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Humoral Immune Response to Herpes Simplex Virus Type 2 Glycoproteins in Patients Receiving a Glycoprotein Subunit Vaccine

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Serial serum specimens from 22 herpes simplex virus (HSV)-seronegative recipients of an HSV type 2 (HSV-2) glycoprotein subunit vaccine were analyzed by radioimmunoprecipitation and polyacrylamide gel electrophoresis for the development of antibodies to HSV-2 gB, gD, and g80, a complex of gC and gE. Volunteers received 50 (n = 12) or 100 μg (n = 10) of vaccine at days 0, 28, and 140; sera were drawn weekly for 8 weeks and again at days 140, 147, and 365. Among seronegative volunteers, antibody to gB was detected 2 weeks after the first dose, while antibodies to g80 and gD were detected after the second dose (day 35). Antibodies to nonglycosylated HSV-specific proteins were not detected. A dose-response effect between recipients of 50- and 100-μg doses was observed in the proportion of vaccine recipients seroconverting to g80 and in the proportion of recipients retaining antibodies to both gD and g80 over time. Diminishing complement-independent neutralizing antibody titers occurred after the second dose and were associated with loss or reduction of detectable antibody to gD. Volunteers who were seropositive for HSV-1-specific antibody (n = 11) were also enrolled in the trial and received 50-μg doses of vaccine. Vaccination resulted in conversion to HSV-2 complement-independent neutralizing antibody specificity or indeterminant specificity in 10 of 11 volunteers. These shifts were accompanied by changes in the radioimmunoprecipitation and polyacrylamide gel electrophoresis profile. These changes, which were apparent by 14 days after the first vaccine dose, included de novo appearance or increased levels of antibody to g80 and increased levels of antibody to gD and gB. These studies document the immunogenicity of solubilized glycoproteins gB, gD, gC, and, possibly, gE in humans.

Studies in animals indicate that antibodies to herpes simplex virus (HSV) proteins and glycoproteins have a protective role in preventing acquisition of disease (8, 11, 15, 29), maintaining latency (32), and limiting the extent of neural latency (17, 22, 26, 30). Subunit vaccines containing HSV glycoproteins have been shown to protect mice from challenge with infectious virus (15, 29). In humans, the presence of HSV-specific antibody is associated with milder first episodes of genital herpes (10, 20, 28). While this observation indicates that antibody may have a protective role in humans as well as animals, the specific roles of antibodies to individual HSV proteins are not known. Recently, an HSV glycoprotein subunit vaccine was developed and tested in humans to determine its immunogenicity and side effects (18). The vaccine was tolerated well and shown to elicit specific immune responses as measured by in vitro assays, including complement-independent neutralizing antibody activity (CINA), lymphocyte transformation activity, and antibody-dependent cell cytotoxicity (ADCC). This report describes the frequency of seroconversion to individual glycoproteins, the sequence of appearance of these antibodies, the effect of vaccine dose on the protein-specific humoral response, and the association between antibodies to individual glycoproteins and the development of CINA in recipients of this HSV type 2 (HSV-2) subunit vaccine.

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MATERIALS AND METHODS

Patient population and study design. Thirty-three volunteers who had no reported contact with persons with genital herpes were enrolled at the University of Washington Herpes Research Clinic. Vaccine recipients were divided into three groups according to dose received and immune status for HSV-1 antibodies at the beginning of the study. Individuals in the first group (group A; n = 12) were seronegative on entry and received 50 μg per dose. Ten additional seronegative individuals (group B) received 100 μg per dose. Group C (n = 11) was composed of eight individuals who had HSV-1-specific neutralizing antibody (CINA) in their prevaccination sera and three individuals who had CINA of indeterminant specificity in their prevaccination sera. These individuals received 50 μg per dose. Three doses of vaccine were administered to all study patients at days 0, 28, and 140. Volunteers were followed for 1 year, with serum drawn at 1-week intervals for 8 weeks and again at days 140, 147, and 365. Details of the protocol have been described previously (18). Four vaccine recipients were dropped from analysis during the course of the study because they reported sexual exposure to someone with genital herpes. One group A vaccine recipient was also lost to follow-up after 147 days. Thus, the figures reported represent 11 group A vaccine recipients at days 56, 140, and 147 and 8 vaccine recipients at day 365. Nine group B vaccine recipients were analyzed on days 140, 147, and 365.

