew faster, increased the proportion in the S phase, and recovered their ability to release virus (Fig. 5A). Correspondingly, when the proportion of cells in the S phase was reduced by deprivation of serum, the cells became less permissive for virus (Fig. 5B). In agreement with this, the susceptibility of the cells to growth inhibition by the virus was low when they were recently recovered from tumors, but they became more susceptible after they had started to grow faster (Fig. 6). As a result of these experiments, it does not seem necessary to hypothesize that either the immune system, the structure of the tumor, or the existence of subsets of tumor cells are responsible for nonpermissivity of tumors to the virus. Rather, the low susceptibility of AT-84 tumors can be explained by the slow growth and small replicating fraction in a tumor in vivo compared with a cell monolayer in vitro. Although one of the advantages often claimed for HSV-1 as a vector for gene therapy is its ability to infect nonreplicating cells (19), it is possible that this independence from the cell cycle has been...
over stated in the past. Indeed recently, there has been interest in the fact that cell cycle inhibitors might be used to treat herpesvirus infections (20, 21).

The present data suggest that HSV-1 is unable to inhibit tumors when the percentage of cells that are in the S phase is less than around 20%. Because human oral cancers show a percentage of cells that are in the S phase of between 15% and 20% (22), it seems that the virus would not be expected to be very effective against human oral cancers despite its effectiveness against human oral cancer cell lines. The human tumors with the highest S-phase fraction do tend to be more aggressive (22, 23), which implies that therapy with HSV-1 might be most likely to be effective in the more aggressive tumors.

In different types of tumors, there are probably different relative roles of cell cycle and immune response in regulating the effect of HSV-1. Thus, in glioblastomas, where the virus does replicate well in the tumors, immunity is important (3, 4). In oral cancer, it seems that the metabolic state of the tumor cells is the critical limiting feature of viral replication. Future improvements of oncolytic therapy for this tumor should probably be directed toward improving the replication of the virus in the tumour cells.

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References

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