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Crossed Immunoelectrophoretic Identification of Partially Purified Type Common and Type Specific Herpes Simplex Virus Glycoprotein Antigens (39934)

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Introduction. Several investigators have reported the presence of herpes simplex virus (HSV) type common and type specific precipitating antigens (1-4). Crossed immunoelectrophoretic analysis of HSV antigens obtained from infected cells solubilized with the nonionic detergent Triton X-100 has demonstrated the presence of a group of HSV glycoproteins (5).

Several independent studies have shown that HSV glycoproteins play an important immunological role by demonstrating that antibodies against these antigens are able to neutralize the virus particle (6-9). In a previous study we have shown that the membrane-bound HSV glycoprotein antigens could be partially purified and separated from other HSV antigens by ion-exchange chromatography (10).

The present study was done to determine the type specificity and antigenicity of chromatographically purified HSV glycoproteins because the antigens are useful in human serology (Vestergaard, in preparation) and might also be of potential use as vaccines.

Materials and methods. Viruses, cell cultures, reference antigens, and antibodies. HSV type 1 (MacIntyre) and HSV type 2 (MS) were propagated in a rabbit cornea cell line (11). The cells were scraped off the glass surface 24 hr after infection with HSV type 1 or type 2 at a multiplicity of 5 and solubilized in 5% Triton X-100, as described earlier (4, 5). This crude antigenic preparation had a protein content of about 10 mg/ml and is referred to as reference antigen. Radioactively labeled HSV antigens were made similarly, except that the isotope (^{14}C-labeled protein hydrolysate, Amersham, England) was added to the maintenance medium in a concentration of 1 μCi/ml 3 hr after infection. Rabbit antisera against HSV types 1 and 2 were made in rabbits, as described earlier (4), and a purified immunoglobulin preparation was made from pools of whole serum by the method of Harboe and Ingild (12). This preparation is referred to as reference antibodies.

Ion-exchange chromatography. Reference antigen was fractionated on DEAE-cellulose columns, as described earlier (10). The antigens eluted immediately after void volume in the first three fractions represented the major part of the membrane-bound HSV glycoproteins, and this partially purified preparation is referred to as pool 1.

Crossed immunoelectrophoresis. This technique has been described in detail elsewhere (4, 5, 10), but short descriptions of methodology are included in the legends to figures.

Rabbit antisera against pool 1. Pool 1 from HSV type 1 or 2 was mixed with an equal amount of incomplete Freund's adjuvant and inoculated intracutaneously on the back of each of three rabbits. Three injections of 0.2 ml each were given 2 weeks apart and the animals bled 1 week after the last injection.

Plaque reduction multiplicity analysis. This was done by mixing approximately 1000 plaque-forming units (PFU) of HSV type 1 or type 2 with twofold dilutions of antiserum. After 30 min of incubation at 37°, the plaque reduction obtained at the different dilutions was assayed on rabbit cornea cells overlaid with maintenance medium containing 1% methyl cellulose and 2% human serum with high titer antibodies against HSV types 1 and 2.

Guinea pig antisera. An area of about 5 cm^2 on the backs of adult female guinea pigs was depilated. The skin was scarified with sandpaper and rubbed with a cotton-tipped stick soaked in tissue culture fluid from HSV type 1- or type 2-infected cell cultures. The animals were bled 2 to 3

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weeks later, when the resulting skin lesions were healed, and sera from animals infected with the same virus type were pooled and tested in crossed immunoelectrophoresis with intermediate gel (13, 14). By this method the antiserum to be examined is incorporated in a gel slab placed between the first- and second-dimensional electrophoresis. Specific antibodies present in such an intermediate gel will cause a lowering of the corresponding precipitates in the crossed immunoelectrophoretic profile.

