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Herpes Simplex 1716—an ICP 34.5 Mutant—Is Severely Replication Restricted in Human Skin Xenografts *in Vivo*

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HSV-1716 is a replication-restricted, neuroattenuated ICP 34.5 gene mutant of herpes simplex virus type 1 (HSV-1). Because of the attenuated phenotype of ICP 34.5 mutants in rodent models of HSV disease, they have been promoted as potential vaccine strains and gene therapy vectors and have been used by us and others as therapeutic agents for the treatment of experimental malignant tumors. However, all data on the phenotype of HSV-1716 and other ICP 34.5 mutants are from animal model systems, while humans are the natural hosts of HSV-1. To achieve an initial characterization of the phenotype of 1716 in human tissue, we have studied its replication in mature human skin xenografts on SCID mice. We find that replication of 1716 is severely restricted in such human skin grafts relative to both parental wild-type HSV-1 strain 17^+ and the HSV-1716 revertant virus 1716R, in which the 759-bp ICP 34.5 gene deletions have been repaired. Moreover, the replication of both 1716 and 17^+ is significantly better in the human skin grafts than it is in mouse skin. The implications of these findings are discussed.

HSV-1716 has a 759-bp deletion in both copies of the ICP 34.5 gene (*1*). Deletion or mutation of the ICP 34.5 gene results in HSV-1 variants that are incapable of replicating in the central nervous system of mice and do not cause encephalitis when inoculated via various routes (*1–3*). This is in sharp contrast to wild-type HSV-1, which grows exponentially in brain and kills mice within days of inoculation (*1*).

In vitro, ICP 34.5 HSV-1 mutants grow as well as wild-type virus on dividing cells of most established cell lines (*1, 2, 4*). However, on nondividing cells such as confluent primary mouse embryo cells, these mutants show impaired replication (*4*). In previous studies, we, and others, have examined the replicative phenotype of ICP 34.5 HSV-1 mutants *in vivo* in nonneuronal tissues in mice. These mutant viruses showed limited replication in the footpad (*5*), and replication in the eye could not be demonstrated following intraocular inoculation (*6, 7*).

We have recently shown that 1716 induced regression of preformed experimental intracranial melanoma and significantly improved survival times of treated tumor bearing mice (*8*). Similar results with other types of brain tumors have been demonstrated by others using comparable HSV-1 ICP 34.5 mutants (*9, 10*). The exciting potential of HSV-1 ICP 34.5 mutants as vaccine strains, and therapeutic agents for treatment of brain tumors, has prompted us to explore the *in vivo* phenotype of 1716 in more detail. Human skin xenograft systems have been employed to achieve replication of varicella zoster virus, human papilloma virus, and molluscum contagiosum virus, which are fastidious human dermatotrophic viruses (*11–13*). In the case of HSV-1, the use of a human skin xenograft system allows for studies in a prototypic human tissue of natural acute infection.

We find that the replication of both HSV-1716 and wild-type HSV-17^+ is much more efficient in the human skin than it is in mouse skin. Moreover, we find that relative to wild-type HSV-17^+, and the revertant virus HSV-1716R, the replication of HSV-1716 is severely restricted in human skin xenografts *in vivo.*

The titration data shown in Fig. 1 demonstrate the restricted nature of HSV-1716 in the human skin xenografts relative to wild-type HSV-17^+ and the revertant virus HSV-1716R. HSV 1716 shows only a slow increase in titer over the 3 days examined. In contrast, HSV-17^+ demonstrates exponential growth in the human skin over the same time period. To confirm that the phenotype of 1716 in the human skin was attributable to deletion of the ICP 34.5 gene, and not an additional unknown mutation, we also examined the replication of a revertant virus—1716R—in which the 759-bp ICP 34.5 gene deletions have been repaired (*14*). As shown in Fig. 1, 1716R demonstrated brisk replication in the human skin grafts and thus has a phenotype like that of wild-type 17^+.

