Detection of Asymptomatic Initial Herpes Simplex Virus (HSV) Infections in Animals Immunized with Subunit HSV Glycoprotein Vaccines

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The evaluation of herpes simplex virus (HSV) vaccine efficacy will require methods to detect asymptomatic acquisition of HSV infection and to assess the risk of recurrences in these patients. HSV-infected vaccinees should develop antibodies to HSV polypeptides not included in subunit vaccines. Sera from 57 HSV glycoprotein-vaccinated guinea pigs that had asymptomatic initial infections after genital HSV type 2 challenge were collected after vaccination but before HSV challenge and again 30 days after HSV challenge to determine the antibody response to HSV polypeptides. Antibodies to nonvaccine HSV polypeptides were detected in sera collected after viral challenge from 32 (56%) of these 57 animals. Twenty-six (81%) of the 32 animals with detectable antibody developed recurrent disease; however, recurrences also developed in 11 (44%) of the remaining 25 that did not show detectable antibody to nonvaccine HSV polypeptides. The magnitude of vaginal viral shedding during the initial disease period following challenge was significantly lower in animals that did not develop antibody to nonvaccine polypeptides compared with those that did develop antibody (area under the viral shedding curve, 5.2 ± 3.2 versus 18.1 ± 5.8 ; $P < 0.0001$). These data suggest that detection of antibody to nonvaccine HSV polypeptides will identify the majority (70%) of initially asymptomatic vaccinees that develop recurrent disease but that latency can be established even with markedly reduced levels of viral replication that did not induce a detectable antibody response.

Several subunit herpes simplex virus (HSV) glycoprotein vaccines have shown efficacy in animal studies (4, 5, 12, 14, 21), and human clinical trials have been initiated (16). Vaccine efficacy has classically been evaluated by the ability to prevent acute symptomatic disease. However, an effective HSV vaccine should not only modify acute infection, but also prevent or reduce the establishment of latency to prevent or reduce subsequent recurrent disease. Latent infections can be established in asymptomatically infected individuals or in those with atypical or unrecognized symptoms (11, 13, 17). Initially asymptomatic patients can develop symptomatic recurrent diseases (6) and serve as a source of infection for their sexual partners (15, 19) or, in the case of pregnant women, their newborn infants (18).

In our initial animal trials, candidate HSV glycoprotein vaccines reduced the severity of initial disease, many animals developing initially asymptomatic infection. Vaccination also reduced but did not eliminate recurrent disease even in initially asymptomatic animals (21; L. R. Stanberry et al., *J. Gen. Virol.*, in press). Therefore, it appears that evaluation of HSV vaccine efficacy will require methods to detect asymptomatic acquisition of HSV infection to allow assessment of the risk of recurrences.

Infected vaccinees should produce antibodies to HSV polypeptides not included in the subunit vaccine. We have recently reported that animals immunized with HSV glycoproteins that developed symptomatic infection after HSV type 2 (HSV-2) vaginal inoculation reliably produced antibody to the nonglycosylated ICP-35 group of HSV proteins and that this served as a useful prognostic indicator to identify animals that developed more severe recurrent dis-

ease (9). Rises in neither neutralizing nor enzyme-linked immunosorbent assay titers identified even symptomatic animals. The sensitivity for identifying initially asymptomatic animals that developed recurrent disease, using detection of ICP-35 antibody, was only 47%, however. In this report, we have extended our evaluation from the 35 animals that had asymptomatic initial infections after genital HSV-2 challenge reported previously (9) and have used other procedures to identify antibody to HSV polypeptides (2, 3) as a means to predict animals that develop recurrent disease.

MATERIALS AND METHODS

HSV vaccines. The subunit glycoprotein vaccines and their administration have been described previously (20-22; Stanberry et al., in press). This report includes animals that received HSV-1 glycoprotein B prepared by recombinant DNA technology in Chinese hamster ovary cells (21), glycoprotein D prepared in either yeast (21) or Chinese hamster ovary cells (20, 22), and lectin-purified HSV-1 or HSV-2 total glycoproteins (21).