Results. Figure 1A shows the precipitating pattern of HSV type 1 antigens. The numbering of antigens is in accordance with previously published papers (4, 5, 10). Figure 1B shows that antigens Ag6, Ag7, Ag8, and Ag11 are present in pool 1 from HSV type 1. Figure 1C shows the precipitating pattern of HSV type 2 antigens. The num-

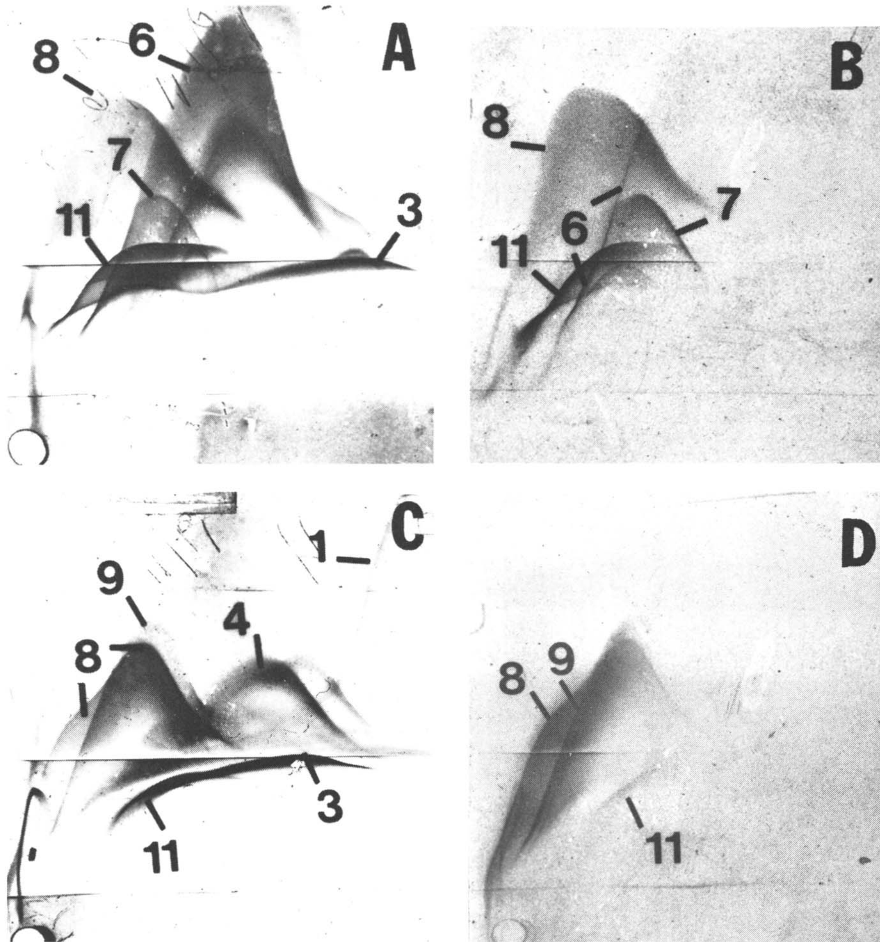

FIG. 1. Crossed immunoelectrophoresis of mixtures of reference HSV antigens and ^{14}C-labeled pool 1. Gel: 1% (w/v) agarose (HSB, Mr = −0.10 Litex, Denmark) dissolved in 0.180 *M* Tris–0.060 *M* Barbital, pH 8.6, containing 1% Triton X-100. First-dimensional electrophoresis: 30 μl of reference antigen plus 5 μl of pool 1 coelectrophoresed in 1.5-mm-thick gel at 10 V/cm for 90 min, anode to the right. Second-dimensional electrophoresis: Performed on 7 × 7-cm glass plates for 16 hr at 1.5 V/cm in 1-mm-thick gel containing 12 μl/cm^2 of the reference anti-HSV type 1 and 2 antibody preparation. Intermediate gel: Gel slab, 7 × 1.5 cm large and 1.25 mm thick, without antibodies. (A) Coomassie brilliant blue (CBB) staining of HSV type 1 reference antigen coelectrophoresed with ^{14}C-labeled pool 1 from HSV type 1. (B) Autoradiographic picture of the same plate. (C) CBB staining of HSV type 2 reference antigen coelectrophoresed with pool 1 from HSV type 2. (D) Autoradiographic picture of the same plate.

