Detection of Asymptomatic Herpes Simplex Virus Infections after Vaccination

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Twenty-two volunteers seronegative for antibodies to herpes simplex virus (HSV) were enrolled in a trial to determine tolerance and immunogenicity of an HSV-2 glycoprotein subunit vaccine. Vaccine was administered at days 0, 28, and 140, and sera were obtained on days 0, 7, 14, 21, 28, 35, 49, 56, 140, 147, and 365 for determination of HSV neutralizing antibody activity and antibody-dependent cell cytotoxicity (ADCC). Sera were also tested by immunoprecipitation of radiolabeled HSV-2-infected cell proteins and polyacrylamide gel electrophoresis to identify the viral proteins which elicited antibody responses in vaccine recipients. After vaccination two male volunteers presented with atypical first-episode genital herpes: patient 1 with a culture-negative genital lesion at day 53 and patient 3 with urethritis at day 68. Seroconversion to wild-type viral proteins not present in the vaccine was detectable by radioimmunoprecipitation-polyacrylamide gel electrophoresis within 10 days in both patients. Two additional volunteers, one a sex contact of patient 1, seroconverted asymptotically to nonvaccine proteins during the trial. All four vaccine breakthrough patients were indistinguishable from the other volunteers in the time required to develop neutralizing and ADCC antibodies, in the titer of these antibodies, and the time to seroconversion to gB and gD vaccine proteins. However, only one of the four breakthrough patients had antibodies to g80 (a complex of gC-2 and gE) after vaccination as compared with 15 of the other 18 volunteers ($P = 0.05$). Neither neutralizing antibody nor ADCC titers consistently identified acquisition of wild-type viral infection; therefore, protein-specific serologies were required to detect wild-type antibodies in these four patients. These data underscore the importance of using serologic assays which will distinguish naturally acquired infection from the immune response to vaccination.

Vaccines for immune prophylaxis of genital herpes simplex virus (HSV) infection have recently been developed and are currently being tested for immunogenicity and efficacy in humans (1, 14, 21). Traditionally, the efficacy of a vaccine has been determined by its effect on preventing disease. However, HSV infection may occur in the absence of symptoms or in the presence of atypical or unrecognized symptoms (9, 17, 23). Asymptomatic infection with HSV type 1 (HSV-1) and HSV-2 can result in neural latency with subsequent reactivation of the virus in salivary or genital secretions weeks or months after the primary infection (6, 15). In addition, studies have shown that many HSV-seropositive persons do not recognize or report symptomatic disease (24). Evaluation of vaccine efficacy, therefore, must include measures not only of its effect on reducing clinical disease but also on reducing asymptomatic acquisition of infection. Use of serologic markers characteristic of natural infection but not of vaccination is one strategy for determining whether wild-type infection has occurred after vaccination (25). We recently developed a method (radioimmunoprecipitation-polyacrylamide gel electrophoresis [RIP-PAGE]) to evaluate the immune response to HSV-specific polypeptides (3) and have used this assay to differentiate between the limited antibody response to an HSV-2 glycoprotein subunit vaccine and the broader antibody response to wild-type virus. In this report, we describe the role of this assay in identifying acquisition of asymptomatic and atypical genital HSV infection in 4 of 22 healthy adults receiving this

subunit vaccine. These individuals were enrolled in an open trial to test the tolerance and immunogenicity of the vaccine (14) and were followed for 1 year to describe their humoral and cellular responses to vaccine.

MATERIALS AND METHODS

Study design. Vaccine was administered at days 0, 28, and 140 to healthy HSV-seronegative adults with no history of genital herpes as described previously (14). All subjects denied having a sexual partner with genital herpes at enrollment. Twelve subjects received 50 µg per dose, and 10 subjects received 100 µg per dose. Sera were obtained on days 0, 7, 14, 21, 28, 35, 49, 56, 140, 147, and 365 and were tested for neutralizing antibody activity, antibody-dependent cell cytotoxicity (ADCC), and for reactivity with radiolabeled antigen (RIP-PAGE).

Vaccine. The subunit vaccine (lot no. 806; Merck Sharp & Dohme, Inc., West Point, Pa.) was prepared as reported elsewhere (14). HSV-2-infected chicken embryo fibroblasts were detergent disrupted, treated with DNase, and subjected to chromatography on lens culinaris agglutinin to select for glycoproteins. For biochemical studies on the vaccine, the glycoprotein-enriched fraction was chromatographed on Sephadex G-50, and aliquots were frozen for use in the biochemical analyses described in this report. For use as vaccine, the preparation was treated further with Formalin and alum.