Vaccination and inoculation. Young adult, female, Hartley guinea pigs (Charles River Breeding Laboratories, Wilmington, Mass.) were immunized 60 and again 30 days before intravaginal HSV-2 inoculation. Animals received the vaccine with either Freund complete adjuvant or alum, and both the subcutaneous and hind-footpad routes were used for immunization (21; Stanberry et al., in press). Table 1 summarizes the number of animals immunized with each vaccine protocol. Following immunization, animals were inoculated intravaginally with $5.7 \log_{10}$ PFU of HSV-2 strain MS (ATCC VR-540) (21). All animals included in this analysis had HSV-2 isolated from the vagina at least 24 h after viral challenge but did not develop genital lesions during the initial

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TABLE 1. Immunization of guinea pigs with subunit HSV vaccines

Vaccine ^a	Adjuvant ^b	Route	Total no. vaccinated	No. initially asymptomatic ^c
gD	CFA	Footpad	8	6
	Alum	Subcutaneous	8	5
gB	CFA	Footpad	7	6
	Alum	Subcutaneous	6	2
gB + gD	CFA	Footpad	8	4
	Alum	Subcutaneous	8	2 ^d
gp 1	CFA	Footpad	7	6
	None	Footpad	7	3 ^d
gp 2	CFA	Footpad	13	13
	None	Footpad	10	4
	Alum	Footpad	8	6
	Alum	Subcutaneous	6	2

^a The first glycoprotein D (gD) vaccine listed was derived from *Saccharomyces cerevisiae* containing a partial gD gene. The remaining glycoprotein B (gB) or gD vaccines were derived from stable Chinese hamster ovary cell lines that constitutively secrete gB or gD. gp 1, Lectin-purified glycoprotein from HSV-1 strain Patton; gp 2, lectin-purified glycoprotein from HSV-2 strain 333.

^b CFA, Complete Freund adjuvant; alum, aluminum hydroxide.

^c These animals did not develop lesions during the acute period.

^d Sera from two animals, one receiving gB plus gD subcutaneously and one receiving HSV-1 glycoproteins without adjuvant, were not available for study.

14-day interval after inoculation, the period when unvaccinated animals develop and recover from their initial lesions (7, 23).

Sera were collected 30 days after the second immunization, immediately prior to intravaginal HSV-2 inoculation, and again 30 days after viral challenge. We have shown previously (7) that HSV-2 antibody response peaks in HSV-2-infected guinea pigs at about 30 days after viral inoculation and that antibody to HSV polypeptide ICP-35 is present in all animals at this time. Sera were stored frozen at -70°C until use. Of the 59 serum pairs from initially asymptomatic animals, 57 were available for analysis by immunoblotting.

Evaluation of initial and recurrent disease. All animals were evaluated daily from days 1 through 60 for genital lesions (7, 23), recurrent disease occurring from days 15 to 60 (24). The titer of virus in the vaginal vault was determined from daily vaginal swab samples obtained on days 1 to 7 (18). Clinical recurrences are reported as both lesion days (defined as total number of days with any lesion) and recurrent episodes (defined as a new lesion appearing after a lesion-free day) (8).

Immunoblot assay. The immunoblot procedures were performed with ^{125}I -labeled protein A (procedure A) (9) or peroxidase-conjugated anti-guinea pig sera (procedure B) (3) as described previously. There were insufficient sera to perform procedure B on three animals. Briefly, procedure A utilized HSV-2 strain MS- and HSV-1 strain McIntyre-infected HEp-2 cells as antigen, while procedure B utilized HSV-2 strain 333- and HSV-1 strain E115-infected human diploid fibroblast cells as antigen. Infected-cell polypeptides were then solubilized and boiled prior to electrophoresis in sodium dodecyl sulfate-polyacrylamide gels, cross-linked with *N,N*-diallyltartardiamide in procedure A and bisacrylamide in procedure B. Proteins were then transferred to nitrocellulose, and nonspecific binding was blocked. Sera diluted 1:50 (procedure A) or 1:100 (procedure B) were then incubated overnight. After washing, blots were incubated

with either ^{125}I -labeled protein A or peroxidase-labeled goat anti-guinea pig immunoglobulin G (IgG; Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Electroradiographic images of ^{125}I blots were made on Kodak XAR-5 film, while peroxidase-labeled blots were developed with a solution containing 4-chloro-1-naphthol. Procedures A and B were performed in separate laboratories on blind, coded, pre- and postchallenge serum pairs.