Serologic assays. Sera were analyzed for the presence of HSV antibody by CINA. Briefly, serial dilutions of sera were mixed with HSV-1 (strain 2931) or HSV-2 (strain MS-2)

and then added to human embryonic tonsil cells in microtiter plates. After 5 days, plates were read for viral cytopathic effect. Antibody titer was determined as the highest dilution of serum inhibiting 50% of the viral input, as calculated by the Reed-Meunch formula. Virus-specificity of CINA antibody was determined by calculating the potency of neutralization as described for HSV-1 (pN_1) and HSV-2 (pN_2) (19, 27). HSV-1 specificity was defined as $pN_1 - pN_2 > 0.5$. HSV-2 specificity was defined as $pN_1 - pN_2 < 0.05$. Sera with values for $pN_1 - pN_2$ between 0.05 and 0.5 were considered to be of indeterminant specificity. Positive CINA was defined by a titer to HSV-1 or HSV-2 $\geq$1:8 or by a titer to both HSV-1 and HSV-2 of $\geq$1:4.

RIP-PAGE. Analysis of antibodies to individual glycoproteins was performed by radioimmunoprecipitation (RIP) of [^{35}S]methionine-labeled cytoplasmic extracts from human diploid fibroblasts infected with HSV-1 (strain E115) or HSV-2 (strain 333). Cytoplasmic extracts were obtained as previously described (2), with the following modifications: 10^6 infected, radiolabeled cells were lysed with 5 ml of P-RIPA buffer (10 mM phosphate buffer [pH 7.2] containing 15 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 1% aprotinin) for 30 min at 4°C, clarified by low-speed centrifugation (5 min at 200 × *g*), and briefly sonicated. The cell lysate was further clarified at 280,000 × *g* for 30 min and frozen at −70°C in 1.0-ml aliquots prior to use. Immunoprecipitation was performed for 30 min at 4°C; immune complexes were collected on protein A-Sepharose beads as described previously (2), and proteins were denatured and subjected to polyacrylamide gel electrophoresis (PAGE) and fluorography (7). Determination of the presence or absence of HSV-specific antibodies was made under standardized fluorography conditions, including input counts per minute of 150,000 cpm per reaction and exposure of X-ray film for 5 days. Radiolabeled antigen preparations were tested with positive control sera prior to use to assure uniform radiolabeling of viral glycoproteins in each preparation.

Glycoprotein subunit vaccine. Preparation of the glycoprotein subunit vaccine has been previously described (18). A portion of the same lot number of the vaccine preparation was reserved prior to Formalin treatment and the addition of alum. This extract, a gift of Arlene McLean and Vivian Larson (Merck Sharp & Dohme Research Laboratories), was held at −70°C until immediately prior to use in assays designed to determine the composition of the vaccine.

Western blots. Components of the vaccine were analyzed by immunodetection of separated immobilized proteins. Immunoprecipitated glycoproteins were separated by PAGE and transferred to nitrocellulose by electrophoresis at 200 mA in a TE52 transfer chamber (Hoeffer Scientific, San Francisco, Calif.) for 16.5 h in transfer buffer (25 mM Tris hydrochloride–20% methanol–0.19% glycine). Nitrocellulose sheets were cut into strips, blocked with 5% bovine serum albumin in phosphate-buffered saline for 1 h at 20°C (6, 14), and reacted with rabbit anti-HSV-2 antiserum for 3 h at 20°C with constant rocking. After extensive washing with 0.05% Tween 80 in phosphate-buffered saline, biotinylated anti-rabbit immunoglobulin was added (Vecta Stain, Vector Laboratories, Burlingame, Calif.), and insert directions were followed for staining the bound antibody with avidin-biotin complex-bound horseradish peroxidase.

Antibodies. Rabbit hyperimmune antiserum against HSV-2 was obtained from Dako Corporation, Santa Barbara, Calif. Mouse monoclonal antibodies against HSV-2 gC-2 (188-8), gE (III347), gB (59-6), and gD (114-4) were gifts of P. G. Spear, University of Chicago. Mouse monoclonal antibody against gG (AP5) was a gift of A. C. Minson, University of Cambridge, United Kingdom.