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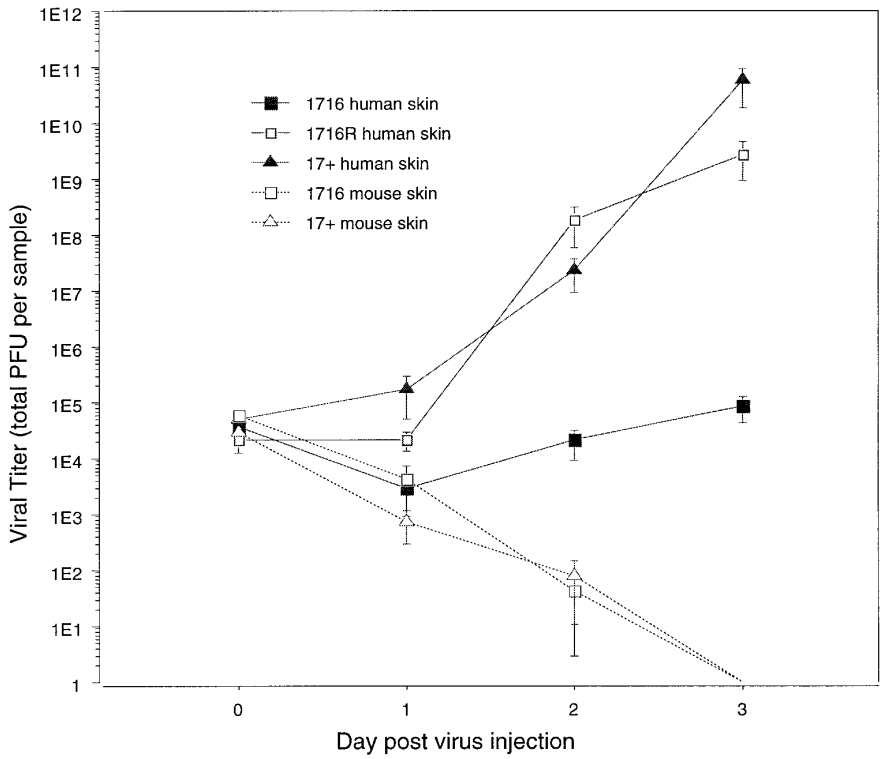

FIG. 1. Replication of HSV 17, 1716R, and 1716 in mouse skin and mature human skin xenografts. Full-thickness human foreskin xenografts were produced on SCID mice as previously described (*17*). Defatted full-thickness human skin grafts, ~1.5 cm in diameter, consisting of neonatal foreskin from elective circumcision, were grafted onto the flank of mice after removing full-thickness skin at the recipient site down to fascia. The grafts were sewn on, covered with an adhesive bandage clipped to the mouse, and covered with cloth tape. The grafts were left undisturbed until they had matured for 4–6 weeks. For inoculation directly into mouse skin, an ~5-cm^2-area patch of hair was removed from one flank using a chemical depilatory agent 1 day prior to viral inoculation (Magic Shaving Powder, Carson Products Co., Savannah GA). Mice were anesthetized with im ketamine/xylazine, and 5×10^6 PFU of HSV 17$^+$, 1716R, or 1716, in a total volume of 50 μl, were injected intracutaneously into either their flank skin or within mature human skin xenografts using a Hamilton syringe and a disposable 30-gauge needle. At the various times shown, mice were sacrificed by lethal injection of anesthesia, and the entire xenograft, or the ~5-cm^2 patch of mouse flank skin surrounding the site of inoculation, was removed aseptically to the level of fascia and frozen in liquid nitrogen. At the time of viral titration assay, each tissue sample was rapidly thawed in a 37° water bath, and the tissue was homogenized in viral culture medium at a 10% weight/volume ratio using a Pyrex Ten Broeck tissue grinder. The homogenates were centrifuged at 3000 g for 10 min at 4°. The supernatant of each tissue homogenate was diluted logarithmically in media, and the viral titer of each was determined in triplicate by plaque assay on BHK cells (*18*). Each data point represents the mean ± standard error of two tissue samples, and the experiment shown was repeated with similar results.

Comparison of the replication of 1716 and 17$^+$ virus in the human skin xenografts to that in SCID mouse skin demonstrates that both viruses replicate much better in the human skin. As shown in Fig. 1 intracutaneous inoculation of SCID mouse flank skin resulted in a level of replication of both 1716 and 17$^+$ that was just at the threshold of our titration assay. Despite a continual drop in titer over the 3 days examined, we believe that the rather slow decline most likely represents low-level replication rather than a complete absence of replication.

The restricted nature of HSV-1716 replication relative to that of wild-type 17$^+$ in the human skin xenografts is further supported by gross and histologic examinations of the tissues at various times postinfection. Hematoxylin and eosin sections are paired with sections stained immunohistochemically for HSV-1 antigens. HSV cytopathic effect and antigen staining within the human skin xenografts subsequent to HSV-1716 infection were scant, focal, and restricted to the epidermis at all times examined. In fact, the sections shown in Fig. 2 for 1716 were selected specifically to show evidence of 1716-induced pathology. The majority of tissue sections of 1716-infected human skin xenografts examined did not show any histopathologic or immunohistochemical evidence of HSV infection.