HSV protein nomenclature. Glycoproteins are named according to conventions agreed upon at the 9th International Herpesvirus Workshop (Oxford, 1983): gB (130,000 and 110,000 molecular weight) and gD (60,000 molecular weight). A glycoprotein complex at 80,000 molecular weight is des-

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ignated g80 and contains three glycoproteins, gC-2 (5, 26), gE (18, 19), and gG (12), which are not readily resolved in the gel system described here. Work described previously indicated that all three of these glycoproteins are contained in the vaccine and that antibody to the g80 complex is directed primarily to gC-2 (4).

The major nucleocapsid protein of 148,000 molecular weight and a second nonglycosylated protein of 88,000 molecular weight are designated p148 and p88, respectively. Finally, a phosphoprotein of 66,000 molecular weight which was previously called vp66 (3) has been identified as VP16 (10, 13).

RIP-PAGE. [³⁵S]methionine-labeled antigen preparations from HSV-1-, HSV-2-, and mock-infected cells were prepared as previously described (3) and adjusted to 150,000 cpm/50 μ l with lysis buffer (10 mM Tris [pH 8.0], 0.1 mM EDTA, 0.1 M NaCl, 1% Nonidet P-40, 1% sodium deoxycholate). Sera were centrifuged at $2,000 \times g$ for 2 min, and 25 μ l of each supernatant was incubated with 50 μ l of antigen and 25 μ l of 10 mM Tris (pH 8.0) for 2 h at 4°C. Immuno-precipitates were collected on protein-A coated beads (Pharmacia, Inc., Piscataway, N.J.) and washed by centrifugation. Proteins were denatured and then separated by electrophoresis in 9% polyacrylamide slab gels as described previously (3). Radiolabeled proteins were visualized by fluorography (8).

Neutralizing antibody. HSV-1 and HSV-2 neutralizing antibody activity was determined in a complement-independent microneutralization assay (16, 20). Antibody specificity to HSV-1 or -2 was determined by calculating the potency of neutralization (pN) of HSV-1 minus the pN of HSV-2 (pN1 - pN2). Anti-HSV-1 specificity was defined as a pN1 - pN2 of >0.5 and HSV-2 specificity as a pN1 - pN2 of <0.05 . Antibody with a pN1 - pN2 of between 0.05 and 0.5 was classified as indeterminate. Titers of $>1:8$ to HSV-1 or HSV-2 or titers to both HSV-1 and HSV-2 of $>1:4$ were considered positive in this assay. Titers cited in this report are those obtained by neutralization of HSV-2.

ADCC. ADCC anti-HSV antibody was quantitated by microtiter assay as described in detail previously with HSV-1 infected, ⁵¹Cr-labeled Chang liver cells as targets, peripheral blood lymphocytes from normal HSV-seronegative nonvaccinated volunteers as effectors, and serial dilutions of plasma (1). Endpoint titers were determined by taking the highest test-plasma dilution to give a specific release at least 5% greater than the same dilution of plasma from three negative control subjects (14, 22).

RESULTS

After receiving vaccine, 2 of the 22 vaccinees developed signs and symptoms of atypical genital HSV infection, and 2 additional vaccinees developed serologic evidence by RIP-PAGE of naturally acquired asymptomatic HSV infection. None of the other 18 individuals developed clinical or serologic evidence of naturally acquired HSV infection. The clinical and epidemiologic characteristics of these four patients are presented below.

Patient 1 received 50- μ g doses of vaccine on days 0 and 28. On day 56 he related a 3-day history of thigh paresthesias, a penile lesion, and bilateral swollen inguinal lymph nodes. On exam, he had an erythematous area (2 by 1 cm) with a central 3-mm lesion from which no viruses were isolated. His source contact from 10 days earlier had no clinical or virologic evidence of genital or oral HSV infection but had HSV neutralizing antibodies indicative of past

infection with both HSV-1 and HSV-2. Serum obtained on day 56 precipitated HSV-2 p148, p88, and VP16, proteins not found in the vaccine. Eight months later, he presented with two vesicles on the shaft of the penis from which HSV-2 was isolated.

Patient 2 is a 26-year-old female who received two 50- μ g doses of vaccine on the same days as her husband, patient 1, plus a third dose on day 140. Her sera had precipitating antibodies solely to HSV glycoproteins through day 147 despite her husband's symptoms and seroconversion to wild-type proteins on day 56. She presented to the clinic with her husband on day 298 when he developed typical recurrent genital lesions. Genital examination revealed no genital lesions, and vaginal and cervical cultures were negative. However, serum drawn at this time had antibodies which precipitated nonvaccine proteins p148, p88, and VP16. She noted no symptoms in the period between days 147 and 298, and subsequent monthly follow-up exams and cultures through day 400 revealed no evidence of symptomatic expression of HSV in the genital tract. Her only reported sexual partner between days 147 and 298 was patient 1.