RESULTS

Composition of the subunit vaccine. To determine the composition of the vaccine, a competition experiment was first performed in which a [^{35}S]methionine-labeled HSV-2 antigen preparation was reacted with hyperimmune rabbit anti-HSV-2 antiserum in the presence of increasing amounts (0.8 and 3.2 μg) of the vaccine preparation. Levels of immunoprecipitated, radiolabeled gB, gD, and g80 diminished, while levels of precipitated, nonglycosylated polypeptides were not reduced with added vaccine. These data indicated that gB, gD, and at least one glycoprotein migrating between 75,000 and 95,000 daltons (g80 complex) were present in the vaccine.

At least three HSV-2 glycoproteins have been reported in the 75,000- to 95,000-dalton range: gE at 75,000 (23, 24), gC-2 at 80,000 (4, 34, 36), and gG at 92,000 (16) daltons. To determine which of these three proteins comprised the g80 complex, the vaccine was reacted with monoclonal antibodies to HSV-2 gE, gC, and gG. The resulting pelleted immune complexes and the unbound proteins in the supernatants were subjected to Western blot analysis with hyperimmune rabbit anti-HSV-2 antiserum to detect transferred, immobilized HSV-2 proteins. Figure 1 shows the results of the studies detecting gC (Fig. 1A), gG (Fig. 1B), and gE (Fig. 1C) in the vaccine.

Glycoprotein gC was precipitated in sufficient quantity to result in a prominent band on the Western blot (Fig. 1A), and gG could be distinguished as a narrow band migrating slightly slower than gC (Fig. 1B). Glycoprotein gG was also detected in the glycoprotein preparation by the binding of AP5 monoclonal antibody to Western blots of the electrophoresed vaccine (data not shown). Immunoprecipitated, transferred gE appeared as a faint band migrating slightly faster than gC (Fig. 1C) and as a pronounced band migrating slightly slower than gD. A fifth band migrating at approximately 65,000 daltons (noted with an arrow in panel B in Fig. 1) appeared in the pellets of all three immunoprecipitates. Its identity is unknown. The apparent comigration of gD and the lower-molecular-weight form of gE prompted concern that the RIP-PAGE system would not allow the detection of antibodies specific to gD. However, precipitation with monoclonal antibody showed that the lower-molecular-weight form of gE was detectable in [^{3}H]glucosamine-labeled preparations but was not detected with [^{35}S]methionine radiolabeling. Since [^{35}S]methionine-labeled gD was easily visualized, immunoprecipitated protein with an apparent molecular mass of 60,000 daltons was scored as gD.

Sequential response to HSV-2 glycoproteins in initially seronegative vaccine recipients. Comparison of the relative migration of the proteins precipitated by the sera of the vaccine recipients with the migration of those precipitated by mouse monoclonal antibodies to HSV-2 gB, gD, gE, and gC indicated that gB, gD, and at least one glycoprotein of the 75,000- to 95,000-molecular-weight complex were precipitated by human sera. Because of the close migration of gE and gC, we have designated the radiolabeled target in this region as g80 (Fig. 2). gG was not radiolabeled by [^{35}S]methionine and, as such, was not detected in [^{35}S]methionine-labeled immunoprecipitates.

The immune response to the HSV-2 subunit vaccine was limited to the HSV-2 glycoproteins, gB, g80, and gD (lanes B through I) (Fig. 2). Nonglycosylated polypeptides precipi-