Radioimmunoprecipitation-polyacrylamide gel electrophoresis. Sera were reacted with [^{35}S]methionine-labeled, HSV-2-infected cell proteins as described previously (2). Briefly, HSV-2 strain 333-infected cells were radiolabeled for 5 to 16 h postinfection and then detergent solubilized and diluted 1:2 in Tris-buffered saline. Sera were diluted 1:200 in the radio-labeled protein mixture and incubated overnight at 4°C . Immune complexes were collected on protein A-coated Sepharose (Sigma Chemical Co., St. Louis, Mo.) and then subjected to electrophoresis and fluorography as described before (1).

Statistics. Comparison of means was performed by utilizing a two-tailed Student's *t* test. Comparisons of frequency utilized the Fisher exact test.

RESULTS

Guinea pig vaccination. Table 1 summarizes the immunization regimens for the animals evaluated in this study. The protective efficacy against acute clinical disease for the vaccines evaluated ranged from 25 to 100% depending on the adjuvant, route of administration, and composition of vaccine. Only animals with virus isolated at 24 h that did not develop acute genital lesions were included in the studies reported here. This was done because intravaginal inoculation of guinea pigs does not result in 100% "take" even in control animals. To differentiate animals who were unsuccessfully inoculated (due to the inherent difficulties in the procedure) from the effect of vaccine or antiviral agents, we have used this definition (7, 10, 21). It is possible that we have therefore underestimated the effect of vaccination by eliminating these animals. Of 21 vaccinated animals with initially symptomatic disease, 20 developed antibody to nonvaccine polypeptides after HSV-2 inoculation (9), while all 20 infected control animals (no prior vaccination) evaluated had antibody to nonglycosylated HSV-2 polypeptides 30 days after HSV-2 inoculation (data not shown).

Serologic investigation of initially asymptomatic animals. A total of 57 serum pairs from vaccinated animals (before and 30 days after HSV-2 inoculation) were evaluated by Western blot (immunoblot), using ^{125}I -labeled protein A for identification of antibodies to HSV polypeptides. Antibody to HSV polypeptides not identified in the postvaccine prechallenge sera (nonvaccine polypeptides) was identified in 29 sera obtained after intravaginal HSV-2 challenge. Examples of the antibody response to HSV-2 polypeptides before and after HSV-2 intravaginal inoculation in immunized animals that did and did not develop detectable antibody to nonvaccine proteins are shown in Fig. 1. Although antibody to several nonvaccine HSV polypeptides was identified, antibody to the ICP-35 group of polypeptides (9, 10) was the most easily detected and was identified in all 29 animals that developed antibody to nonvaccine HSV polypeptides.

Fifty-four serum pairs were also evaluated by an immunoblotting technique that uses peroxidase-conjugated anti-guinea pig IgG to identify HSV antibody. Examples of the antibody response to both HSV-1 and HSV-2 polypeptides are shown in Fig. 2. The initial response was stronger to the

FIG. 1. Immunoblot analysis using ^{125}I -labeled protein A to identify antibody to HSV-2 polypeptides. Immunoblots of sera obtained after HSV glycoprotein vaccination but before HSV-2 vaginal inoculation are labeled A, and those obtained from the same animal 30 days after HSV-2 inoculation are labeled B. Antibody to nonvaccine polypeptides was identified in the post-viral challenge sera represented in lanes 2, 6, and 10 but not in lanes 4, 8, and 12. gB, glycoprotein B; gD, glycoprotein D; CFA, complete Freund adjuvant; alum, aluminum hydroxide.

HSV-1 polypeptides included in the vaccine than in the corresponding HSV-2 polypeptides. Following HSV-2 challenge, however, the major new antibodies detected reacted against HSV-2 polypeptides, most prominently ICP-35. Similar results were found with ^{125}I -labeled protein A, although the difference between HSV-1 and HSV-2 reactivity was not as marked.

Comparison of the two immunoblotting techniques demonstrated a high level of correlation (Table 2). Only two sera

TABLE 2. Comparison of immunoblotting procedures to identify antibody to nonvaccine HSV polypeptides following HSV challenge^a

Procedure A ^b	Procedure B (no. of animals) ^b		
	Nonvaccine polypeptide antibody detected	Nonvaccine polypeptide antibody not detected	Indeterminate
Nonvaccine polypeptide antibody detected	27	0	1
Nonvaccine polypeptide antibody not detected	2	18	6

^a A total of 54 sera were available for assay by each method. Sera were assayed without knowledge of the clinical or virologic results or the results of the other assay method. One serum that reacted to nonvaccine polypeptides and two that did not, using ^{125}I , were not available for analysis with peroxidase-conjugated anti-guinea pig IgG.

