Isolation of the Major Herpes Simplex Virus Type 1 (HSV-1)-Specific Glycoprotein by Hydroxylapatite Chromatography and Its Use in Enzyme-Linked Immunosorbent Assay for Titration of Human HSV-1-Specific Antibodies

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A 131,000 molecular weight herpes simplex virus type 1 (HSV-1) glycoprotein designated antigen number 6 (Ag-6) was previously shown to possess almost exclusively HSV-1-specific antigenic sites. Fused rocket and crossed immunoelectrophoresis of fractions obtained from hydroxylapatite chromatography of crude HSV-1 antigen (Triton X-100-solubilized, infected tissue culture cells) showed that a subtraction of Ag-6 could be separated from the other HSV antigens. Enzyme-linked immunosorbent assay with the isolated Ag-6 showed that sera from rabbits infected with HSV-1 and HSV-1 human antisera contained antibodies to Ag-6, whereas sera from HSV-2-infected rabbits and sera from patients with primary HSV-2 infections did not react with Ag-6. Enzyme-linked immunosorbent assay of 852 human sera for antibodies to HSV type-common glycoproteins, Ag-6, and HSV-2-specific antigens showed that 139 sera which reacted negatively with HSV type-common glycoproteins also did not react with Ag-6 or with HSV-2-specific antigens. The 713 sera reacting positively to HSV type-common antigens either reacted with Ag-6 (328 sera) or with HSV-2-specific antigens (31 sera) or both (354 sera). This means that Ag-6 might be useful in large-scale human serology for the detection of past infection with HSV-1, irrespective of whether or not past infection with HSV-2 has occurred.

Exact identification and titration of herpes simplex virus (HSV) type-specific antibodies in human sera is necessary to study the epidemiology of HSV type 1 (HSV-1) and HSV-2 infections and to correlate these infections with premalignant and malignant changes in the uterine cervix (6).

Crossed immunoelectrophoretic analysis of the antigenic composition of HSV-1 and HSV-2 has shown that the two virus types have two major glycoproteins in common, whereas the type-specific antigenic sites are distributed on four other glycoproteins (12, 16). One of these glycoproteins, designated antigen number 6 (Ag-6), was shown to possess HSV-1-specific antigenic determinants almost exclusively (15). Ag-6, which is a 131,000 molecular weight glycosylated polypeptide (15), is probably identical to the “C” polypeptide described by Spear (8) and the HSV-1-specific envelope polypeptide described by Courtney and Powell (1).

Quantitative immunoelectrophoretic analysis of human antibodies to the type-common and type-specific HSV glycoproteins showed that humans had antibodies to Ag-6 (9). Ag-6 therefore fulfilled the two criteria necessary for its usefulness in human HSV type-specific serology, namely type specificity and immunogenicity.

This paper describes a simple procedure for isolation of a subtraction of Ag-6 and the results obtained with 852 sera titrated for antibodies to Ag-6 by enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Virus, cell culture, and crude antigen. HSV-1 (F) was propagated in a rabbit cornea cell line (4). Solubilized viral antigens were prepared from the cells infected at a multiplicity of 5 plaque-forming units per cell. The cells were scraped off the glass surface 24 h after infection and solubilized in 5% Triton X-100 as previously described (10). The crude antigen preparation had a protein content of about 10 mg/ml.

Hydroxylapatite chromatography. A column (5.3 by 3.5 cm², Bio-gel HTP, Bio-Rad Laboratories, Richmond, Calif.) prepared according to the recommendations of the manufacturer was equilibrated with a phosphate buffer (5 mM K2HPO4, 5 mM NaH2PO4, 15 mM NaN3, 0.05%, wt/vol, Triton X-100, pH 6.8). A 5-ml amount of the crude HSV-1 antigen was dialyzed against the equilibration buffer overnight and applied to the column. The eluted proteins were monitored at 280 nm by Uvicord equipment (LKB, Bromma, Sweden), and 2.5-ml fractions were collected with an Ul-
torac (LKB) fraction collector. The first 12 fractions were eluted with the equilibration buffer. Fractions 13 to 40 were eluted with a continuous phosphate gradient from 0.01 to 0.50 M. The gradient was formed by mixing the equilibration buffer with increasing amounts of 0.50 M of the same buffer in an Ultrograd apparatus (LKB). The flow rate was 15 ml/h (Fig. 1).