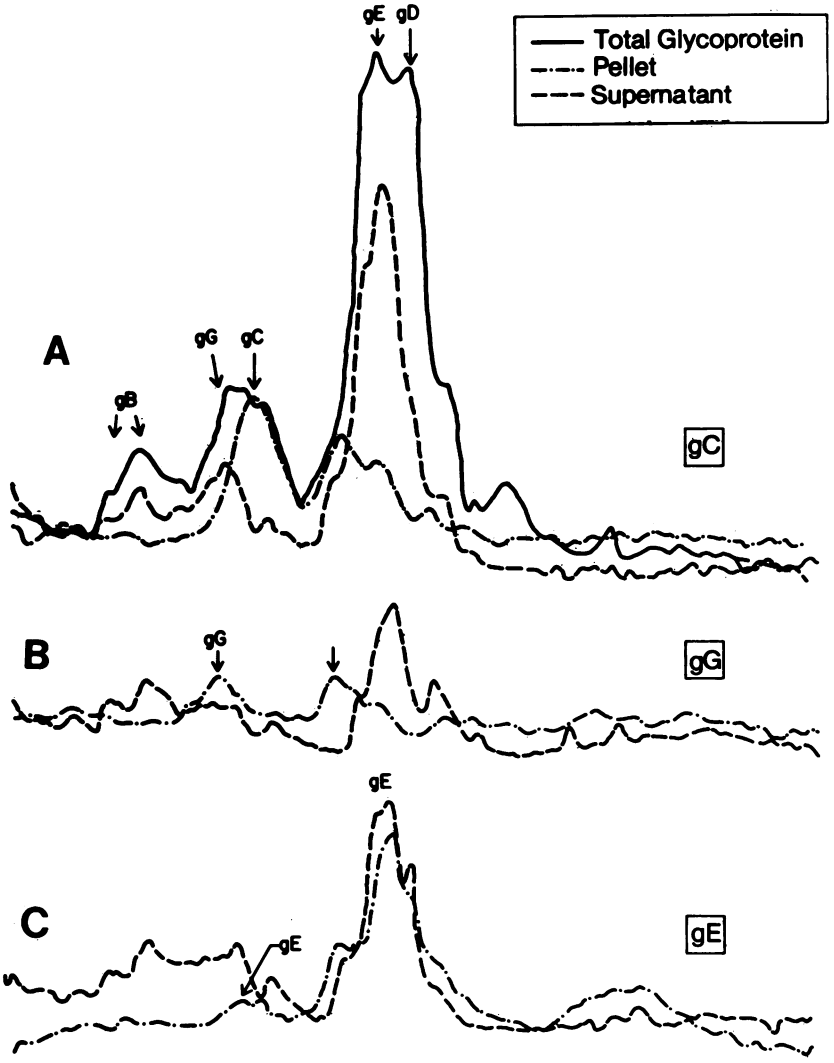

FIG. 1. Detection of vaccine glycoproteins by precipitation with mouse monoclonal antibodies. Mouse monoclonal antibodies to gC (A), gG (B), and gE (C) were reacted with the glycoprotein vaccine. The immunoprecipitates were pelleted, and both pellets and unprecipitated proteins in the supernatants were denatured and electrophoresed into a single gel. The untreated glycoproteins in the vaccine were run in the gel for comparison. Proteins in the gel were transferred to nitrocellulose, reacted with rabbit anti-HSV-2 hyperimmune serum, and visualized by avidin-biotin complex stain with horseradish peroxidase. Precipitation of gC and gG resulted in a depletion of the respective protein in the supernatant. This effect was not as apparent with gE, in part, because of the predominance of the 65,000-dalton gE species which comigrated with gD. One protein, marked with an arrow in panel B, persisted in both pellet and supernatant fraction in all three experiments. Its identity is not known.

tated by convalescent sera from patients with naturally acquired genital HSV-2 infections (Fig. 2, lane J) were not precipitated by sera from any of the vaccine recipients. Antibody to HSV-2 gB was detected in the sera of all vaccine recipients before antibody to either gD or g80, as shown in a representative series of antibody profiles from a volunteer who received 50-μg doses (Fig. 2). Antibody to gB was first noted at day 14 in 9 of 12 patients receiving 50 μg of vaccine on day 0 (Fig. 3A). All vaccine recipients had seroconverted to gB by day 35, 1 week after the second dose. Antibody to gB was detectable in sera from all patients from this time through the remainder of the study. Antibody to gD was first detected on day 35 in half (6 of 12) of the patients studied (Fig. 3A). All patients had seroconverted to gD by 1 week after the third dose of vaccine (day 147). Antibody to g80 was first noted in 2 of 12 patients at day 35. After three vaccine doses, 8 of 11 had seroconverted to g80; three patients failed to develop detectable antibody to g80 during the study.

