

A Genetically Inactivated Herpes Simplex Virus Type 2 (HSV-2) Vaccine Provides Effective Protection against Primary and Recurrent HSV-2 Disease

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A glycoprotein H (gH)-deleted herpes simplex virus type 2 (HSV-2) was evaluated as a vaccine for the prevention of HSV-induced disease. This virus, which we term a DISC (disabled infectious single cycle) virus, can only complete one replication cycle in normal cells and should thus be safe yet still able to stimulate broad humoral and cell-mediated antiviral immune responses. A gH-deleted HSV-2 virus that has been tested as a vaccine in the guinea pig model of recurrent HSV-2 infection was constructed. Animals vaccinated with DISC HSV-2 showed complete protection against primary HSV-2-induced disease, even when challenged 6 months after vaccination. In addition, the animals were almost completely protected against recurrent disease. Even at low vaccination doses, there was a high degree of protection against primary disease. A reduction in recurrent disease symptoms was also observed following therapeutic vaccination of animals already infected with wild type HSV-2.

An effective vaccine against primary and recurrent disease caused by herpes simplex virus (HSV) is an important goal because the incidence of genital herpes is high and increasing worldwide [1]. We have recently shown that HSVs lacking the essential glycoprotein H (gH) gene have considerable potential as vaccines [2, 3]. These viruses can be grown in a complementing cell line expressing the gH protein [4]. Viruses produced from the complementing cell line can infect normal cells, undergoing a single cycle of replication, but the virus particles emerging from these normal cells lack gH and are therefore noninfectious. Thus, this virus, which we have called a DISC (disabled infectious single cycle) virus, is similar to a traditional killed vaccine, in that being genetically inactivated, it cannot spread within the host. In other respects, however, it has the properties of a live vaccine, in that it should be capable of generating humoral and cell-mediated immune responses against all virus-encoded antigens.

We have shown that DISC gH-deleted HSV-1 can protect against HSV-1 challenge in the mouse ear model. The DISC virus, by virtue of its capacity for a single-round of replication in normal cells, is considerably more potent than a nonreplicating, inactivated virus preparation [2]. This seems likely to result from its ability to provoke effective cell-mediated immune responses as measured by assays for both delayed-type hypersensitivity [2] and cytotoxic T cell responses (McLean CS, unpublished data) against HSV antigens.

The gH-deleted DISC HSV-1 has also been tested in the guinea pig model of vaginal HSV infection [3], which is the

most appropriate model for primary and recurrent genital disease in humans [5]. Guinea pigs infected intravaginally (ivag) with HSV-2 develop vesicular lesions on the external genital area. After recovery from this primary disease episode, which lasts for ~2 weeks, animals show spontaneous recurrent lesions at varying frequencies. Vaccination with DISC HSV-1 by ivag and intraepithelial routes provided effective protection against challenge with HSV-2. When animals were vaccinated during the recurrent stage of disease (therapeutic vaccination), recurrences were moderately reduced. The heterotypic protection afforded by a DISC HSV-1 vaccine against HSV-2 challenge was encouraging, but it seemed likely that the use of a homotypic DISC HSV-2 as a vaccine might provide even more effective protection.

To develop a vaccine against HSV-2, the major cause of recurrent genital herpes infections, we constructed a DISC HSV-2 virus lacking gH sequences and a complementary cell line expressing the HSV-2 gH. Herein, we report studies to assess the efficacy of this vaccine in the guinea pig model of genital HSV-2 infection.

Materials and Methods

Viruses and Cells

Several plaque-purified isolates of HSV-2 strain HG52 (gift from Moira Brown, MRC Institute of Virology, Glasgow) were tested for neurovirulence by intracranial injection into BALB/c mice, and the isolate with the lowest neurovirulence characteristics, HG52-D, was used for the construction of the DISC HSV-2 virus.

The virus used for challenge in the guinea pig studies was the MS strain of HSV-2 (supplied by L. R. Stanberry, University of Cincinnati College of Medicine). Virus was propagated and titrated on Vero cells as described previously [3].

Vero cells (no. 88020401; European Collection of Animal Cell Cultures [ECACC], Porton Down, UK) were from a World Health Organization (WHO) cell line approved for the manufacture of

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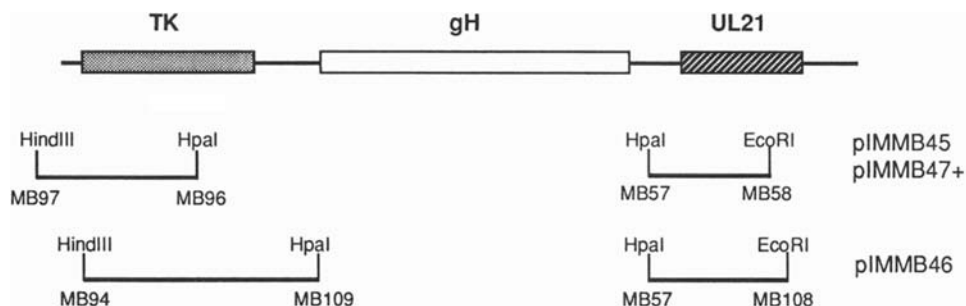


Figure 1. Flanking sequences cloned by polymerase chain reaction for vectors pIMMB45, pIMMB47+, and pIMMB46, showing restriction sites incorporated and oligonucleotides used.

biologics for human use. The cells are available to organizations wishing to produce products such as vaccines, in accordance with the recommendation of the Expert Committee on Biological Standardisation [6]. BHK *tk*⁻ cells were also obtained from ECACC (no. 85011423).

The challenge virus, HSV-2 MS strain, was used as a crude sonicated stock. The DISC HSV-2 virus, grown in gH-expressing CR2 cells, was purified by centrifugation through 36% sucrose and resuspension in Eagle MEM. A mock virus stock was prepared from CR2 cells in the same way as the DISC HSV-2 virus stocks.

Plasmid Construction

First-stage recombination vector, pIMMB47+. Flanking sequences to either side of the HSV-2 gH gene were amplified from viral DNA by polymerase chain reaction (PCR) using Vent DNA polymerase (New England Biolabs, Beverly, MA). Figure 1 shows the regions cloned by PCR and the oligonucleotides used. Fragments amplified with oligonucleotides MB97-MB96 and MB57-MB58 were digested with the appropriate restriction enzymes and cloned into *EcoRI*-*HindIII*-cut pUC119. This vector was designated pIMMB45. A repaired *HindIII* fragment from pMV10 [7] containing the *lacZ* gene driven by the cytomegalovirus immediate early 1 (CMV IE) promoter was inserted at the *HpaI* site of pIMMB45. This vector was designated pIMMB47+.

