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Vaccine Potential of a Herpes Simplex Virus Type 1 Mutant with an Essential Glycoprotein Deleted

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Several approaches to the production of vaccines to human herpesviruses have been proposed. Subunit vaccines, subunits delivered by live vectors, and rationally attenuated vaccines have all been shown to be efficacious in animal models but suffer from uncertainties as to the roles of individual genes involved in pathogenesis and the most relevant components of the immune response required for protection in humans and the target antigens involved. With these problems in mind, we examined the vaccine potential of a fully disabled herpes simplex virus type 1 mutant that is capable of only a single round of replication, since a virus of this type should induce the full spectrum of immune responses but has no pathogenic potential. A virus has been described which lacks essential glycoprotein H (gH) and can be propagated in a cell line which supplies gH in *trans* (A. Forrester, H. Farrell, G. Wilkinson, J. Kaye, N. Davis-Poynter, and T. Minson, J. Virol. 66:341–348, 1992). Infection of normal cells with this mutant is indistinguishable from a wild-type infection, except that the resulting progeny are gH negative and noninfectious: the virus is self-limiting. Infection of mice by the ear pinna route was similarly self-limiting in that input infectivity decreased rapidly at the inoculation site and no infectivity was detected in sensory ganglia. Animals given a wide range of doses of the gH-negative mutant produced both humoral and T-cell responses to herpes simplex virus type 1 and proved solidly resistant to challenge with a high dose of wild-type virus. The gH-negative mutant is presumably capable of establishing a latent infection, but since no infectious virus was detected in numerous attempts to reactivate the mutant, the risk of a pathogenic outcome is minimal.

There is ample evidence, based primarily on studies using animal models, that both the humoral and cell-mediated arms of the immune response can function in the prevention and control of herpes simplex virus (HSV) primary and recurrent infections (14, 20). Thus, a number of studies have demonstrated that administration of passive antibody modulates HSV type 1 (HSV-1) infection in mice (18, 21); nevertheless, B-cell-suppressed mice recover normally from infection (9, 22). A variety of immunization and vaccination protocols have been effective in protecting rodents from HSV-1 infection. These include administration of inactivated virions or infected cell extracts, immunization with purified or recombinant glycoproteins, or vaccination with antigens delivered by live vectors (reviewed in reference 4), but there is conflicting evidence as to the major protective antigens and the immune mechanism involved in the resulting protection. Furthermore, given the relative ease with which protection can be achieved, particularly in inbred mouse strains, there is significant doubt as to the relevance of the mechanisms for immunity to HSV-1 in humans. In the face of this uncertainty as to the nature of the protective immune response to HSV-1 in humans, the development of rationally attenuated vaccines offers some attraction, because live herpesvirus vaccines have proved successful against veterinary diseases of economic importance (5, 17) and attenuated vaccines elicit a natural immune response, thus reducing the need, in vaccine design, for a detailed understanding of the immune response to infection. The complexity of the HSV genome and the presence of a large number of genes that are dispensable for growth in vivo provide a variety of opportunities for rational attenuation. Deletion or interruption of a number of genes, including the thymidine kinase gene (6), several dispensable glycoprotein genes (2, 24), and the protein kinase gene (16), has yielded viruses with attenuated phenotypes that elicit a protective response in experimental animals, but we cannot be certain that these viruses will prove equally attenuated in humans. An alternative approach to attenuation is the use of a fully disabled virus that is replication defective. Although viruses of this type synthesize only a subset of viral proteins and are unable to produce virus particles, Nguyen et al. (15) showed that HSV-1 mutants lacking either immediate-early gene ICP4 or ICP27 or early gene ICP8 were capable of eliciting T-cell responses in BALB/c mice and were capable of protecting mice from subsequent lethal intraperitoneal challenge with wild-type HSV-1. Replication-incompetent viruses of this type, however, fail to replicate the viral genome and, in consequence, do not synthesize significant quantities of late proteins, many of which are known to elicit protective immune responses (11, 19, 23). An alternative approach is to make use of a virus that is replication competent but produces noninfectious particles by virtue of the absence of a single essential virion protein. A single-cycle virus with these characteristics should present all of the virus-specific proteins, apart from the absent one, to all arms of the immune response.