Rabbit antibodies. Rabbits were inoculated intracutaneously with HSV-1 (F)-infected or HSV-2 (G)-infected rabbit cornea cells as previously described (10). None of the rabbits were pretreated for cross-reacting antibody to HSV. The HSV-1 and HSV-2 rabbit antiserum (designated Ra-1 and Ra-2) used in ELISA came from animals that had received several booster inoculations. The rabbit HSV-1 antibodies used in the fused-rocket immunoelectrophoresis (Fig. 2A) were purified from rabbit antiserum Ra-1 by a method similar to that of Harboe and Ingild (3).

Human sera. The anti-HSV-1 human serum pool, designated Hu-1, was made from 12 anti-HSV, high-titer prepuperty sera from children. None of the sera gave any reaction in our HSV-2-specific ELISA (13). The anti-HSV-2 human serum pool, designated Hu-2, consisted of three sera, one serum from a 1-year-old child surviving neonatal herpes, and two convalescent-phase sera from adults with primary genital herpes. In all three cases, the virus was isolated and found to be HSV-2.

The 852 sera tested in ELISA came from adult women; 480 sera were from Danish women for whom a history of past herpes lesions was unknown, and 372 sera were from women in the United States with known genital herpes.

Fused-rocket immunoelectrophoresis. The use of fused-rocket immunoelectrophoresis for identification of antigens subjected to separation procedures has been described elsewhere (11). In the present experiment, electrophoresis was carried out on a glass plate (9 by 11 cm) covered with a 1.5-mm-thick gel of 1% (wt/vol) agarose (HSB, M, = ~0.10; Litex, Denmark). The first-dimensional electrophoresis was done in 1% 36 mM Tris (Hepes, pH 7.4)- and 36 mM MOPS (pH 7) buffer, pH 7.6, with 3 mM NaCl, 0.4 mM calcium lactate, and 1% (vol/vol) Triton X-100. A 30-μl amount from each fraction was placed in each of the 40 holes in the sample gel (see Fig. 2A). The samples were then electrophoresed into the antibody-containing gel (12.5 μl of purified rabbit immunoglobulin to HSV-1 per cm²) at 1.5 V/cm for 16 h, after which the plate was pressed, dried, and stained with Coomassie brilliant blue.

Crossed immunoelectrophoresis. Crossed immunoelectrophoresis was carried out as described in previous publications (10, 15, 16). The immunoelectrophoreses shown in Fig. 3 were done on glass plates (7 by 10 cm), using the same agarose and buffer described above. The first-dimensional electrophoresis was done in a 1.50-mm-thick gel slab (2 by 7 cm) at 10 V/cm for 90 min. The intermediate gel (2 by 7 cm) was 1.25 mm thick, and the second-dimensional gel (6 by 7 cm) containing 10 μl of human immunoglobulin G (IgG) per cm² (5% solution of Gamma Venin, Behringwerke, Germany), was 1 mm thick. The second-dimensional electrophoresis was done at 1.5 V/cm for 16 h.

ELISA. ELISA was performed in microtiter plates. A 100-μl amount of a 1:20 dilution of each fraction was added to the wells after the plate had been pretreated with bovine serum albumin and glutaraldehyde as previously described (14). A 100-μl amount of rabbit or human serum diluted 1:100 was applied to the wells for 1 h at 37°C. A 100-μl amount of peroxidase-conjugated swine anti-rabbit IgG (P2190, DAKO-immunoglobulins Ltd., Copenhagen) diluted 1:100 or peroxidase-conjugated rabbit anti-human IgG (P1090, DAKO-immunoglobulins Ltd., Copenhagen) diluted 1:200 was applied for 1 h at 37°C for the detection of rabbit or human IgG, respectively. Washings were done with phosphate-buffered saline, 0.05% Tween-20, and sera, and conjugates were diluted in the same buffer with 0.5% bovine serum albumin. The plates were washed 12 times by hand between each step. The substrate was orthophenylendiamine/acetobrodrochloride as previously described (14). The ELISA for HSV type-common antibodies was done as described previously (14). The ELISA for HSV-2-specific antibodies was done by the blocking method described recently (13). Ag-6 (the pooled fractions 5, 6, and 7) was employed at a dilution of 1:20.