Second-stage recombination vector, pIMMB46. DNA fragments amplified by PCR with oligonucleotides MB94-MB109 and MB57-MB108 were digested with the appropriate restriction enzymes and cloned into *EcoRI*-*HindIII*-cut pUC119. This vector was designated pIMMB46.

HSV-2 gH gene. The HSV-2 gH gene was constructed from two plasmids carrying adjacent cloned *Bam*HI fragments from the genome of HSV-2 strain 25766. The plasmids were pTW49, containing the *Bam*HI R fragment, and pTW54, containing the *Bam*HI S fragment, both cloned into the *Bam*HI site of pBR322. The plasmids were a gift of A. C. Minson (Division of Virology, Department of Pathology, University of Cambridge, UK). The 5' end of the HSV-2 gH gene was excised from pTW54 using *Kpn*I and *Bam*HI; the 3' end was excised from pTW49 using *Bam*HI and *Sal*I. The two fragments were cloned by a three-way ligation procedure into pUC119, which had been digested with *Sal*I and *Kpn*I. This plasmid, containing the entire HSV-2 gH gene, was designated pIMMB24.

gH expression vector. A 4.3-kb *Sst*I fragment encoding the HSV-1 (strain HFEM) gH gene and upstream HSV-1 gD promoter (-392 to +11) was excised from the plasmid pgDBrgH [4] and

cloned into pUC119 to produce plasmid pUC119gH. A *Not*I site was introduced by site-directed mutagenesis [8] 87 bp downstream of the gH stop codon, approximately half way between the gH and UL21 open-reading frames. The resulting plasmid, pIMC03, was used to generate a *Not*I-*Sst*I fragment, which was repaired and ligated into the eukaryotic expression vector pRc-CMV (Invitrogen, San Diego), which was predigested with *Not*I and *Nru*I to remove the CMV IE promoter. This plasmid was designated pIMC05. Plasmid pIMC08, containing the HSV-2 (strain 25766) gH gene, was constructed as follows. Plasmid pIMMB24 was digested with *Nco*I and *Bst*XI, and the partial fragment containing the central portion of the gH gene was purified from an agarose gel. The 5' end of the gene was reconstructed from two oligonucleotides, CE39 and CE40, which form a linking sequence bounded by *Hind*III and *Nco*I sites. The 3' end of the gene was reconstructed from two oligonucleotides, CE37 and CE38, which form a linking sequence bounded by *Bst*XI and *Not*I sites. The two oligonucleotide linkers and the purified *Nco*I-*Bst*XI gH fragment were cloned in a triple ligation into *Hind*III-*Not*I-digested pIMC05, thus replacing the HSV-1 gH gene by the HSV-2 gH gene. The resulting plasmid was designated pIMC08 and contains the HSV-2 gH gene under the transcriptional control of the virus-inducible gD promoter and bovine growth hormone poly A. It also contains the neomycin resistance gene for selection of G418-resistant stable cell lines.

Oligonucleotide primers. The oligonucleotide primers included MB57, TCAGTTAACGCTCTGTTCCCTTCCCTTC; MB58, CAGAATTCGAGCAGCTCCTCATGTTTCGAC; MB94, TCAAAGCTTATGGCTTCTCACGCCGGCCAA; MB96, TCAGTTAACGGACAGCATGGCCAGGTCAAG; MB97, TCGAAGCTTCAGGGAGTGGCGCAGC; MB108, TCAGAATTCGTTCCGGGAGCAGGCGTGGA; MB10, TCAGTTAACTGC-ACTAGTTTAAATTAATACGTATGCCGTCCGTCGCCGGCTGCCAGTC; CE37, GTGGAGACGCCGAATAATCCGCGAGC; CE38, GGCCGCTCGCGATTATTCGCGTCTCCACAAA; CE39, AGCTTAGTACTGACGAC; and CE40, CATGGTCGTCAG-TACTA.

Recombination Procedures

First stage. Sodium iodide-purified viral DNA (10 μ g) [9] and plasmid DNA (0.5 μ g) were transfected into gH-expressing F6 cells [4] using a standard calcium phosphate protocol [10] with an additional glycerol shock. When an extensive cytopathic effect was observed, cells were harvested and virus was released by sonication. Progeny virus was titrated in the presence of 300 μ g/

mL X-gal (5-bromo-4-chloro-3-indoxyl β -D-galacto-pyranoside). Blue plaques were picked and subjected to three rounds of plaque purification.

Second stage. Recombination was done as above, but virus progeny was passed three times in BHK gH⁺/tk⁻ cells, in the presence of 600 pM methotrexate to select for tk⁺ virus. White plaques were picked in the presence of X-gal and subjected to three rounds of plaque purification and a limiting-dilution cloning stage.

Vaccination Experiments

Animals. Female Dunkin-Hartley guinea-pigs (350-400 g) were obtained from Davis Hall (Darley Oaks Farms, Newchurch, UK).

Vaccination, challenge, and scoring. Animals were challenged ivag with 50 μ L of 10^{5.2} pfu of HSV-2 (MS strain) adsorbed onto gelatin sponge as described previously [3]. ivag vaccination was done using 50 μ L of inoculum adsorbed onto gelatin sponge. Subcutaneous (sc) vaccinations were administered using 0.1 mL of virus inocula diluted in PBS. Disease monitoring and scoring were also done as described [3]. In brief, animals were scored daily during the primary and recurrent disease stages for the number of lesions on the external vaginal area and the extent of erythema (using a scale of 1–5). All scoring was done using a blinded procedure. This was achieved by randomization of cages by an independent worker. When appropriate, vaginal swab samples were obtained from each animal after challenge. Swabs were placed in 1 mL of Dulbecco's MEM and frozen at –80°C for later plaque assay on Vero cells. Serum samples were obtained from a tarsal lateral vein 14 days after the final vaccination and 14 days after challenge.

Serum antibody analysis: neutralization test. Serial 2-fold dilutions of sera were placed in microtiter wells with 100 pfu of HSV-2 (MS strain) and incubated for 2 h at 37°C, and residual infectious virus was assayed by the addition of Vero cells as described previously [3]. Titers were expressed as the highest dilution of serum required to lyse 50% of the cells in the well. A standard control serum was included in all tests.