We have described previously the construction of an HSV-1 mutant in which glycoprotein H (gH) coding sequences are replaced with the *Escherichia coli lacZ* gene and which is propagated in a cell line supplying gH in *trans* (7). Infection of normal cells results in a complete lytic cycle and the production and secretion of normal numbers of noninfectious enveloped

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virions. We therefore decided to investigate the ability of this virus to induce a protective response in mice by a single-cycle infection.

MATERIALS AND METHODS

Viruses. The parent HSV-1 strain used in all of the experiments was the SC16 strain and is hereafter referred to as wtSC16. This virus was propagated on BHK-21 cells as described previously (6). The construction of two HSV-1 mutants derived from the SC16 parental strain in which the *E. coli lacZ* gene replaced either US5 coding sequences (SC16ΔUS5) or gH coding sequences (SC16ΔgH) has also been previously described. The SC16ΔUS5 mutant is indistinguishable from the parent virus with respect to growth and dissemination in *vitro* (7, 25) and in *vivo* (1) and was used to facilitate detection of virus-infected cells in mice. The SC16ΔgH mutant was propagated on a supporting Vero cell line (F6) which supplies gH in *trans* upon infection with HSV-1 (7). Thus, progeny virions are phenotypically gH positive (having derived cellular gH) and possess a particle-to-PFU ratio equivalent to that of the wtSC16 virus. Viruses used for in vivo experiments were partially purified by the following method. Extracellular virus was recovered from cell cultures infected 48 h previously at a multiplicity of infection of 0.01 and centrifuged for 90 min at 85,000 × *g* for 1 h. The virus pellets were resuspended in 3 ml of TES (1 mM Tris [pH 7.4], 1 mM EDTA, 100 mM saline, 0.5% fetal calf serum) and loaded onto discontinuous 20 to 40% sucrose gradients. Following centrifugation at 25,000 × *g* for 1 h, the viruses were harvested from the 20 to 40% interface, diluted in TES, and centrifuged at 85,000 × *g* for 1 h. The virus pellets were resuspended in TES, and aliquots were stored at −70°C. Purified wtSC16 was inactivated with β-propiolactone (0.05% [vol/vol]; 30 min at room temperature); the absence of live virus was demonstrated by plaque assay.

Mice. Four-week-old female BALB/c mice were obtained from Tucks Ltd. and inoculated at 5 to 6 weeks of age. Lightly anesthetized mice were vaccinated by scarification of the left ear pinna with an inoculum of either live or inactivated purified virus in 2 μl of phosphate-buffered saline (PBS). Control mice were inoculated with 2 μl of PBS only. At 14 days after vaccination, mice were challenged by infection of the right ear with wtSC16 at the doses indicated in the figure legends.

Measurement of virus titers in mouse tissues. Ear pinnae and pooled innervating sensory ganglia (CII, CIII, and CIV) were dissected and stored at −70°C. Samples were later homogenized and assayed for the presence of virus on BHK-21 or F6 cells as described previously (6).

Enzymatic detection of SC16ΔUS5 and SC16ΔgH mutants in cells infected in vitro and in vivo. The method used to detect β-galactosidase in cells infected in vitro with recombinant viruses carrying the *lacZ* gene with the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) has been previously described (7). The same procedure was used to detect the mutants in 5-μm frozen sections of ear pinnae from inoculated mice, except that as a final step, the tissue was counterstained with hematoxylin and eosin.

Detection of neutralizing antibodies to HSV-1. Neutralizing antibodies were detected by a plaque reduction assay in the presence of 5% fresh guinea pig serum as a source of complement. Individual serum samples were serially diluted in 500-μl volumes and incubated with an equal volume of wtSC16 (10^3 PFU/ml) for 1 h at 37°C. Nonneutralized virus at the end of the incubation was detected by plaque assay on BHK-21 cells. The titer was calculated as the reciprocal dilution of serum required to achieve a 50% reduction in plaque numbers relative to the number of plaques obtained in the absence of mouse serum. Included as a positive standard in each assay was a complement-dependent neutralizing monoclonal antibody (designated AP7) which is directed against HSV-1 glycoprotein D (13).