Patient 3 received 100- μ g doses of vaccine on days 0 and 28. He developed severe dysuria without urethral discharge on day 68, 8 days after sexual contact with a new female partner. Evaluation on day 70 revealed no evidence of genital lesions, but HSV-2 was isolated from urethral and first-voiding urine specimens obtained on days 68, 70, 73, 76, 78, 80, and 106. Symptomatic dysuria persisted in the absence of genital lesions for 13 days. Antibodies to p148, p88, and VP16 were first detected in serum drawn on day 78 (Fig. 1, lane B). Subsequent follow-up over the next 12 months revealed no recurrences of either urethritis or ulcerative genital lesions.

Patient 4 did not experience any symptoms suggestive of genital, oral, or rectal infection with HSV during the study period. However, serum obtained on day 342 had antibodies to nonvaccine proteins p148 and VP16 (Fig. 1, lane E). A subsequent sample taken on day 411 had these antibodies

FIG. 1. Antibody profiles of sera from two vaccinees who acquired HSV infection. Radiolabeled HSV proteins were immunoprecipitated by sera drawn from patient 3 on day 56 (lane A), day 78 (lane B), and day 106 (lane C). Onset of symptoms in patient 3 occurred on day 68. Antibody profiles are shown for sera drawn from patient 4 on days 56 (lane D), 342 (lane E), and 411 (lane F). Patient 4 was not symptomatic until day 579. For comparison, a serum profile from a patient with culture-proven wild-type genital HSV-2 is shown (lane G).

and also antibody to p88 (Fig. 1, lane F). He reported 10 sexual contacts with a male partner with known oral-labial herpes between days 180 and 240. On day 579 after enrollment he developed a clinically symptomatic facial lesion from which HSV-1 was isolated.

Neutralizing antibody responses to glycoprotein subunit vaccine. All four patients developed detectable neutralizing antibody activity to HSV-1 and HSV-2 before acquiring wild-type infection. Neutralizing antibody activity was first detectable at day 21 in patients 1 and 4 and at day 35 in patients 2 and 3. Most (13 of 18) of the other vaccinees also developed neutralizing antibody by day 35. The neutralizing antibody titers for the four patients before their seroconversion to wild-type HSV proteins did not serve to distinguish them from the other members of the study group. At day 35, patients 1 and 2 had neutralizing titers of 1:16, while patients 3 and 4 had titers of 1:4 and 1:8, respectively. By comparison, titers at day 35 for the other 18 vaccinees ranged from <1:4 (7 of 18) to 1:16 (geometric mean titer of 4.3 ± 2.0).

ADCC responses to glycoprotein subunit vaccine antibody. The first detectable ADCC antibody activity was noted at days 7, 14, and 35 in patients 1, 2, and 4, respectively. Patient 3 developed ADCC antibody at day 72, shortly after acquiring wild-type HSV infection. Of the other 18 vaccine recipients, 12 developed ADCC antibody by day 35 after vaccination. Thus, with the possible exception of the relatively late ADCC antibody development in patient 3, none of the four patients reported here had unusually early or late ADCC responses as compared with the vaccine recipients who did not acquire wild-type infections. ADCC titers for the four patients were similar to those of the 18 patients who did not seroconvert to wild-type HSV proteins. At day 35, patients 1, 2, and 4 had titers of 1:100. The geometric mean titer for the other 18 vaccinees at day 35 was $21.52 (\pm 4.4)$.

Development of RIP-PAGE antibodies to vaccine glycoproteins. All four patients seroconverted to viral glycoprotein gB in the RIP-PAGE assay within the same time frame as vaccinees who did not break through (Table 1). Patients 1, 2, and 4 seroconverted to gB at day 14; all other vaccinees seroconverted to gB by day 35. All four of the vaccine breakthrough patients seroconverted to gD in the RIP-PAGE assay before the acquisition of wild-type disease. Seroconversion to the g80 complex was seen in only one of the four breakthrough patients before their acquisition of

TABLE 2. Serologic responses to acquisition of HSV infection

Patient	Day of onset of symptoms	Day of seroconversion to VP16, p88, p148	Neutralizing antibody titer		ADCC antibody titer	
			Before onset ^a	After onset	Before onset ^a	After onset
1	53	56	16	32	100	1,000
2	None	298	8	4	100	5,000
3	68	78	4	64	<10	500
4	579	342 ^b	8	32	250	100

^a For asymptomatic patients (nos. 2 and 4), titers from day 147 were used.