Serum antibody analysis: ELISA. Plates were coated with a crude extract from HSV-2-infected Vero cells (strain HG52), as described [3]. Negative control plates were coated with an extract of infected Vero cells in the same manner. All sera were tested using 2-fold serial dilutions, and bound antibody was detected by the addition of peroxidase-conjugated rabbit immunoglobulins to guinea pig immunoglobulins (Dako, Carpinteria, CA). All sera were titrated to end point, which was defined as the dilution giving an optical density of 0.1 at 492 nm, and results were normalized to a standard serum carried on all ELISA plates.

Statistical analysis. Clinical scores were compared using a Mann-Whitney nonparametric ranking test in the therapeutic vaccination experiments and Fisher's exact test in the prophylactic vaccination experiments. All serologic assay results and challenge virus titers were compared using Fisher's z test.

Results

Construction of Complementing Cell Lines for DISC HSV-2 Vaccine Production

Forrester et al. [4] reported the construction of a Vero cell line (F6) carrying the HSV-1 gH gene. Although this cell line

can support the growth of a gH-deleted HSV-2 (unpublished observations), we wished to create a new complementing cell line that would meet the stringent criteria desirable for manufacture of a human vaccine and that would minimize the risk of reacquisition of the gH gene by the DISC virus through homologous recombination. We therefore constructed a cell line carrying the HSV-2 gH gene, using an established stock of Vero cells approved for vaccine manufacture by the WHO, and a strategy designed to eliminate all overlap of sequence between the virus sequences present in the cell line and the disabled virus. Within the virus, the gH deletion extends from 3 bases upstream of the start of the gH gene to 96 bases downstream of the stop codon (a point halfway between the gH and UL21 genes); the gH sequences introduced in the CR2 cell line (expressing HSV-2 gH) start precisely at the ATG of the gH gene and end precisely at the stop codon.

The complementing cell lines were created by CaPO₄-mediated transfection using the gH-expressing vectors pIMC05 (HSV-1 gH) and pIMC08 (HSV-2 gH). These vectors carry the neomycin-selectable marker gene, which confers resistance to the antibiotic G418. A clonal cell line was isolated after selection in the presence of G418 and limiting-dilution cloning. Following expansion and freezing, cells were seeded into 24-well plates and tested for their ability to support the growth of gH-negative virus by infection with the gH-deleted HSV-1 SC16 Δ gH [4] at 0.1 pfu/cell. Virus plaques were observed 3 days after infection, confirming expression of the gH gene. Both HSV-1 and -2 gH-expressing cell lines, which supported the growth of SC16 Δ gH, were isolated. These were designated CR1 and CR2, respectively. The HSV-1 gH expression vector (pIMC05) was also transfected into BHK tk⁻ cells, using the same technique to make a gH⁺/tk⁻ cell line. This cell line was required for the second stage in construction of the gH-deleted virus.

Construction of gH-Deleted HSV-2

Our aim was to produce a virus with a simple deletion in the gH gene without any additional nonviral genes. However, since engineering of the genome can be done most efficiently using selectable marker genes, such as *lacZ*, whose expression can be recognized with a color-based assay (using the substrate X-gal), we designed a two-stage recombination procedure for deletion of the gH gene of HSV-2. During the first stage, the gH gene was replaced with the *lacZ* gene. The desired recombinant virus can be recognized because, in the presence of X-gal, it forms blue plaques against a background of white nonrecombinant plaques. The second stage of the procedure involved deleting the *lacZ* gene from the first-stage recombinant virus; this virus can be recognized as a white plaque against a background of blue plaques. Since it is generally difficult to identify single white plaques among a large number of blue plaques, we used a selection procedure using the *tk* gene to facilitate the second stage (figure 2). This took advantage of the fact that the *tk* gene lies adjacent to the gH gene on the HSV genome, and therefore, the first-stage vector could be

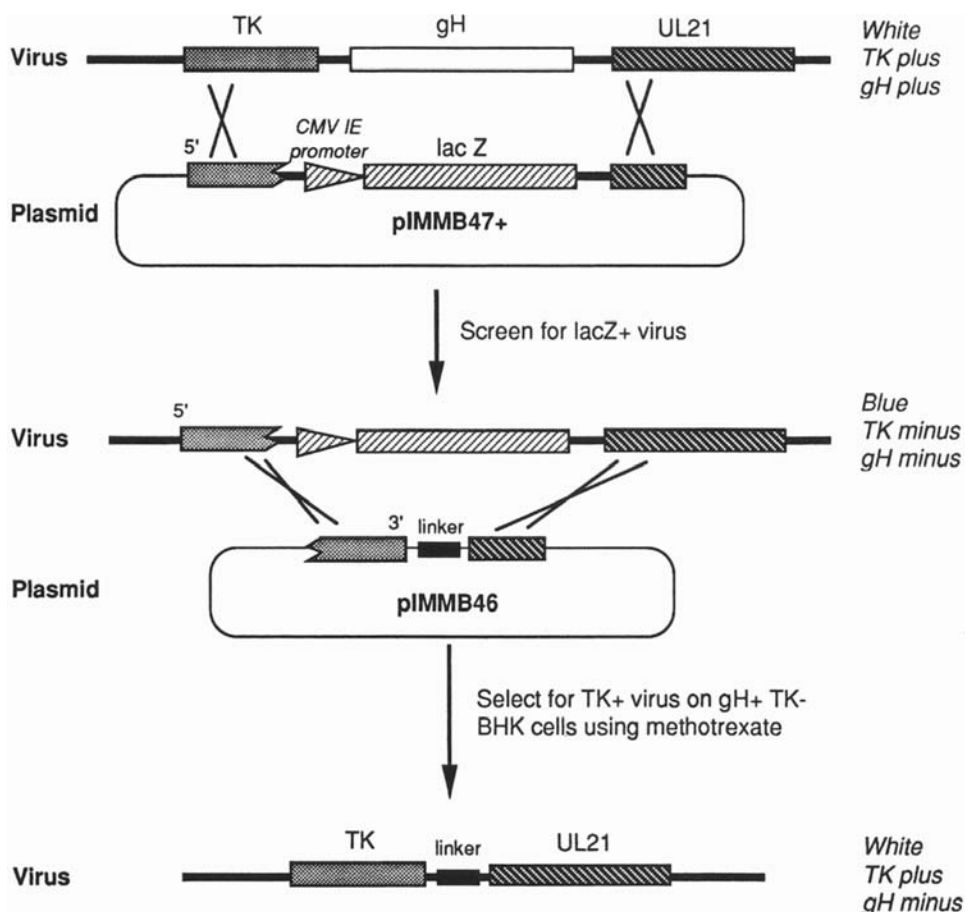


Figure 2. Deletion of HSV-2 gH gene using 2-stage, *tk* selection strategy. CMV IE, cytomegalovirus immediate early 1. TK, *tk*.

designed to delete half of the *tk* and gH genes while introducing the *lacZ* gene.