RESULTS

Fractions 3 to 13, which were eluted before the gradient was applied, contained a material with very high absorption at 280 nm (Fig. 1). This absorption peak was caused almost exclusively by the excess Triton X-100 in the crude HSV-1 antigen because the detergent was not removed by dialysis. A similar absorption pattern was seen when control antigen (Triton X-100-solubilized, noninfected cells) or plain equilibrium buffer with 5% Triton X-100 was passed through the column.

After the excess Triton X-100 had passed through the column and the gradient was applied, two major broad protein peaks appeared, one with maximal absorption at fractions 27 and 28 and one with maximal absorption at fractions 36 and 37 (Fig. 1).

Fig. 1. Hydroxylapatite chromatography of crude HSV-1 antigen. Elution profile at 280 nm (——); phosphate gradient (——)
Figure 2. Immunoprecipitating profile and ELISA of 40 fractions obtained by hydroxylapatite chromatography of crude HSV-1 antigen. (A) Fused-rocket immunoprecipitation pattern (Fig. 2A) clearly shows that a single precipitating antigen eluted together with the Triton X-100 peak (fractions 5 to 11). After the gradient was applied, a number of HSV antigens appeared, one of which showed the reaction of antigenic identity with the antigen eluted together with the Triton X-100.

Only Ra-1 reacted with the antigen eluted in fractions 5 to 11 (Fig. 2B). As soon as the other HSV antigens appeared from fractions 15 and on, both Ra-1 and Ra-2 showed positive reactions.

The two human serum pools had reactions similar to those of Ra-1 and Ra-2 (Fig. 2C). However, it can be seen that, whereas Ra-1 shows a broad reaction with fractions 5 to 11, the human HSV-1 antibodies reacted with fractions 5, 6, and 7 only.

Figure 3A shows the crossed immunoelectrophoretic pattern of crude HSV-1 antigen with human antibodies. As shown previously (9), strongly reacting antibodies are present to the two HSV type-common glycoproteins Ag-8 and Ag-11, whereas antibodies to Ag-6 are somewhat weaker.

Figure 3B shows the identification of Ag-6 in the precipitating profile of the human sera. A comparison of plates A and B shows that incorporation of monospecific rabbit antibodies to Ag-6 into the intermediate gel caused a lowering of the Ag-6 immunoprecipitate.

Immunoelectrophoresis with the pooled fractions 5, 6, and 7 produced only a single immunoprecipitate migrating with the same first dimensional electrophoretic velocity as the anodic part of the Ag-6 precipitate (Fig. 3C).

A comparison of plates D and B shows that addition of the pooled fractions 5, 6, and 7 to the crude HSV-1 antigen resulted in a rise of the Ag-6 immunoprecipitate. This indicates that the antigen present in the pooled fractions was antigenically identical to the Ag-6 present in the crude HSV-1 antigen. A comparison of plates D and B also shows that Ag-6 was the only precipitate which was enhanced. This means that the pooled fractions contained Ag-6 exclusively.

Figure 4 shows that 170 sera did not react with Ag-6. 139 of these sera were completely negative for HSV antibodies, whereas the remaining 31 sera had antibodies to HSV-2-specific antigens. The distribution of antibody titers to Ag-6 of the remaining 682 sera shows that most of the sera had a medium titer and that fewer sera had either a very low or a very high titer. Figure 4 also shows the distribution of Ag-6 antibody titers of the 328 sera without HSV-2 antibodies and the 354 sera with HSV-2 antibodies. It can be seen that sera without HSV-2 antibodies reacted more weakly with Ag-6 (arithmetic mean titer, 0.48) than did sera with dual antibodies (arithmetic mean titer, 0.60). The difference in the distribution of titers to Ag-6 in the two groups of sera was evaluated by the chi-square test and found to be statistically significant (P < 0.01).

Fifteen sera had a very low titer of Ag-6 antibodies (optical density values ranged from 0.05 to 0.09). Of these sera 13 had no antibodies to HSV-2 and had a very low antibody titer to HSV type-common glycoproteins.

The 139 sera (of the 852 sera tested) that reacted negatively with the HSV type-common antigens also reacted negatively both with Ag-6 and HSV-2-specific antigens. The remaining 713 sera reacting positively with HSV type-common antigens either reacted with Ag-6 (328 sera) or with HSV-2-specific antigens (31 sera) or both (354 sera).