^b Immunoblotting procedure A used ^{125}I -labeled protein A, while procedure B used peroxidase-conjugated anti-guinea pig IgG to detect bound antibodies, as described in the text.

gave discrepant results, both identified as containing antibody to nonvaccine polypeptides only when reacted in the system that used peroxidase-conjugated anti-guinea pig IgG. Seven sera gave an indeterminate result in this assay, only one of which was reactive to nonvaccine polypeptides when probed with ^{125}I -labeled protein A. A result was categorized as indeterminate when a weak reaction to only a single nonvaccine HSV polypeptide (usually the 40-kilodalton polypeptide of the ICP-35 group) was observed. By combining the results from both assays, 31 animals developed antibody to nonvaccine polypeptides, while 7 additional animals had some evidence of antibody production.

We next used radioimmunoprecipitation to examine the antibody response in four serum pairs from animals with recurrences that were not identified by either immunoblotting assay and two sera that had given discrepant results in the two immunoblot assays. Antibody to nonvaccine poly-

FIG. 2. Immunoblot analysis using peroxidase-conjugated anti-guinea pig IgG to identify antibody to HSV polypeptides. Immunoblots of sera obtained after HSV glycoprotein vaccination but before HSV-2 vaginal inoculation are labeled A, and those obtained from the same animal 30 days after HSV-2 inoculation are labeled B. Immunoblots labeled I contain HSV-1 polypeptides, and those labeled II contain HSV-2 polypeptides. Antibody to nonvaccine HSV-2 polypeptides was identified in the post-viral challenge sera represented in lanes 4, 8, and 12 but not in lane 16. gB, glycoprotein B; gD, glycoprotein D; CFA, complete Freund adjuvant; alum, aluminum hydroxide.

TABLE 3. Development of detectable antibody to nonvaccine HSV polypeptides after HSV-2 inoculation among initially asymptomatic animals

Antibody detection	Vaginal viral shedding (area under the curve of mean log ₁₀ virus titer per day) ^a	Recurrent episodes ^b	Recurrent lesion days ^b	No. (%) of animals with recurrence
Antibody detected (n = 32) ^c	18.1 ± 5.8	3.0 ± 2.6	5.2 ± 5.0	26 (81)
Antibody not detected (n = 25)	5.2 ± 3.2	1.2 ± 1.9	1.4 ± 2.0	11 (44)
<i>P</i> ^d	<0.0001	<0.005	<0.001	<0.005

^a Calculated through day 7; mean ± standard deviation.^b The definitions of recurrent episode and lesion days are given in Materials and Methods.^c Antibody to nonvaccine HSV polypeptides was detected by evaluating sera obtained from vaccinated animals 30 days after intravaginal HSV-2 inoculation. Animals with antibody detected by any procedure were included in this group.^d Comparison of means was performed by utilizing a two-tailed Student's *t* test. Comparison of the frequency of animals with recurrences utilized the Fisher exact test.

peptides was identified in the postchallenge sera from one of the four sera not identified by immunoblotting and in both sera that had given discrepant results. The sera that reacted to nonvaccine polypeptides by only this assay were obtained from one of two animals with an area under the log viral titer per day curve of >12 (see below) that had not developed antibody to nonvaccine polypeptides by Western blot analysis. With the results for all three assays, a total of 32 of the 59 animals had detectable antibody to nonvaccine HSV polypeptides.

The magnitude of acute vaginal viral replication appeared to determine which animals responded to nonvaccine HSV polypeptides. Antibody to nonvaccine polypeptides was detected in 27 of 28 animals with an area under the log viral shedding curve of ≥12 (including the animal positive only by immunoprecipitation), while only 4 of 29 with lesser virus-shedding values developed detectable antibody. Vaginal viral replication was significantly greater (*P* < 0.0001) in animals that developed antibody than in those that did not (Table 3).

Recurrent disease in initially asymptomatic animals. If all vaccinated animals that developed a latent HSV infection produced detectable antibody to nonvaccine polypeptides after viral challenge, then recurrences should only have developed in animals with antibody to nonvaccine polypeptides. We found that recurrences developed in 26 of 32 (81%) animals that produced antibodies to nonvaccine HSV polypeptides but also in 11 of 25 (44%) animals that did not develop such antibodies (*P* < 0.005; Table 2). Both the number of recurrent episodes (*P* < 0.005) and the number of recurrent lesion days (*P* < 0.001) were significantly greater in animals with detectable antibodies to nonvaccine HSV polypeptides (Table 2).