The second-stage recombination vector (pIMMB46) was designed to reconstruct a functional *tk* gene within the virus genome, while simultaneously deleting the *lacZ* gene. We took the opportunity to insert in its place a short (28-bp) noncoding linker sequence that could provide a convenient “tag” for recognition of the final recombinant virus by nucleic acid hybridization or by restriction enzyme digestion. Second-stage recombinants could thus be selected on the basis that they could grow on gH⁺/*tk*⁻ BHK cells in the presence of methotrexate; under these conditions, *tk*⁻ HSV cannot replicate. Correctly configured recombinants should also form white plaques. The methotrexate selection step was highly successful, increasing the percentage of white *tk*⁺ recombinants from ~0.1% to 50%. Several plaques were picked and purified by plaquing and limiting dilution in CR2 cells. These were characterized by Southern blot analysis (data not shown), and a virus isolate with the expected genetic structure was selected. The DISC HSV-2 virus was designated dH2A. Stocks of the virus were prepared in CR2 cells for vaccination studies.

Vaccination Experiments

The efficacy of the DISC HSV-2 vaccine was tested in the guinea pig model of HSV-2-induced disease [5]. In this model,

virus challenge leads to severe primary disease that lasts for 2 weeks and can be scored using several parameters, including the number of lesions and degree of erythema. Following resolution of primary disease, the infected animals experience recurrent episodes of disease for up to ~3 months. Thus the effect of vaccination on both primary disease and recurrent disease can be assessed.

Prophylactic protection against primary disease (experiment 1). Groups of 12 animals were vaccinated ivag or sc in the flank with either 10⁶ or 10⁷ pfu of DISC HSV-2 or a mock, virus-free preparation. Two identical vaccinations were administered 3 weeks apart. There were no clinical signs or symptoms observed after immunizations by either route. Three weeks after the second immunization, animals were challenged with 10^{5.2} pfu of the MS strain of HSV-2. Primary disease symptoms were scored from days 0 to 14 (figure 3). Animals given either dose of vaccine sc were completely protected from primary disease symptoms (*P* < .0001 compared with mock vaccination). ivag vaccination was less effective but still provided a high degree of protection, reducing the total number of lesions by 85% at a vaccination dose of 10⁶ pfu (*P* < .02) and by 92% at a vaccination dose of 10⁷ pfu (*P* < .001) compared with the mock-vaccinated controls. The difference between the two vaccination routes was statistically significant for the groups receiving 10⁶ pfu (*P* < .005) and for those given 10⁷

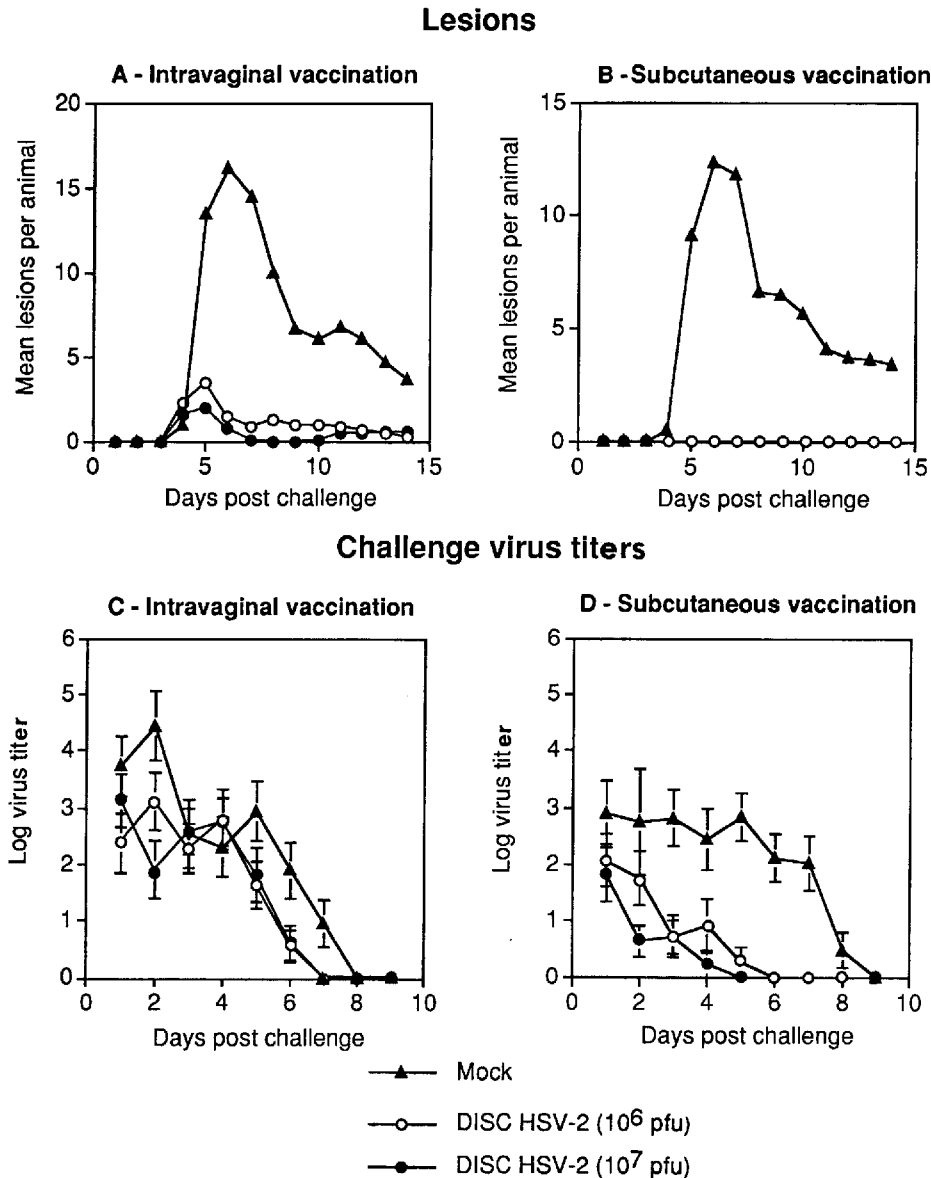


Figure 3. Effect of vaccination on HSV-2-induced primary disease. **A** and **B**, Mean no. of lesions/animal. **C** and **D**, Mean titers of HSV-2 challenge virus. Animals were vaccinated intravaginally (**A**, **C**) or subcutaneously (**B**, **D**) with 10⁶ or 10⁷ pfu of disabled infectious single cycle (DISC) HSV-2 or a mock preparation.

pfu ($P < .05$). There was no statistically significant difference between the 2 mock control groups.