Effect of vaccine dose on immune response to HSV-2 glycoproteins. The frequency and time of seroconversion to gB were similar between seronegative patients receiving 50 (group A; Fig. 3A) and 100 μg (group B; Fig. 3B). Most patients, regardless of dose, required two doses of vaccine to seroconvert to gD, with 50 and 80% of 50- and 100-μg recipients, respectively, seroconverting by day 35. All patients seroconverted to gD by 7 days after the third dose (day 147). Five of ten (50%) 100-μg recipients versus two of twelve (17%) 50-μg vaccine recipients seroconverted to g80 by day 35, while all of the 100-μg recipients versus 73% of the 50-μg recipients seroconverted to g80 by day 147. Similarly, four of nine 100-μg recipients (44%) versus only

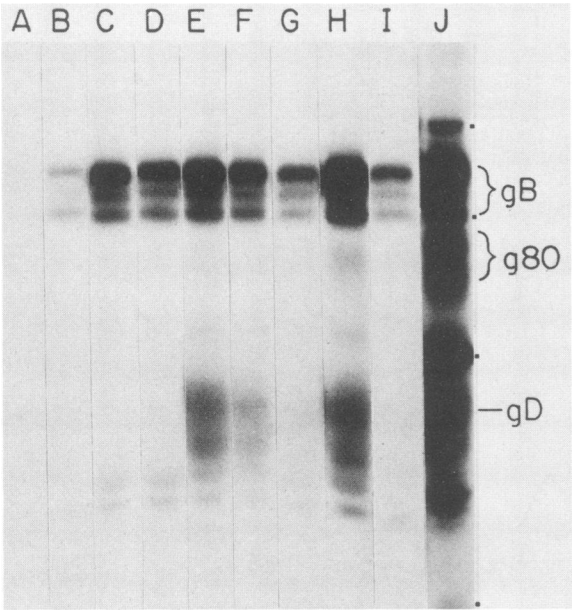

FIG. 2. Sequential appearance of antibodies in response to vaccine. RIP-PAGE reactions with [^{35}S]methionine-labeled HSV-2-infected cell lysates are shown in the indicated lanes with sera drawn at days 7 (A), 14 (B), 21 (C), 28 (D), 35 (E), 56 (F), 140 (G), and 147 (H) and at 1 year (I). Vaccine was given at days 0, 28, and 140. Antibody to gD and g80 first appeared at day 35. Apparent levels of these antibodies waned with time but were recovered after the third dose (lane H). Lane J is a profile from a patient with naturally acquired HSV-2 infection. Nonglycoproteins, which were not precipitated by the sera of the vaccine recipients, are marked with solid squares.

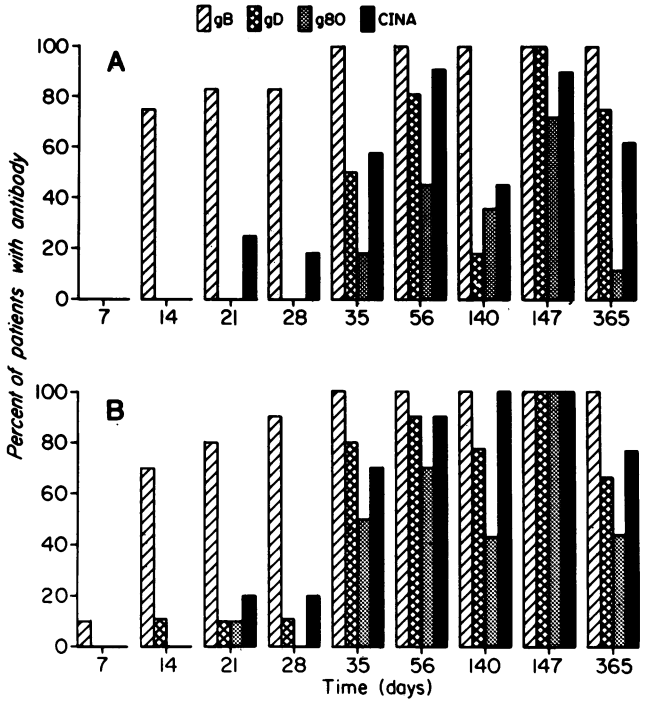

FIG. 3. Dose response of glycoprotein-related antibodies and neutralizing antibody. The proportion of patients with antibodies to gB, gD, and g80 and those with CINA were tallied for each day that serum was drawn for group A patients, receiving 50-μg doses (A) (n = 12 for days 0 through 35; n = 11 for days 56, 140, and 147; n = 8 for day 365), and for group B patients, receiving 100-μg doses (B) (n = 10 for days 0 through 56; n = 9 for days 140, 147, and 365). Vaccine was administered on days 0, 28, and 140. All patients were seronegative for HSV-2 antibodies at entry to the study.