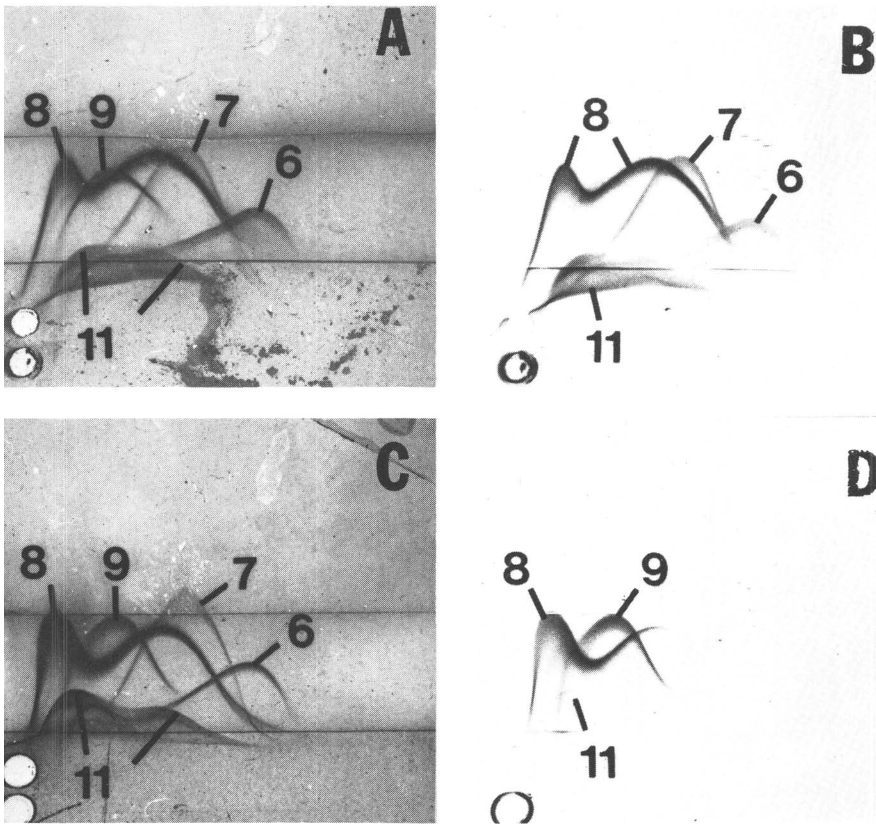

FIG. 2. General technique as in Fig. 1. Both intermediate gel and second-dimensional gel contain 12 μl/cm² of the reference anti-HSV type 1 and 2 antibody preparation. (A) CBB staining of 15 μl of ^{14}C-labeled pool 1 from HSV type 1 coelectrophoresed with 15 μl of nonlabeled pool 1 from HSV type 2. (B) Autoradiographic picture of the same plate. (C) CBB staining of 15 μl of nonlabeled pool 1 from HSV type 1 coelectrophoresed with 15 μl of ^{14}C-labeled pool 1 of HSV type 2. (D) Autoradiographic picture of the same plate.

bering of antigens is in accordance with previously published papers (4, 5, 10). Figure 1D shows that antigens Ag8, Ag9, and a trace of Ag11 are present in pool 1 from HSV type 2.

Figure 2A shows that all the identified antigens in pool 1 from HSV type 1 and type 2 are present as Coomassie brilliant blue-stained precipitates. The autoradiogram of this plate (Fig. 2B) shows that Ag9 is not present among the labeled antigens of pool 1 from HSV type 1. Figure 2C shows likewise that all antigens from both virus types are present, but the autoradiogram of this plate (Fig. 2D) shows that Ag6 and Ag7 are not present among the labeled pool 1 antigens from HSV type 2.

Figure 3A shows that the precipitates of Ag6, Ag7, Ag8, and Ag11 start at the borderline between the first-dimensional gel and the intermediate gel, indicating that the guinea pig antiserum contains antibodies reacting with these antigens. The precipitate of Ag9, however, only extends halfway down the intermediate gel (caused by the cathodic migration of rabbit immunoglobulins from the second-dimensional gel), indicating that serum from guinea pigs infected with HSV type 1 does not contain antibodies reacting with Ag9. Figure 3B shows that Ag6 and Ag7 have moved up from the borderline between the first-dimensional gel and the intermediate gel. The anodic part of the Ag8 precipitate has also been lifted, but the cathodic part of the precipitate still reaches the baseline and so does the precipitate of Ag11. This indicates that guinea pigs infected with HSV type 2 developed