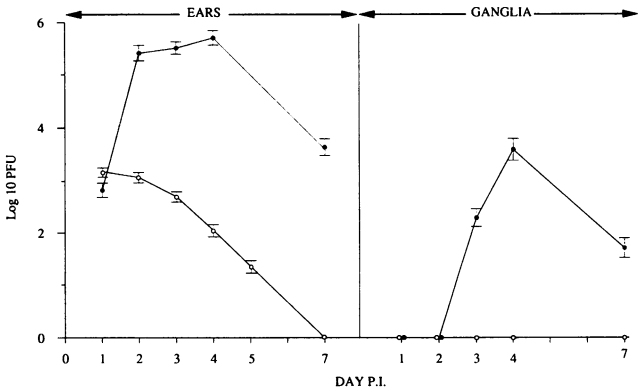

FIG. 1. Clearance of HSV-1 *lacZ* insertional mutants in the ears and ganglia of mice. Groups of four mice were infected with either SC16ΔgH (○) or SC16ΔUS5 (●), and virus titers were measured on days 1, 2, 3, 4, 5, and 7 postinfection (P.I.). Virus titers in mice infected with the SC16ΔgH mutant were determined on F6 cells, while titers in mice infected with the SC16ΔUS5 mutant were assayed on BHK-21 cells. The data are presented as geometric means with the standard errors of the means. All virus was of the β-galactosidase-positive phenotype.

Delayed-type hypersensitivity (DTH) assays. Groups of six mice were vaccinated in the left ear pinna with either the live SC16ΔgH mutant, inactivated SC16ΔgH, or live wtSC16 at the doses indicated in Fig. 4. A control group of mice was inoculated with PBS only. At 14 days postvaccination, mice were challenged with 10^6 PFU of wtSC16 by scarification of the right ear pinna. The thicknesses of both ears were measured at 24 and 48 h postchallenge. The results are expressed as differences in thickness between challenged and unchallenged ears.

RESULTS

Determination of the level of nondefective virus in SC16ΔgH progeny. Replication-defective deletion mutants are occasionally rescued by recombination with the viral gene resident in the helper cell line. Before initiating protection experiments, it was therefore important to determine the levels of nondefective virus in SC16ΔgH virus stocks and to assess the possible impact of this component on the experiments. Following two rounds of cloning by limiting dilution, working stocks of SC16ΔgH virus were titrated on gH-inducible F6 cells and on Vero cells and the relative titers were estimated as approximately 5 × 10^6:1. Plaques formed in Vero cells were β-galactosidase negative, implying that the nondefective phenotype had resulted from replacement of the *lacZ* gene by the gH-encoding gene resident in the cell line. Mice were then infected by the ear route with 5 × 10^7 PFU of SC16ΔgH virus, and titers at the inoculation site and in innervating dorsal root ganglia were measured by plaque assay on F6 cells. The results are shown in Fig. 1, in which, for comparison, the virus titers resulting from parallel infections with 5 × 10^5 PFU (i.e., a 100-fold lower dose) of SC16ΔUs5 are also shown. One day after infection, less than 0.01% of the input SC16ΔgH inoculum was recovered from the inoculation site and this residual

inoculum was cleared during the subsequent 7 days. No infectivity was recovered from dorsal root ganglia. All of the detectable virus had the β-galactosidase-positive phenotype and was not detected by assay on BHK-21 cells. Thus, although each animal received an estimated 10 PFU of replication-competent virus, this component was never detected and was presumably overwhelmed by the huge excess of defective virus. Histological examination revealed that after 5 days, when only about 0.0001% of the input inoculum can be detected, large numbers of β-galactosidase-positive cells were present at the inoculation site (Fig. 2a). Thus, inoculation with phenotypically gH^+ virus had successfully established infection but, in contrast to the SC16ΔUs5 virus (Fig. 2b), had not spread beyond the inoculation site. At 1 month after infection, the cII, cIII, and cIV dorsal root ganglia were dissected from 12 mice infected with SC16ΔgH and were incubated in tissue culture medium for 5 days to allow reactivation of latent virus. The tissue was then homogenized and assayed for infectivity. None was found.