In contrast to HSV-1716, wild-type 17$^+$ showed gross, histopathologic, and immunohistochemical evidence of robust replication and pathologic effects. By Day 3 postinoculation focal epidermal histopathologic and immunohistochemical effects attributable to HSV were clearly seen. By Day 7 postinoculation with 17$^+$, near total destruction of the human skin grafts was seen. As is evident in the representative gross photograph in Fig. 2, full thickness epidermal ulceration is present in the majority of the xenograft. The representative histologic section from Day 7 post 17$^+$ infection shows complete loss of the epidermis with necrotic crust present. Immunohistochemical evaluation shows HSV antigen staining at the

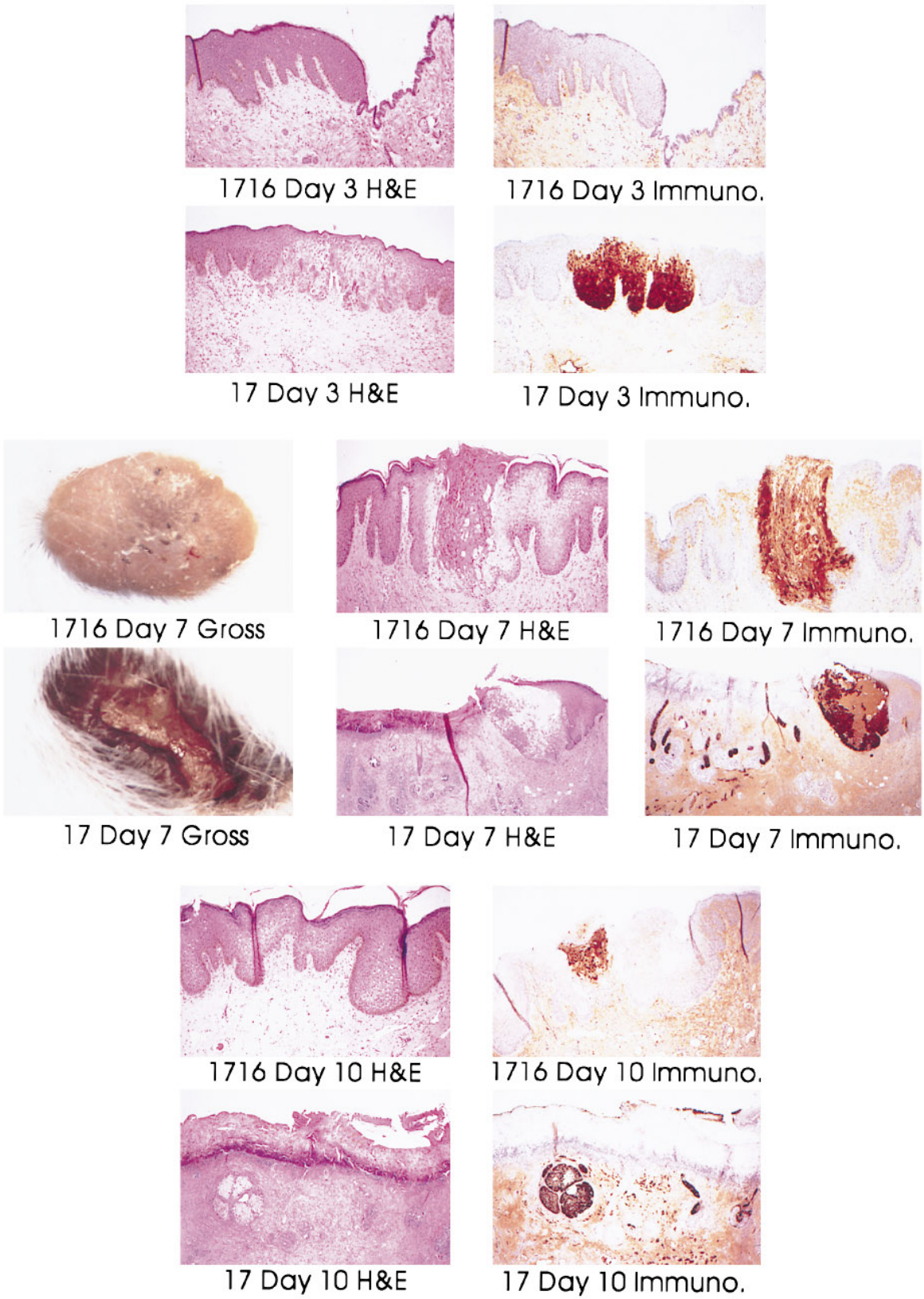

FIG. 2. Comparison of the gross and histologic changes caused by HSV-17 and 1716 in mature human skin xenografts. Full-thickness human foreskin xenografts were produced on SCID mice as described in the legend to Fig. 1. Mice were anesthetized with im ketamine/xylazine, and 5×10^6 PFU of HSV 17^+ or 1716, in a total volume of 50 μl, were injected intracutaneously within mature human skin xenografts using a Hamilton syringe and a disposable 30-gauge needle. At the various times shown, mice were sacrificed by lethal injection of anesthesia, and the entire xenograft was removed aseptically to the level of fascia and fixed in 10% neutral buffered Formalin. Histologic sections for light microscopy were stained with hematoxylin and eosin. Parallel sections were stained for HSV antigen expression using an indirect avidin–biotin immunoperoxidase method (Vector Labs, Burlingam, CA) as specified by the manufacturer with slight modifications developed in our laboratory (*19*). Rabbit antiserum to HSV-1 (Dako Corp., Carpinteria, CA) was used at a dilution of 1:1000. Sections were counterstained with hematoxylin. As a control for the specificity of immunostaining, nonimmune rabbit serum was substituted for the primary HSV-1 antiserum (data not shown). Magnification is 200× in all histologic sections.

edge of the xenograft and staining of adnexal structures within the dermis. By Day 10 post 17^+ infection, all sections examined exhibited complete epidermal destruction and significant dermal adnexal infection by immunohistochemistry.

When HSV-1716 or HSV-17^+ was inoculated into depilitated mouse skin, no gross alteration was seen surrounding the site (data not shown).

This study provides initial information on the phenotype of the HSV-1 ICP 34.5 mutant 1716 in human tissue. Although the restricted nature of the replication of this virus in neuronal tissues is well established (*1, 2, 4*), the replication of this and other ICP 34.5 mutants in nonneuronal tissues is less well documented. A study examining the replication of 1716 in mouse footpad found that the peak viral titer on Day 1 postinfection was comparable to that of wild type, but that the duration of recovery of titerable virus was several days shorter for the mutant (*5*). The