Replication of challenge virus after prophylactic vaccination. It was of interest to assess the impact of vaccination with the DISC HSV-2 virus on challenge virus replication in the guinea pig vagina. Vaginal swab samples were therefore collected daily throughout the scoring period for experiment 1, and the amount of virus was titrated by plaque assay (figure 3). ivag vaccination resulted in a modest reduction (~10 fold) in challenge virus titers. A much greater reduction in challenge virus replication was observed in the animals vaccinated sc. In mock-vaccinated animals, titers between 10² and 10³ pfu/mL were detectable for the first 7 days after infection. In the vaccinated animals, there was a substantial reduction in virus titers, and the virus was cleared to below detectable levels by day 5. In the animals vaccinated twice with 10⁷ pfu, there was a 100-

fold reduction from days 2 to 7 ($P < .05$ on day 2 and $P < .001$ on days 3–7, compared with mock group), and the animals became clear of virus several days before the mock-vaccinated controls; on day 5, none of the vaccinated animals had detectable virus in the vagina, while 10 of 12 mock vaccinated animals still had virus present.

Antibody responses in vaccinated animals. The serum antibody response was measured at three time points. Blood samples were obtained from 6 animals from each group before the start of the experiment, 2 weeks after the second vaccination, and 2 weeks after challenge. Sera were analyzed by ELISA and a neutralization test, with similar results. Neutralizing antibody titers are shown in figure 4. Anti-HSV-2 antibody responses were present in both groups vaccinated sc with DISC HSV-2 ($P < .001$ compared with mock group). Subsequent challenge with wild type HSV-2 did not significantly increase antibody

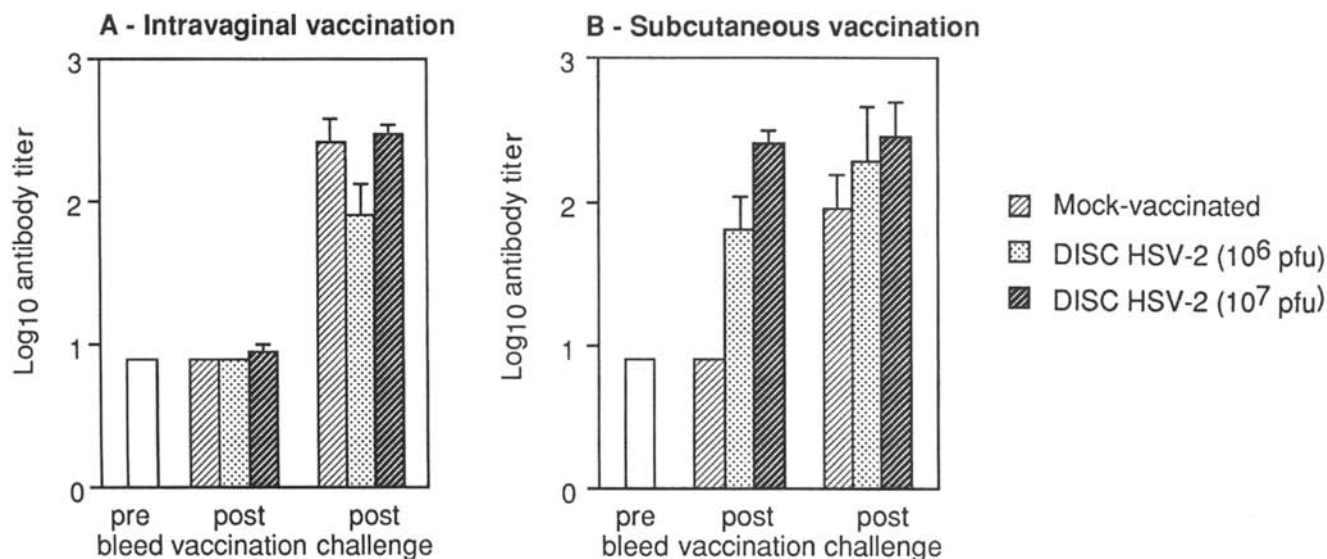


Figure 4. HSV-2 neutralizing antibody titers in guinea pig sera in intravaginally (A) or subcutaneously (B) vaccinated and challenged animals. Sera were obtained before vaccination (pre bleed), 2 weeks after second vaccination (post vaccination), and 2 weeks after challenge with wild type virus (post challenge). DISC, disabled infectious single cycle.

titers in these animals. In contrast, none of the groups vaccinated ivag showed a detectable antibody response, either by ELISA or neutralization test. Only after challenge with wild type HSV-2 did antibody titers become detectable. None of the tested sera showed any binding to control plates coated with uninfected Vero cells.

Protection against recurrent disease by prophylactic vaccination (experiment 2). A second experiment was set up to test whether the DISC HSV-2 vaccine could protect animals not only against the primary disease but also against recurrent disease. Groups of animals were vaccinated sc in the flank with 10^7 pfu of the DISC HSV-2 (24 animals) or with a mock, virus-free preparation (60 animals). Two identical vaccinations were administered 3 weeks apart, and 3 weeks later animals were challenged with $10^{5.2}$ pfu of the MS strain of HSV-2. Primary disease symptoms were scored from days 0 to 14. The DISC HSV-2 vaccine again provided complete protection against primary disease symptoms (figure 5A). Animals that recovered sufficiently from primary infection to be suitable for recurrency scoring (all of the vaccinated group and 36 of the mock-vaccinated group) were monitored from weeks 3 to 9 after challenge (figure 5B). Vaccination with DISC HSV-2 clearly protected extremely well against recurrent disease: There were no lesions in the vaccinated group for the first 5 weeks of scoring, after which 2 animals each had one lesion. This represents a 98.6% reduction in recurrent disease symptoms compared with the mock-vaccinated group ($P < .0001$).