Induction of protective immunity by SC16ΔgH virus. Experiments were designed to compare the protective efficacy of equivalent doses of inactivated virus with that of replication-defective virus over a broad dose range. Protection was assessed by measuring the growth of wild-type virus in the ear pinna 5 days after challenge, the point at which peak titers are observed in this model (Fig. 1). Groups of four mice were vaccinated by left-ear scarification with SC16ΔgH or β-propiolactone-inactivated virus at doses ranging from 5×10^2 to 5×10^7 PFU. The particle-infectivity ratio of the purified virus preparation was approximately 30:1, and the maximum dose used therefore was about 10^9 virions, equivalent to about 1 μg of virion protein. After 14 days, mice were challenged by inoculation of the right ear with 2×10^6 PFU of wtSC16. The results of two experiments are shown in Fig. 3, which gives the geometric mean titers of challenge virus 5 days after inoculation. At the higher doses used, vaccination with live SC16ΔgH or with inactivated virus resulted in complete clearance of the challenge virus at 5 days postinfection, while at the lower dose no effect was observed with live or killed vaccine. At all intermediate doses, vaccination with the live gH^- virus suppressed challenge virus growth with greater efficiency. Comparison of the data suggests that a 100-fold-higher dose of inactivated virus is required to achieve an equivalent effect in this assay.

Induction of neutralizing antibodies by the SC16ΔgH mutant. Although different studies have suggested different mechanisms of immunity to HSV challenge, a comparison of the efficacies of different glycoprotein antigens delivered with a live vector suggested that protection correlated with neutralizing antibody induction. Injection with a live gH-negative virus results in a single-cycle infection and significant amplification of the delivered antigen compared with vaccination with an equivalent dose of killed virus. We therefore predicted higher neutralizing antibody titers in animals that received SC16ΔgH than in those that received inactivated virions. Serum samples taken from individual mice prior to challenge in the protection experiment shown in Fig. 3 (experiment 2) were assayed for neutralizing antibody to HSV-1 (Table 1). At the lowest vaccine dose for which sera were tested (5×10^4 PFU), SC16ΔgH infection induced detectable neutralizing antibody, whereas inactivated virions did not. Nevertheless, the neutralizing antibody titers in the sera of mice that received higher vaccine doses were not significantly different and it seems unlikely that preexisting antibody titers can account for the considerable difference in protection afforded by vaccination with the live disabled virus and the inactivated virus. For comparison, the neutralizing antibody titers of animals injected in parallel with 2×10^6 PFU of wtSC16 are shown. This dose of wild-type virus affords complete protection against subsequent challenge with the same dose. As expected, the antibody titers induced in these animals were higher although not dramatically so.

Induction of DTH responses by the SC16ΔgH mutant. It is widely accepted that T-cell responses are crucial in controlling HSV infection, and while uncertainty remains as to the relative contributions of different T-cell subsets, there is strong evidence that $CD4^+$ T-cell responses are effective in virus clearance in the mouse ear model. DTH responses to wtSC16 in vaccinated mice were therefore measured as described in Materials and Methods. The experimental design was similar to that described for the protection study in that a range of doses of the vaccinating virus was employed and the responses induced by equivalent doses of an inactivated vaccine were included for comparison. In addition, groups of mice which received equivalent doses of wtSC16 as the vaccinating virus were also included. The results are presented in Fig. 4. At vaccinating doses of 5×10^3 and 5×10^4 PFU, the SC16ΔgH mutant induced DTH responses at levels on day 2 postinfection which were comparable to those induced by wtSC16, while the inactivated vaccine at these doses was completely ineffective. In a separate experiment, a dose of 10^2 PFU of either the live-disabled or killed vaccine failed to elicit a DTH response (data not shown). Nevertheless, DTH responses were detected in mice given higher doses of the inactivated vaccine, with responses which were equivalent to those induced by the SC16ΔgH mutant at the highest vaccine dose used. While these results do not, of course, show that the basis of the efficacy of a live disabled virus, by comparison with a killed virus, lies in its ability specifically to induce a DTH response, they are consistent with the general view that a live vaccine recruits a T-cell response more efficiently than does an equivalent dose of killed antigen.