peak vaginal viral titer of an HSV-1 strain F ICP 34.5 mutant was shown to be approximately 100-fold less than that of wild type on average over Days 1 to 8 post intravaginal infection in mice and guinea pigs (*7*). No replication of two ICP 34.5 mutants engineered from different parental strains was detectable in the eyes of mice infected via scarified cornea (*6, 7*). The difference in titer of recovered virus between 1716 and wild-type 17^+ seen in human skin xenografts is striking and parallels the restricted replication of ICP 34.5 mutants seen in neuronal and nonneuronal tissues of rodents. Immunohistochemical evaluation of infected human skin xenografts for HSV-1 antigen demonstrates that antigen staining is focal in nature and occurred only in the epidermis. The polyclonal antibody used for immunohistochemistry was raised to an infected cell lysate and detects both structural and nonstructural HSV antigens. The titration data document production of progeny HSV-1716. Thus, we feel that the focally limited antigen staining observed is best interpreted as quantitatively limited, but qualitatively typical, viral replication rather than an abortive infection. It has been shown that replication of 1716 in stationary state mouse 3T6 cells *in vitro* is severely restricted and that a defect in virus maturation and egress from the nuclei of infected cells occurs (*15*). It is possible that this same defect occurs in the human skin xenografts and accounts for our findings.

A somewhat unexpected finding of this study was the inability to detect brisk replication of wild-type 17^+ replication in SCID mouse flank skin following intracutaneous infection. We and others have shown replication of 17^+ and other wild-type HSV-1 isolates in peripheral tissues of rodents (*5–7, 16*). Generally, significant trauma to the tissue such as preinjection of the footpad with hypertonic saline, abrasion of the footpad or lip with an emery board, scarification of the cornea with a needle, or abrasion of the vaginal mucosa with a cotton applicator stick is necessary to successfully inoculate virus at peripheral sites (*6, 7, 16*). For the purpose of this study a relatively nontraumatic intracutaneous injection was used. Although this is clearly sufficient to establish an ongoing infection in the human skin xenografts, it is apparently not sufficient to do so in murine flank skin in the case of 17^+. We are presently investigating the phenotype of other "wild-type" clinical isolates and mutants in human skin xenografts.

While the dramatic difference in replication between 1716 and 17^+ in the human xenograft skin offers encouragement as to the attenuated nature of ICP 34.5 HSV-1 mutants in nonneuronal human tissues, the impressive differences in replication found between the human and mouse skin for both mutant and wild-type HSV offer yet another reminder that the mouse is a far from perfect model for study of a human pathogen such as HSV-1.

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