one of eight (12.5%) 50-μg recipients retained detectable antibody to g80 at 1 year after vaccination. A dose-response effect was also noted in the response to gD. By day 140, 16 weeks after the second vaccine dose, seven of nine (78%) 100-μg recipients versus four of eleven (18%) 50-μg recipients had detectable antibody to gD. Analysis of 1-year follow-up sera, however, revealed that a similar proportion of 50- and 100-μg recipients had lost detectable antibody to gD (two of eight and three of nine, respectively).

Association of neutralizing activity with specific antibody development. In both dosage groups, CINA developed 7 to 42 days after antibody to gB was first detected. One patient did not develop CINA during the study but did develop antibody to gB. The percentage of CINA-positive patients was similar at each time point for the two dosage groups until day 140. At that time, all of the 100-μg recipients were CINA seropositive, while only 45% of the 50-μg recipients were seropositive (P = 0.01; Fisher exact test; two tailed). In the 50-μg group, five of the eleven patients who were CINA positive at day 56 lost CINA by day 140; all regained CINA by day 147, 1 week after the third dose. The five individuals who became CINA negative at day 140 also lost detectable antibody to gD (Fig. 2, lane G and Fig. 3A). Three others had reduced CINA titers at day 140 accompanied by loss of detectable antibody to gD or reduced levels of antibody to gD, as indicated by densitometric scans of fluorograms (data not shown). Fluctuations in CINA titer were not reflected in apparent changes in levels of antibody to gB. Similarly, although the incidence or apparent levels of antibody to g80 fluctuated with time and vaccine boosters, the changes did not appear to be directly associated with changes in CINA.

Immune response of seropositive recipients. Eight volunteers with HSV-1-specific CINA and three with CINA of indeterminant type were enrolled onto the 50 μg-per-dose vaccination protocol. CINA type specificity shifted from HSV-1 to HSV-2 or type indeterminant after vaccination in serum samples from eight of eight HSV-1-positive patients by 7 to 28 days after vaccination. Of the three patients who entered the study with CINA of indeterminant type, one remained indeterminant and two seroconverted to HSV-2-type-specific CINA.

RIP-PAGE assays were performed on sera from all 11 patients to determine whether the shifts in CINA type specificity seen after vaccination were accompanied by apparent changes in antibody profiles. All eight of the patients who had shifts from HSV-1 to indeterminant or HSV-2 CINA showed clear changes in RIP-PAGE profiles by 28 days after the first dose. These changes included the de novo appearance of immunoprecipitated g80 in four patients, increased levels of precipitated g80 in two patients, and increased levels of precipitated HSV-2 gD in six patients. Three patients had increased levels of immunoprecipitated gB. Only two of the patients with altered profiles had evident changes in all three antibodies. Serum profiles for one of these two patients are shown in Fig. 4. While levels of precipitated proteins from HSV-1-infected cells changed little after vaccination, increased levels of gB, gD, and g80 were precipitated from HSV-2-infected cell lysates.

Increases in RIP-PAGE antibody to gB, g80, and gD and de novo appearance of antibody which precipitated g80 were