DISCUSSION

It is generally accepted that live vaccines offer considerable advantages over killed or subunit vaccines in terms of both the range of responses induced and the longevity of the response. The ability of HSV to establish life-long latency is, however, a significant issue in contemplating human vaccination with an attenuated HSV. A disabled virus capable of a single cycle of infection may be an attractive alternative. The findings presented here demonstrate that the SC16ΔgH HSV-1 mutant is able to establish a self-limiting infection in vivo that induces both humoral and T-cell responses and protects mice against subsequent challenge with wild-type HSV-1. Low doses of the SC16ΔgH mutant induced levels of DTH responses in mice which were comparable to those induced following infection with wtSC16. In addition, the SC16ΔgH virus elicited neutralizing antibodies with titers which remain elevated well beyond clearance of the virus from the animals. In comparison with an inactivated vaccine, the SC16ΔgH mutant was more effective in inducing DTH responses and in conferring protection during acute infection. Although live-virus vaccines have been shown to elicit cellular immunity more readily than killed-virus or subunit vaccines, the mechanism(s) by which this is achieved is unknown. A simple explanation for our results is that the amplification of viral genes produced during the single cycle of replication of the SC16ΔgH mutant in vivo and the natural presentation of appropriate viral targets elicit responses which, in both quantity and quality, are more effective in conferring protection than are the responses induced by killed-virus