^b Anti-p88 was not detected until day 411.

wild-type infection. Patients 1 and 3 seroconverted to g80 only in conjunction with seroconversion to wild-type proteins, while patient 2 seroconverted at day 147 before showing signs of asymptomatic wild-type infection. Patient 4 did not seroconvert to g80 even after seroconverting to wild-type HSV proteins. In contrast, 12 of the other 18 vaccinees (66.7%) had seroconverted to g80 by day 56, and 15 of 18 (83.3%) had seroconverted by day 147 (Table 1; $P = 0.05$ by Fisher's exact test for comparison between breakthrough and nonbreakthrough patients).

Serologic evidence for wild-type infection. Antibodies to nonglycosylated polypeptides VP16, p148, and p88 characteristic of wild-type HSV-2 infection were detectable by RIP-PAGE within 10 days of clinical symptoms in patients 1 and 3 (Table 2). In patients 2 and 4, antibodies to p148, p88, and VP16 were detected by RIP-PAGE despite the absence of signs or symptoms of HSV infection. None of the other 18 vaccine recipients had evidence of seroconversion to p148, p88, or VP16. Neutralizing antibody titers in three of four patients rose after the development of clinically symptomatic disease or the presumed time of acquisition of HSV infection (Table 2). However, only in patient 3 could the postinfection titer (1:64) be considered high compared with that of other vaccine recipients. Vaccinees who did not acquire wild-type HSV infection also experienced fourfold rises and falls in their neutralizing antibody titers over the course of the study (14). Postinfection ADCC titers rose 10-fold for patient 1 and 50-fold for patients 2 and 3. Patient 4, however, did not develop a rise in ADCC titer after seroconverting to nonvaccine proteins (Table 2). By comparison, ADCC titers above 1:500 were not obtained from vaccinees who did not acquire wild-type HSV infection.

Specificity of RIP-PAGE assay. To rule out the possibility that detection of antibodies to the nonglycosylated proteins of 148,000, 66,000 (VP16), and 88,000 molecular weight were related to an immune response to contaminants in the vaccine, we designed a competition experiment in which a [³⁵S]methionine-labeled HSV-2 antigen preparation was reacted with hyperimmune rabbit anti-HSV-2 antiserum in the presence of 0.8 or 3.2 µg of the vaccine preparation. After fluorography, densitometry tracings were made of the resulting RIP-PAGE antibody profiles and superimposed for comparison (Fig. 2). Levels of immunoprecipitated radiolabeled gD and g80 diminished when 0.8 µg of unlabeled vaccine antigen was present in the reaction mixture. Addition of 3.2 µg of vaccine resulted in greater competition and further reduction in the levels of precipitated ³⁵S-labeled g80 and gD and, in addition, resulted in a decrease in the peak height of gB. Levels of precipitated nonglycosylated polypeptides were not reduced with added vaccine, indicating that polypeptides p148 and VP16 were not present in the vaccine.

In addition, direct detection of VP16 in the vaccine was

TABLE 1. Time to first seroconversion to HSV-2 glycoproteins gB, g80, and gD in recipients of the HSV-2 subunit vaccine

Time after vaccination (days)	% with antibody to glycoprotein ^a					
	gB		g80		gD	
	Patients	Others	Patients	Others	Patients	Others
7	0	5.6	0	0	0	0
14	75	72.2	0	0	0	11.1
21	75	83.3	0	5.6	0	11.1
28	75	83.3	0	5.6	0	11.1
35	100	100	0	38.9	75	50.0
56	100	100	25 ^b	66.7	100	83.3
140	100	100	50	66.7	100	83.3
147	100	100	75	83.3	100	100

^a Cumulative percentage of persons having the specific antibody. $n = 4$ for patients, and $n = 78$ for others.

^b Note that two of four patients seroconverted to g80 only in conjunction with seroconversion to wild-type proteins (patient 1 at day 56 and patient 3 at day 78). Patient 2 seroconverted to g80 at day 147, while patient 4 did not seroconvert to g80 during the study period, despite seroconversion to wild-type HSV proteins and development of an HSV-1-positive lesion.