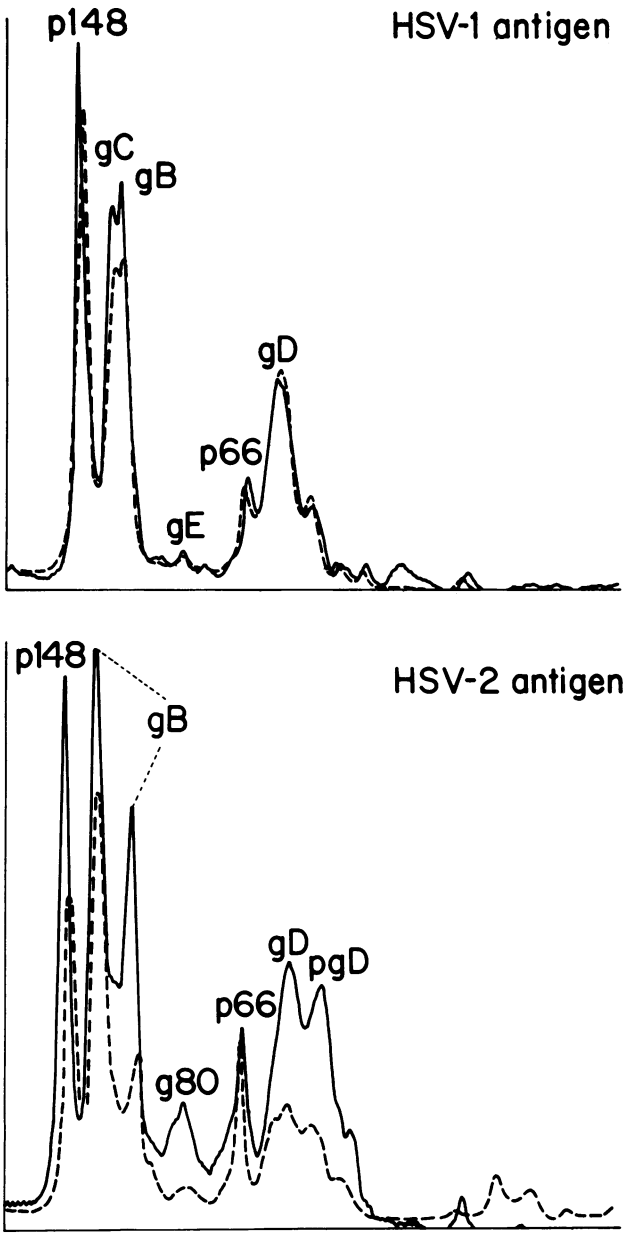

FIG. 4. Changes in antibody profiles in a vaccine recipient who was seropositive at the start of the study. Sera from days 0 (- - -) and 14 (——) after vaccine dose on day 0 were reacted with HSV-1-infected cell lysate (top panel) or HSV-2-infected cell lysate (bottom panel). Densitometry was performed on the fluorograms from these reactions. Note the apparent increase in precipitated gB, g80, gD, and pgD from the HSV-2 lysates. The increased peak height at p148 in both panels may be due to artifact, as a band appeared in this area in negative control reactions with preimmune sera (data not shown).

noted in two of three patients who had CINA of indeterminant specificity at enrollment. The third patient seroconverted to HSV-2 CINA specificity without detectable qualitative or quantitative changes in the antibody profile. Changes in HSV-2 antibody profiles apparent after the second dose of vaccine were limited to two patients and not associated with apparent changes in CINA specificity.

DISCUSSION

Data presented in this study confirm and extend results reported previously that individuals receiving an HSV-2 glycoprotein subunit vaccine mount an immune response to the glycoproteins composing the vaccine (18). Monoclonal antibodies to known glycoprotein targets were used to show that the vaccine contained HSV-2 glycoproteins gB, gG, gC, gE, and gD. In contrast to the broader response to 15 or more proteins which develops after natural infections (3, 35), the response to the subunit vaccine was limited to gB, gD, gC, and, possibly, gE, an observation which may be useful in documenting acquisition of naturally occurring genital HSV infections after vaccination.

Antibodies to individual glycoproteins were made in detectable levels at different times after vaccination. Antibody to gB appeared earlier than antibody to gD and g80 in all vaccine recipients. It is of interest that this pattern reflects the time course of antibody appearance in natural genital HSV-2 infections (3). Since both gB and gD are expressed on the surface of infected cells and are therefore exposed to immune cells, one could speculate that either (i) gB is inherently more immunogenic or (ii) relatively lower titers of antibody to gB can be detected by RIP-PAGE, resulting in its apparent earlier detection. Development of sensitive protein-specific assays to each of these proteins may help answer this question.