All of the animals that developed recurrent disease in the absence of detectable antibodies to nonvaccine polypeptides had areas under the log viral shedding curve of <12. The magnitude of vaginal viral replication was not significantly different in these animals compared with animals that developed neither recurrences nor antibody to nonvaccine poly-

TABLE 4. Magnitude of initial local viral replication related to subsequent recurrent disease and antibody response to nonvaccine HSV polypeptides

Antibody	Area under the log viral shedding curve (mean ± SD) ^a	
	Recurrences detected	No recurrences detected
+ ^b	17.2 ± 5.6	22.1 ± 5.2
—	6.3 ± 2.6	4.4 ± 3.5

^a Calculated as mean log₁₀ titer of virus per day through day 7.^b +, Antibody to nonvaccine polypeptide detected.

peptides (area under the log viral shedding curve, 6.3 ± 2.6 versus 4.4 ± 3.5; *P* = 0.15) (Table 4). Similarly, in animals that developed detectable antibody to nonvaccine polypeptides, the magnitude of vaginal viral shedding was similar whether animals did or did not develop recurrent disease.

DISCUSSION

Because a high percentage of initial HSV infections are asymptomatic or unrecognized (11, 13, 17), methods to detect these infections in vaccine recipients are needed to allow assessment of vaccine efficacy. Vaccines that modify the acute infection but do not alter the frequency of latency establishment would be of limited value because latent infection could reactivate and cause recurrent disease. Following exposure to HSV, many HSV glycoprotein-immunized animals experienced asymptomatic local viral replication (21; Stanberry et al., in press). A portion of these developed recurrent disease (21; Stanberry et al., in press), albeit at reduced rates. Determination of serologic markers to identify those vaccinees that establish a latent viral infection after HSV exposure which could later reactivate is therefore needed. In these studies, we have sought to identify such a marker, reasoning that immunoblotting should allow detection of antibodies to HSV polypeptides not included in the vaccine but which might be expected to be produced after HSV infection. Such markers have been used in human vaccine studies (2). However, the sensitivity of these markers could not be determined because of the uncertainties of postvaccination exposure to HSV in human subjects.

Recurrent disease (a clinical manifestation of latent infection) developed from days 15 to 60 in 37 of the 57 vaccinated animals that had not developed lesions during the acute infection period. Most guinea pigs latently infected with HSV develop recurrent disease during this period of observation (24; our unpublished observation). Of the 37 animals, 25 (68%) developed detectable antibody to nonvaccine polypeptides when evaluated by immunoblotting and an additional animal was identified when evaluated by immunoprecipitation (70%). Furthermore, one of the immunoblotting procedures identified another four of these animals as having a partial response to nonvaccine polypeptides which was classified as indeterminate. Thus, although these procedures identified the majority of asymptotically acquired latent infections that led to recurrences, at least seven animals developed recurrences in spite of developing no evidence of antibody to nonvaccine polypeptides.

As observed previously (9), the magnitude of vaginal viral shedding determined which animals developed detectable antibody to nonvaccine HSV polypeptides. Presumably, a minimal antigenic mass is required to elicit a detectable

antibody response. Of the 11 animals with recurrent disease that did not develop detectable nonvaccine antibodies, all had an area under the viral shedding curve of <12 (mean, 6.3 ± 2.6). Thus, it appeared that virus entered neural tissues early and established a latent infection even though local viral replication was low. Whether levels of antibody that were below the sensitivities of our assays were produced in these animals is unknown.

The most commonly detected antibody to nonvaccine HSV polypeptides was anti-ICP-35 antibody (9). This antibody was detected in all animals that developed antibodies to nonvaccine polypeptides in both immunoblotting assays. It is possible that an enzyme-linked immunosorbent assay directed at this antigen would be more sensitive than the immunoblotting assays reported here, as has proven to be the case in detection of anti-glycoprotein D antibody (8).

Detection of antibody to nonvaccine HSV polypeptides, especially ICP-35, should identify most, but not all, potentially infected subjects immunized with glycoprotein subunit vaccines. Detection of asymptomatic infection following the use of live attenuated vaccines may pose even more difficulties.

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