Prophylactic protection by low vaccine doses (experiment 3). The previous experiment showed that good protection can be achieved with two vaccine doses (10^6 or 10^7 pfu) of DISC HSV-2. It was of interest to know whether lower doses of the vaccine or one vaccination could achieve comparable levels of

protection. To investigate this, the following experiment was set up. Groups of 12 animals were vaccinated sc in the flank twice (17 days apart) with 10^4 , 10^5 , or 10^6 pfu of DISC HSV-2. An additional group received one vaccination with 10^5 pfu. Two mock-vaccinated groups, vaccinated either once or twice, were also included. Animals were challenged 35 days after the first vaccination with $10^{5.2}$ pfu of the MS strain of HSV-2. As shown in figure 6, vaccination with DISC HSV-2 provided good protection from primary disease symptoms, even at very low doses. Two vaccinations with 10^4 pfu gave 90.7% reduction in disease symptoms ($P < .001$), while two doses of 10^5 pfu resulted in a 98.6% reduction ($P < .0001$). Two vaccinations with 10^6 pfu gave complete protection, as before. One dose of 10^5 pfu was also highly effective, providing 94.5% protection against primary disease ($P < .001$). Antibody titers were measured by ELISA and neutralization and showed an increase above those of mock-treated animals in a dose-dependent manner, with double vaccinations resulting in a higher antibody titer than a single vaccination (results not shown).

Long-term protection following prophylactic vaccination (experiment 4). An important measure of the efficacy of a prophylactic vaccine is the durability of the protection. To investigate this, we set up an experiment in which guinea pigs were vaccinated sc with 1, 2, or 3 doses of DISC HSV-2 (10^7 pfu/dose) and challenged after 6 months. An untreated group of animals was used as a control. Table 1 shows the results of this study together with data from experiments 1 and 3 for comparison. Both the double- and triple-dose vaccinations provided 100% protection from disease symptoms, and in the case of the single-dose vaccination, disease symptoms were reduced by $>99\%$ ($P < .0001$ in all cases). Shedding of the challenge virus was also considerably reduced, particularly for the dou-

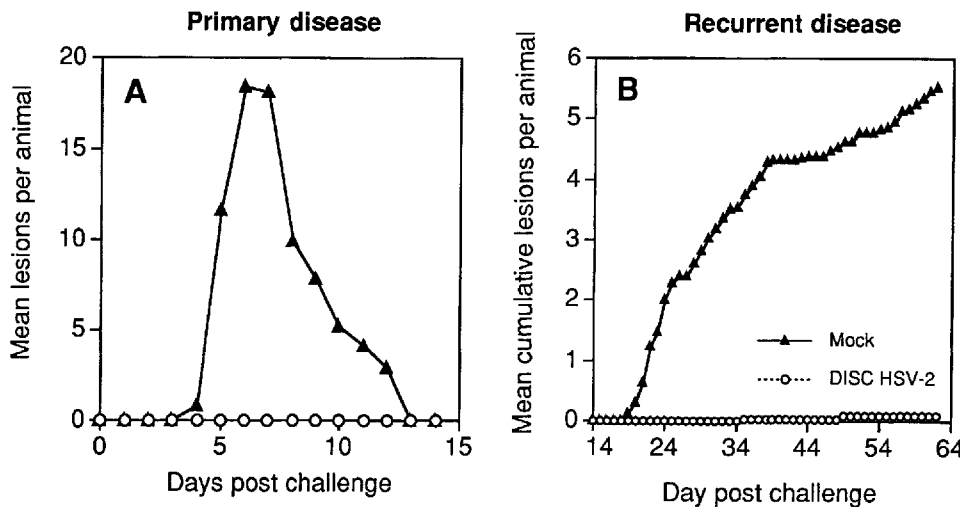


Figure 5. Primary and recurrent disease after prophylactic vaccination. Mean no. of lesions (A, primary disease) and mean cumulative lesions (B, recurrent disease) per animal in groups of guinea pigs vaccinated subcutaneously with 10^7 pfu of disabled infectious single cycle (DISC) HSV-2. Animals were vaccinated 2 times 3 weeks apart, challenged 3 weeks later, and monitored from weeks 1–9.

ble- and triple-vaccination regimes. Virus in the vaccinated groups was cleared by day 6, 3 days earlier than in the mock-vaccinated group (data not shown). In addition, the virus titers were dramatically reduced in the double- and triple-vaccinated groups. This effect can be clearly seen in the virus titers on day 2 after challenge (table 1). Antibody responses in all vaccinated groups were measured by ELISA and a neutralization test and showed a marked increase over mock-vaccinated animals. Antibody levels declined only marginally over the 6 months following vaccination (results not shown).

Protection against recurrent disease by therapeutic vaccination. We previously demonstrated a trend toward reduced frequency of symptom recurrence in guinea pigs vaccinated

with DISC HSV-1 after the onset of HSV-2–induced vaginal disease [3]. It was therefore of interest to test the DISC HSV-2 vaccine using this model. In the first experiment, groups of guinea pigs (13 or 14/group) were vaccinated ivag with 10^7 pfu of DISC HSV-1, DISC HSV-2, or a mock vaccine preparation on days 28, 42, and 56 after infection with $10^{5.8}$ pfu of wild type HSV-2. Animals that had sufficiently recovered from the primary disease phase were scored for the presence of recurrent lesions for 7 weeks after the first vaccination. Figure 7A shows that the mean cumulative lesion score for the group vaccinated with DISC HSV-2 was ~50% less than that for the mock group ($P = .074$). Animals vaccinated with DISC HSV-1 showed a reduction of 22% ($P = .191$), similar to that observed previously [3]. In a second, larger experiment, 2 groups of animals were sc vaccinated therapeutically with 10^7 pfu of DISC HSV-2 (31 animals) or mock vaccine (38 animals) on days 18 and 35 after infection with wild type HSV-2. For this experiment, equal numbers of animals considered to have experienced severe, medium, or mild disease during the primary phase of disease were assigned to the 2 vaccination groups; only animals that had recovered sufficiently from primary disease were included for scoring, as before. In this case, vaccination resulted in a 36% reduction in the cumulative lesion score compared with the control group ($P = .02$) (figure 7B).

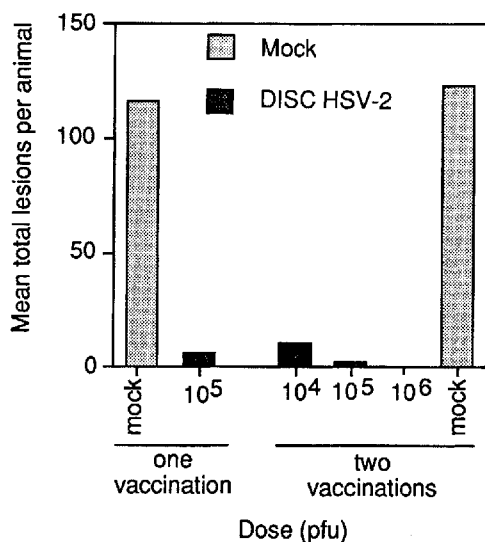


Figure 6. Protection from range of doses of disabled infectious single-cycle (DISC) HSV-2. Mean total lesions/animal, calculated as sum of mean daily lesions during acute phase of disease (days 1–14 after challenge). Animals were vaccinated 1 or 2 times with various doses of DISC HSV-2 or mock preparation.