Antibody to glycoproteins which migrate in the g80 region of polyacrylamide gels appeared in 73% of 50-μg vaccine recipients and 100% of 100-μg vaccine recipients by day 147. These findings were of interest, as the human antibody response to HSV-2 glycoproteins in the g80 complex (gC, gE, and gG) have not been well documented. In contrast, direct evidence for human serum antibody to HSV-1 gC has been obtained by radioimmunoassay (1) and enzyme-linked immunoassay (9, 33) or Western blotting (12). Since the vaccine contained HSV-2 gC, gE, and gG, human serum reactivity to at least one of the three glycoproteins was indicated. In an attempt to determine whether gC-2 or gE were targets of this response, we reacted the sera of vaccine recipients with HSV-2 cell lysates which had been preabsorbed with mouse monoclonal antibodies to gC and gE. By this technique, antibody to HSV-2 gC was shown to be present after vaccination, but antibody to gE was not demonstrated. It will be necessary to utilize purified proteins in specific serologies to directly identify seroconversion to gC and gE in the vaccine recipients.

Antibodies to gG were not demonstrated in this study. As a component of the vaccine, gG appeared to comigrate with gC by Western blot analysis (Fig. 1). Immune precipitates of [^{3}H]glucosamine-radiolabeled gG and monoclonal antibody confirmed that gG comigrates with gC in our system (data not shown). Study of the antibody response to gG is complicated not only by its comigration with at least one other major glycoprotein target of the immune response but by its lack of labeling with [^{35}S]methionine. Using an indirect method to detect antibody to gG, we found that patients with wild-type HSV-2 infections consistently had antibody to gG (R. Ashley, A. Minson, and L. Corey, Program Abstr. 24th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 37, 1984). However, we could not demonstrate this antibody in sera from vaccine recipients. Since the vaccine appears to contain gG, the apparent lack of immune response may be due to altered immunogenicity of the solubilized gG or limited sensitivity of our assay for antibody to gG.

We have previously reported that sera from these patients

react in an ADCC assay (18). One or more of the antibodies detected by RIP-PAGE may be active in this assay. Antibodies to HSV-1 gB, gC, and gD have all been shown to be active in mediating ADCC (21). Studies with mouse monoclonal antibodies to HSV-2 glycoproteins have shown that HSV-2 gB- and gD-specific antibodies can be active in ADCC (5). Data reported here indicate that human antibodies to HSV-2 glycoproteins gB, gD, and possibly gC are also capable of mediating immune cytolysis.

Sera from vaccine recipients were also reactive in in vitro viral neutralization (CINA). Vaccine recipients whose CINA titers waned over time also had waning levels of precipitating gD antibody over time. This apparent association between antibody to gD and CINA activity extends to humans the observations made by others that mouse monoclonal antibodies to gD have neutralizing capability in vitro (13, 25, 31) and in vivo (11). Other antibodies may also be involved in neutralization, as CINA was detectable in some patients after antibody to gB was apparent but before gD-specific antibody was detected. Because we have no independent measure of the sensitivity of RIP-PAGE for antibodies to individual proteins, the relative roles of these antibodies to CINA must be resolved by other types of assays.

It was of interest that CINA and ADCC titers differed between vaccine recipients and patients with natural genital HSV-2 infections. In most cases, CINA titers were two- to fourfold higher and ADCC titers were 20- to 50-fold higher in patients with naturally acquired HSV-2 infections than in those vaccinated with HSV-2 glycoproteins (18). Nonglycoproteins may be required for development of maximal titers of ADCC and CINA antibodies. Alternatively, presentation and dose of antigen in natural infection may differ from the form and amount of antigen presented in an injection of solubilized proteins. For example, gG appears to be more immunogenic in a natural infection than in soluble form in the vaccine. The dose response of CINA as well as that of gD-specific antibody in 50- versus 100-μg vaccine recipients would support the suggestion that dose affects both the magnitude and the duration of antibody detected by CINA and RIP-PAGE.

These studies document the immunogenicity of solubilized HSV-2 glycoproteins gB, gD, gC, and, possibly, gE in humans and describe a sequential antibody response similar to that seen in natural infection. The fluctuations in apparent titer to gD and g80 over time were dose dependent. The possible protective in vivo function of antibodies elicited by the vaccine glycoproteins is largely unknown and will be determined by ongoing double-blind efficacy trials.

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