DISCUSSION

When an isolated viral subtype antigen is used
FIG. 3. Crossed immunoelectrophoresis with human antibodies in the second-dimensional gel. The numbering of antigens is in accordance with earlier publications (12, 13, 16). A and B first dimension: 30 μl of crude HSV-1 antigen. Intermediate gel A: 5 μl of normal rabbit IgG per cm². Intermediate gel B: 5 μl of monospecific rabbit anti Ag-6 antibodies per cm². C first dimension: 45 μl of Ag-6 (pooled fractions 5, 6, and 7) and intermediate gel as B. D first dimension: 30 μl of crude HSV-1 antigen plus 30 μl of Ag-6. Intermediate gel as B.

FIG. 4. Histogram of optical density values of 852 human sera reacting with Ag-6 in ELISA.

in large-scale human serology, several questions arise that should be answered.

First, is the antigen properly identified? On the basis of the results presented it is safe to conclude that the antigen is indeed Ag-6.

Second, is the antigen truly HSV-1 specific? In our previously published work concerning the immunological and biochemical characterization of Ag-6 (15), it was concluded that Ag-6 possesses some very strong HSV-1-specific determinants, but the presence of some weak type-common antigenic determinants on the same protein has not been ruled out.

The first thing to consider in regard to type specificity is the sensitivity of the assay used. A test of low sensitivity (i.e., immunoprecipitation) might show that a given antigen is type specific (16), whereas a high-sensitivity assay might detect cross-reactivity. The ELISA is a very sensitive method for the detection of antigen-anti-
body reactions (17), and sera from HSV-infected rabbits gave an HSV-1-specific reaction to Ag-6 present in the first 11 fractions (Fig. 2B). However, animals experimentally infected with HSV might not develop the same antibody response as the chronically infected natural host, and the results with Ra-1 and Ra-2 can only be considered suggestive. The human serum pool also gave an HSV-1-specific reaction (Fig. 2C), and this result together with the fact that we found 31 individual human HSV-2 antisera that did not react with Ag-6 at all, suggests that Ag-6 possesses almost exclusively HSV-1-specific antigenic determinants. The reason for this high specificity of the Ag-6 in the Triton X-100 peak might be that it represents a subfraction of Ag-6. About half of the Ag-6 eluted with the Triton X-100, and the other half eluted after the gradient had been applied (Fig. 2A). The possibility exists that the latter subfraction of Ag-6 might possess some type-common antigenic determinants.

The third question is whether Ag-6 is representative; i.e., do all humans with HSV-1 infections develop antibodies to Ag-6? In regard to the 682 sera in this study reacting to Ag-6, the answer is yes. Serum from an individual infected with HSV-1 who does not develop Ag-6 antibodies should react to our HSV type-common antigens without reacting to Ag-6 or to the HSV-2-specific antigens. As stated in Results, such a serum was not found.

It should be kept in mind that human sera have higher titers to HSV type-common antigens than to HSV type-specific antigens (9). The reason that we were able to detect Ag-6 antibodies with the same level of sensitivity as antibodies to HSV type-common antigens was that the concentration of Ag-6 in the ELISA was about 10 times higher than the concentration of type-common antigens employed. This calculation was based on quantitative immunoelectrophoretic measurements by the use of monospecific rabbit antibodies to Ag-6 and to the type-common HSV glycoproteins Ag-8 and Ag-11 (16).

We found that sera with dual antibodies had a higher antibody titer to Ag-6 than did sera containing HSV-1 antibodies only. This finding contradicts the findings of Smith et al. (7) and McClung et al. (5), who by using 51Cr release tests reported that sera with dual antibodies had lower titers of HSV-1-specific antibodies than did sera containing HSV-1 antibodies only. However, Forghani et al. (2) reported that their radioimmunoassay for HSV type-specific antibodies showed that infection with one HSV type did not suppress the type-specific antibody response to a later infection with the heterologous HSV type.

The explanation as to why sera with dual antibodies react more strongly to Ag-6 than to pure HSV-1 sera could be that dual infections boost antibodies to common determinants, and it has not been completely ruled out that a few common determinants might not be present on Ag-6. The strong reactivity of sera with dual antibodies to Ag-6 is practical from a serological point of view. Only two of the 354 sera with dual antibodies had such a low titer to Ag-6 that doubts could be raised about past infection with HSV-1.

Our conclusion is that Ag-6 can be used in serology for the detection of past human infection with HSV-1, irrespective of whether infection with HSV-2 has occurred in the past.

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LITERATURE CITED