Discussion

Both live virus and subunit approaches to vaccination against HSV have been investigated in recent years (reviewed in [11]). Subunit vaccines based on glycoproteins D and B have proved effective in animal models both prophylactically and therapeutically [12, 13] and have recently entered human clinical trials [14]. However, it remains uncertain whether such subunit vaccines will provide the efficacy and longevity of response required to protect against human HSV infection in the field.

Genetically modified live attenuated viruses have also been shown to protect well against HSV-induced disease in animal

Table 1. Summary of primary disease symptoms in prophylactic protection experiments.

Experiment	Vaccination regime		Primary disease symptoms		Log ₁₀ virus titers on day 2 after challenge (GMT ± SE)
	Dose (pfu)	No. of doses	Total lesions (mean)	% of control	
1	Mock	2	67.5	100	2.8 ± 0.9
	10 ⁶	2	0	0	1.8 ± 0.5
	10 ⁷	2	0	0	0.6 ± 0.3
3, low dose	Mock	1	116	100	4.1 ± 0.5
	10 ⁵	1	6.1	5.2	2.4 ± 0.4
	Mock	2	123	100	4.2 ± 0.5
	10 ⁴	2	10.5	8.5	3.0 ± 0.5
	10 ⁵	2	1.7	1.4	1.9 ± 0.4
	10 ⁶	2	0	0	1.0 ± 0.4
4, long term	None		105	100	3.8 ± 0.3
	10 ⁷	1	0.2	0.2	3.1 ± 0.3
	10 ⁷	2	0	0	0.6 ± 0.3
	10 ⁷	3	0	0	0.7 ± 0.3

NOTE. GMT, gross mean titer.

models [15]. However, although live attenuated virus vaccines have proved extremely effective in controlling many diseases, due to their capacity for stimulation of broad-based, long-lasting immunity [11], the use of replication-competent viruses inevitably raises safety concerns, particularly in immunocompromised persons, in whom even a substantially attenuated virus may still produce disease. A particular problem associated with herpesviruses is the risk of disease arising from reactivation of latent vaccine virus [16]. The DISC virus vaccine approach, which relies on the use of a virus inactivated by the deletion of an essential gene, offers the immunologic advantages normally associated with a live attenuated virus vaccine

but without the risk of disease resulting from vaccine virus spread.

Previous studies from this laboratory [2, 3] and others [17, 18] have shown that herpesviruses with deletions in essential genes can provide effective protection in animal models of disease. Using a gH-deleted DISC HSV-1 virus, we demonstrated substantial, though not total, protection against primary and recurrent disease in the guinea pig model of genital HSV-2 infection [3]. The results presented here indicate that enhanced protection can be provided in this animal model using a gH-deleted DISC HSV-2 vaccine. This virus was designed specifically for use in humans, in that it was based on an already

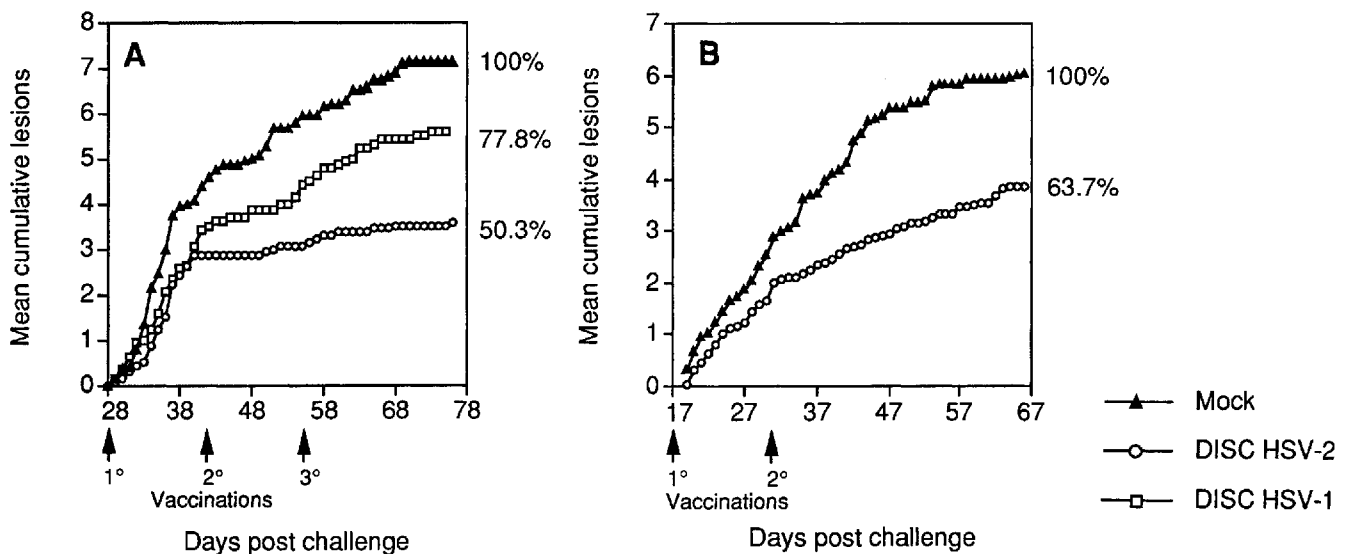


Figure 7. Mean cumulative lesions during recurrent phase of disease for guinea pigs vaccinated 3 times intravaginally with 10⁷ pfu of disabled infectious single cycle (DISC) HSV-1, DISC HSV-2, or mock vaccine on days 28, 42, and 56 after challenge with 10^{5.8} pfu HSV-2 (A) or 2 times subcutaneously with 10⁷ pfu dH2A 18 and 32 days after challenge with 10^{5.2} pfu HSV-2 (B).

attenuated parent HSV-2 virus, contains no extraneous marker genes, and was constructed in a way that minimized the risk of reacquisition of the gH gene during growth in the producer cell. Using an assay capable of detecting replication-competent virus in DISC HSV-2 preparations, we could not detect any replication-competent virus in 10^9 pfu of DISC HSV-2 produced from the complementing cell (Boursnell MEG, Blakeley D; unpublished observations).

sc vaccination with doses of $\geq 10^6$ pfu of DISC HSV-2 vaccine protected guinea pigs completely against primary disease symptoms. ivag vaccination with equivalent doses of the vaccine was less effective, but substantial protection was still observed. The reason for the improved level of protection afforded by the sc route is not clear, but an obvious possibility is that the antigen load is simply higher. Only a fraction of the particles in a virus preparation are infectious, so when a DISC virus preparation is administered directly to a mucosal surface, without trauma, a relatively small proportion of the particles may be available to trigger an immune response. This proportion may be much larger after sc inoculation of the vaccine. An alternative explanation could be that sc delivery allows more effective access to "professional" antigen-presenting cells and hence a more vigorous immune response. A third possibility is that sc vaccination gives access to a different subset of immune cells, leading to a qualitative difference in the immune response and thus to improved protection.