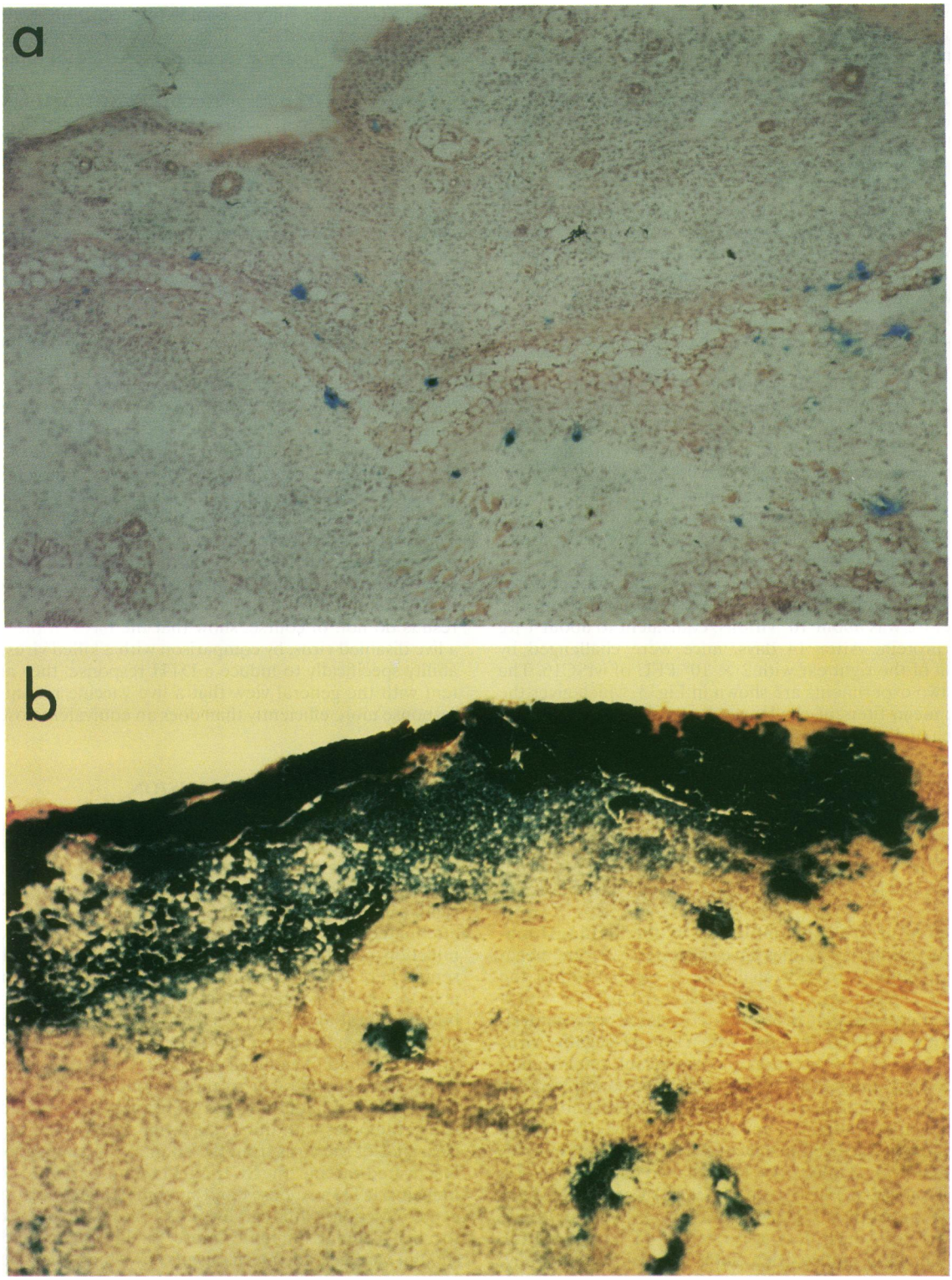

FIG. 2. Histological demonstration of cells infected with HSV-1 *lacZ* insertional mutants in mice (magnification, ×160). Sections of the ear pinnae of mice infected 5 days previously with either SC16ΔgH (a) or SC16ΔUS5 (b) were examined microscopically after being stained with X-Gal.

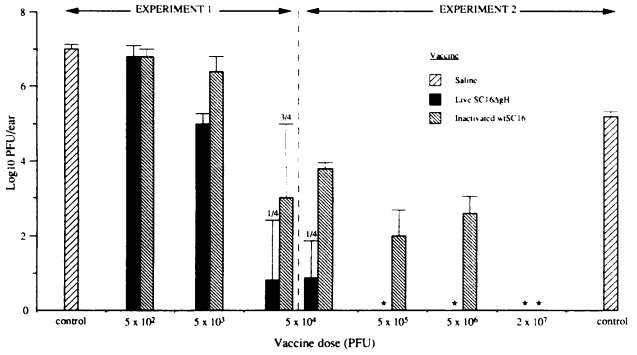

FIG. 3. Clearance of wtSC16 in the ears of mice vaccinated with either SC16ΔgH or β-propiolactone-inactivated wtSC16. Groups of four mice were vaccinated at the doses indicated by scarification of the left ear pinna. Control mice were given saline only. Mice were challenged 14 days postvaccination with 2×10^6 PFU of wtSC16 in the right ear pinna, and virus titers were measured at 5 days postinfection. The data are expressed as geometric means and the standard errors of the means. Asterisks indicate that virus was not detected. In cases in which virus was not recovered from four of four mice, the numbers of mice from which virus was recovered are indicated above the error bars.

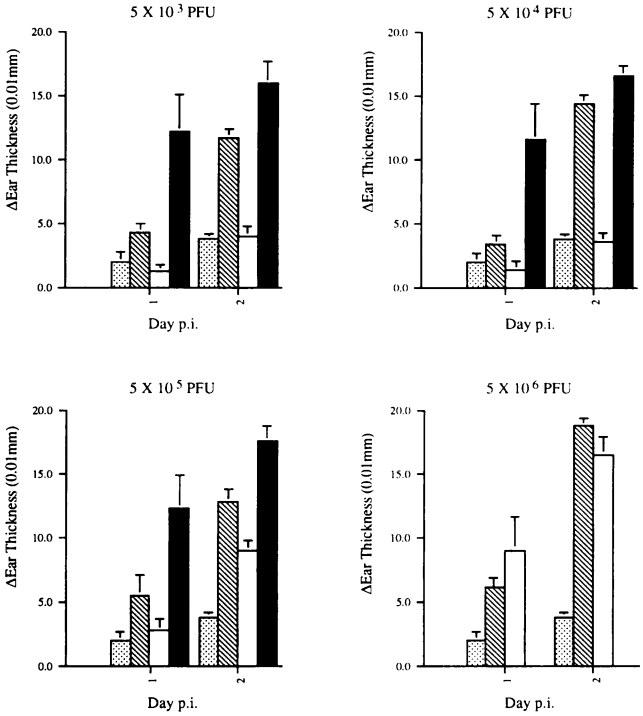

FIG. 4. DTH responses in mice vaccinated with PBS (□), SC16ΔgH (▨), β-propiolactone-inactivated SC16ΔgH (□), or wtSC16 (■). Mice were vaccinated in the left ear pinna at the doses indicated 14 days prior to challenge with 10^6 PFU of wtSC16 in the opposite ear. Note that wtSC16 was not used at the highest vaccine dose (5×10^6 PFU), since this is a lethal dose for 5-week-old BALB/c mice. Ear thickness was measured 24 and 48 h postchallenge and is expressed as the difference between the challenged and vaccinated ears. The data are presented as mean differences in ear thickness and the standard errors of the means. p.i., postinfection.