FIG. 2. Composition of the glycoprotein vaccine. A standard RIP reaction was run with [35 S]methionine-labeled HSV-2-infected cell lysate and rabbit hyperimmune antiserum to HSV-2 (—). Replicate reaction mixtures were run in the presence of 0.8 μ g (—•—) or 3.2 μ g (·····) of the glycoprotein preparation from which the vaccine was derived. Precipitates were electrophoresed and then subjected to fluorography. Films were scanned with a linear densitometer, and tracings were superimposed for comparison. Radiolabeled proteins in the gB region, proteins which migrated to a region designated g80 (at 80,000 daltons), and proteins migrating in the gD region were all immunoprecipitated less efficiently in the presence of the glycoprotein preparation. Proteins p148 and p88 (which appeared only as a shoulder on the gB curve) were not reduced. Proteins contained in the vaccine (inset, left lane) and in HSV-2-infected cell cytoplasmic extracts (inset, right lane) were Western blotted and reacted with a monoclonal antibody, LP1, against VP16. LP1 was not reactive with vaccine proteins. Duplicate Western blots were also reacted with hyperimmune rabbit anti-HSV-2 serum (blots not shown). In these controls, the vaccine-containing blot showed five poorly resolved bands with the migration characteristics of known HSV-2 glycoproteins. The blots with total infected-cell extract showed 18 bands, including VP16.

attempted by using a VP16-specific monoclonal antibody, LP1 (13). The vaccine preparation and a preparation of HSV-2-infected cell proteins were electrophoresed, and proteins were transferred electrophoretically to nitrocellulose (7) and reacted with LP1. LP1 failed to bind to the immobilized vaccine proteins but bound efficiently to VP16 from infected-cell extract (Fig. 2, inset).

DISCUSSION

The serologic and clinical observations in this study have several implications for the design and testing of candidate vaccines for herpes group viruses. As illustrated by patients 1 and 2, asymptomatic or atypical symptomatic acquisition of infection can result in ganglionic latency, reactivation of symptomatic disease, and transmission of genital HSV-2 infection. Furthermore, as shown by patient 4, symptomatic recurrence did not necessarily develop soon after asymptomatic acquisition of infection. Therefore, methods to evaluate HSV vaccines must measure not only the frequency of clinically symptomatic disease during the study period but must also evaluate whether asymptomatic infection has occurred. From a practical point of view, development of serologic markers for distinguishing between wild-type infection and vaccination responses appears to be one of the best ways to approach this problem. This approach has been successfully used in efficacy trials of hepatitis B surface antigen subunit vaccine (25).

Our data indicate that after vaccination with a herpes subunit vaccine, biologic assays such as neutralizing antibody and ADCC assays did not suffice to distinguish the immune response of those who developed wild-type infection from that of other vaccines. Although titer rises occurred after vaccine breakthrough, only the neutralizing antibody titer in patient 3 and the ADCC titer in patients 1 and 2 (Table 2) exceeded the highest levels elicited by vaccine proteins alone. Our previous work suggested that antibodies to p148, p88, and VP16 would be useful markers of vaccine breakthrough since 100% of genital HSV-2 patients were shown to seroconvert to these proteins within a month of their primary episode (2). The four cases presented here indicate that these markers are, indeed, effective means of documenting vaccine breakthrough.

As this study was not designed as a vaccine efficacy trial, the exposure of the 22 vaccine recipients to wild-type virus was intended to be minimal, and data were not collected to document exposure history in the 18 nonbreakthrough subjects. Therefore, documentation of any specific protective effect of one or more antibodies will require development of quantitative protein-specific serologies as well as a larger number of patients at risk of acquiring disease. These preliminary observations do suggest that further studies on the correlation between the immune response to these proteins and the natural history of genital herpes should be pursued. For example, seroconversion to gB and gD in the four patients occurred within the same time frame as the other vaccinees. However, only one of the four vaccine breakthrough patients had seroconverted to g80 (a complex of gC-2 and gE) by day 56 (in conjunction with acquiring wild-type infection) compared with 12 of 18 of the other vaccine recipients.

It should be emphasized that we do not feel that acquisition of wild-type infection in these four subjects should discourage further evaluation of this and other HSV vaccines. Animal studies with similar vaccines have not proven to have 100% efficacy in preventing disease (11). Further, since the trial was not designed to demonstrate efficacy, accurate data were not gathered to compare the frequency of sexual contact with potentially infected HSV-2 patients (i.e., the risk of acquiring infection) between the group of four vaccine breakthrough patients and the cohort of 18 patients who did not develop serologic or clinical evidence of wild-type HSV infection. The determination of vaccine efficacy awaits the outcome of double-blind, placebo-controlled trials on a larger population of individuals at risk.

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