In any event, it was clear that sc vaccination induced much higher serum neutralizing antibody titers than did ivag vaccination. Indeed, the overall titers generated by both the 10^6 and 10^7 pfu sc doses were similar to those induced by challenge virus infection in mock-vaccinated animals. These results, together with the observation that substantial protection could be induced by as little as 10^4 pfu delivered by this route, indicate that the immune system is remarkably sensitive to the DISC HSV-2 vaccine and suggests more broadly that multicycle virus replication may not be required for the efficacy of attenuated virus vaccines. This general finding is supported by studies from other laboratories using nonreplicating poxvirus-based vaccines [19].

The immunologic mechanisms responsible for the potency of the DISC HSV-2 vaccine, however, remain unclear. ivag vaccination did not induce a detectable serum antibody response yet still reduced disease substantially, suggesting that cell-mediated immunity may be an important component. Because DISC virus can undergo a cycle of replication in the host, presentation of virus antigens to T lymphocytes, in the form of processed peptides complexed with major histocompatibility complex classes I and II, should occur in much the same way as with wild type virus. Mice vaccinated with DISC HSV-1 mount effective delayed-type hypersensitivity and cytotoxic T lymphocyte responses against virus antigens, and it appears that these T cell-based effector mechanisms are the basis for the protective immunity observed in the HSV-1 mouse ear challenge model [3] (McLean CS, unpublished data). It is also possible, however, that local antibody responses induced by

direct ivag vaccination, in the form of secreted IgA or IgG, may have been responsible for part or all of the protective effect. Direct analysis of antibody titers in vaginal secretions should help to resolve this question.

The absence of a detectable antibody response after ivag vaccination with DISC HSV-2 contrasts with our earlier findings using a DISC HSV-1 vaccine [3]. Differences in humoral responses between HSV types 1 and 2 have been reported in studies of mice [20], in which 3 different strains of HSV-2 induced much lower humoral responses than did 6 strains of HSV-1.

sc vaccination with the DISC virus provided not only complete protection against primary disease symptoms but also virtually complete protection against recurrent disease. This could be because the amount of virus reaching the innervating ganglia was greatly reduced or abolished by the initial protective effect of the vaccine against challenge virus replication in the vagina. Thus, vaccination may have prevented or reduced the establishment of latency. Alternatively, the immune response stimulated by the vaccine may have been capable of controlling reactivated infection before the symptoms appeared. Both these mechanisms play a role in the observed protection against recurrent disease. It is clear that vaccination with the DISC virus substantially reduced challenge virus replication in the vagina. In addition, in the mouse ear model of HSV-2 infection, we found that vaccination with high doses of DISC HSV-2 blocked the establishment of latency in the cervical ganglia by challenge virus, as judged by explant in vitro reactivation studies (data not shown). It is also clear from our data, however, that immune responses generated by the DISC virus vaccination are sufficiently long lasting that they could control recurrences. Direct analysis of the ganglia innervating the guinea pig vagina for the presence of latent virus will be necessary to resolve this question.

There have been several reports of successful therapeutic vaccination against HSV-2-induced recurrent disease in guinea pigs using HSV-2 antigen preparations [13, 21–23]. In this study, we confirmed our previous observation that ivag vaccination with DISC HSV-1 during the recurrent stage of disease resulted in a modest reduction in recurrence frequency. As might have been expected, therapeutic ivag vaccination with the homologous DISC HSV-2 led to a much larger reduction in the cumulative lesion score (50%), though the numbers of animals involved (13 or 14/group) rendered this result of marginal significance ($P = .074$); however, the data did reveal a more significant reduction ($P = .037$) in the number of days on which disease was observed (not shown). The size of the experiment did not allow us to see a significant difference in the number of lesions between the DISC HSV-1 treatment and the DISC HSV-2 treatment ($P = .34$). In a second therapeutic experiment using a much larger number of animals and an sc route of vaccination, we observed a 36% reduction in recurrent lesions that was highly significant ($P = .02$). The immunologic mechanisms responsible for the observed therapeutic protection are not clear. An increase in anti-HSV-2 antibody titer was evident following vaccination with DISC HSV-2

(results not shown), but previous studies in other laboratories have not revealed a reproducible correlation between antibody titers and the pattern of recurrent disease [21]. Elucidation of the precise immune mechanisms responsible for the control of recurrent HSV infection in the guinea pig model has been hampered by a shortage of useful immunologic reagents and by the outbred nature of the animals used.

Our studies highlighted several aspects of the DISC HSV-2 virus vaccine that make it an attractive candidate for human use. One is its potency. The lowest dose of vaccine used (10^4 pfu), which provided almost complete protection against primary disease, would represent only about 4 ng of virus, calculated on the basis of a particle-to-infectivity ratio of about 200. It is by no means certain that such low doses would be as effective in humans; nevertheless, the data are most encouraging. A second important element is the vaccine's ability to reduce substantially replication of challenge virus. From an epidemiologic perspective, it is considered highly desirable that an HSV vaccine should not only be able to prevent disease but also reduce virus shedding, as this should lead, over time, to a general reduction in disease prevalence even in unvaccinated persons. Third, the vaccine induced a durable immune response, resulting in complete protection against challenge even after 6 months. Long-lasting immunity will clearly be a crucial ingredient of a successful vaccine against HSV. The vaccine also provided strong and durable immunity to challenge even after just one dose. From a practical standpoint, the ability to induce effective protection with one vaccination would represent a considerable advantage. Finally, therapeutic vaccination reduced substantially the subsequent development of HSV-2-induced lesions, suggesting that DISC HSV-2 virus could be used in persons already infected with the virus to control recurrence. We therefore conclude that the DISC HSV-2 has considerable potential as a vaccine against HSV-induced disease in humans.

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