vaccines. However, the protection afforded by the submicrogram doses of killed-virus vaccine illustrates the relative ease with which protection may be achieved in the mouse model. Further work is required to quantify the immune responses generated by this single-cycle vaccine and to determine, by adoptive transfer, which of the responses afford protection. Furthermore, while mice have unique advantages in studies of immune responses, HSV infection in guinea pigs is appropriate for studies of reactivation and recrudescence and experiments are now in progress to evaluate the vaccine potential of a single-cycle virus in this animal model.

Similar studies addressing the vaccine potential of disabled HSV-1 mutants were reported by Nguyen et al. (15), who demonstrated that cellular immunity to HSV-1 and protection from lethal challenge in mice could be achieved by vaccination with replication-defective HSV-1 mutants. The magnitude of T-cell responses to these mutants was proportional to the extent to which viral gene expression occurred in infected cells. However, the replication-defective mutants used in their study were deficient in the expression of all or some of the late gene products, many of which are known to be targets for antibodies and T cells (3, 8, 10, 12). In contrast, infection of cells with the phenotypically gH-positive SC16ΔgH mutant results in a replicative cycle that is normal in all respects except that the progeny are noninfectious. Therefore, we can presume that the SC16ΔgH mutant has the capacity to elicit immune responses with a range of specificities (with the exception of responses to gH) comparable with those induced by the parental wild-type virus.

Given that the SC16ΔgH virus is incapable of producing infectious progeny in cells that are unable to provide gH in *trans*, it is unlikely that infection could spread beyond the cells surrounding the vaccination site in vivo. However, given that disabled HSV-1 mutants have been previously shown to establish a latent infection, it is possible that the SC16ΔgH virus can establish a latent infection as a result of direct entry into sensory nerve endings by the input inoculum. Indeed, preliminary experiments have identified the major latency-associated transcript in the cervical ganglia of mice vaccinated with the SC16ΔgH virus (data not shown). Although detection of the *lacZ* gene is required for formal proof that the SC16ΔgH virus resides in sensory ganglia, numerous attempts to reactivate virus from infected animals have proved unsuccessful, implying

TABLE 1. Quantification of neutralizing antibodies to HSV-1 in mice vaccinated with either live SC16ΔgH or inactivated wtSC16

Vaccine	Vaccine dose (PFU)	Neutralizing antibody titer[a] at:	
		14 days	3 mo
Live SC16ΔgH	0	<50	<50
	5×10^4	79 ± 29	ND[b]
	5×10^5	158 ± 58	ND
	5×10^6	400 ± 0	500 ± 178
	2×10^7	630 ± 162	ND
Inactivated wtSC16	0	<50	<50
	5×10^4	<50	ND
	5×10^5	100 ± 37	ND
	5×10^6	500 ± 257	912 ± 130
	2×10^7	400 ± 0	ND
Live wtSC16	2×10^6	1,600 ± 585	1,600 ± 0

[a] Each individual titer is expressed as the geometric mean of the reciprocal dilution (plus 1 standard deviation) of serum required to neutralize 50% of the virus infectivity obtained in the absence of antibody.
[b] ND, not done.

that the latent virus is unable to produce infectious virus upon reactivation. Furthermore, attempts to rescue β-galactosidase-positive virus by superinfection of latently infected ganglia in vitro were also unsuccessful.

In conclusion, our studies demonstrate that the SC16gH mutant is able to stimulate protective immune responses without the risk of virus dissemination associated with live attenuated HSV vaccines. Although the SC16ΔgH mutant may have the propensity to establish a latent infection, reactivation of the latent virus could result only in the production of noninfectious virus. This vaccination strategy may also be applied to systems in which HSV-1 is used as a live vector for expression of heterologous genes.

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