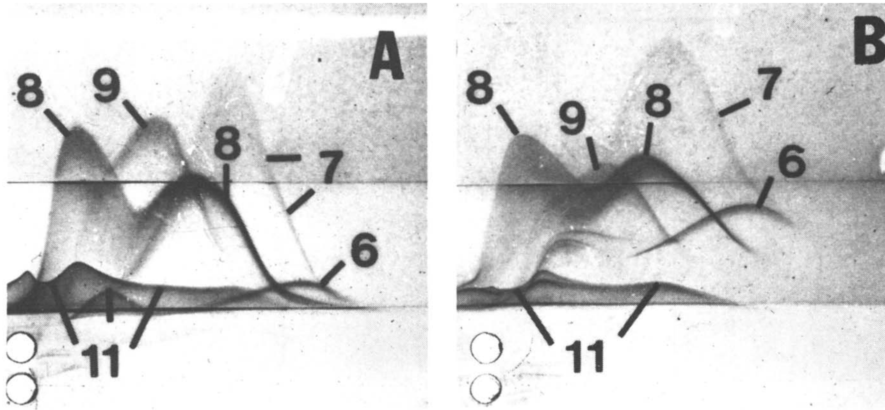

FIG. 3. General technique as in Figs. 1 and 2, with 12 μl/cm² of the reference anti-HSV type 1 and 2 antibody preparation in the second-dimensional gel. CBB staining of 15 μl of pool 1 from HSV type 1 coelectrophoresed with 15 μl of pool 1 from HSV type 2. (A) Intermediate gel with 15 μl/cm² of the guinea pig antiserum pool against HSV type 1. (B) Intermediate gel with 15 μl/cm² of the guinea pig antiserum pool against HSV type 2.

antibodies against Ag11 and the cathodic part of Ag8, but not against Ag6 and Ag7. The peak of the Ag9 precipitate as seen in Fig. 3B has been lowered considerably from the position in Fig. 3A, but the precipitate is not clearly visible at the borderline between the intermediate and first-dimensional gels. This finding suggests that the HSV type 2-infected guinea pigs did produce antibodies against Ag9, but of low avidity.

Figure 4A and B show that animals immunized with pool 1 antigens from HSV type 1 developed antisera with higher neutralizing potency than type 2-immunized animals, and both type 1 and type 2 rabbit antisera showed a greater capacity to neutralize homologous than heterologous virus.

Discussion. Ag8 was found to be an electrophoretically heterogeneous protein with an anodic and a cathodic peak, but the continuous precipitation line shows that there is antigenic identity between the two components (Figs. 2 and 3). Ag8 from HSV type 1 was, in the purified preparation, distributed with an overweight on the anodic part, while Ag8 from HSV type 2 was confined to the cathodic part (Figs. 2B and D). This finding, combined with the fact that antisera from guinea pigs infected with HSV type 2 mainly reacted with the cathodic part of the precipitate (Fig. 3B), suggests that Ag8, besides having strong

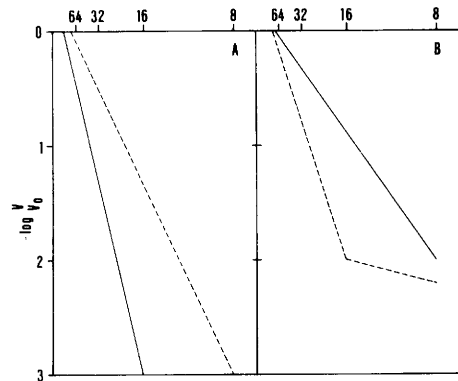

FIG. 4. Multiplicity analysis of the neutralizing potencies of rabbit anti-HSV type 1 and type 2 antisera. Each line represents the average of three individual sera. The same HSV type 1 and type 2 viral preparations were used in all tests. (A) Sera from rabbits immunized with pool 1 from HSV type 1. (B) Sera from rabbits immunized with pool 1 from HSV type 2. HSV type 1 (——); HSV type 2 (-----).

type common antigenic determinants, also might possess some minor HSV type 1 specific determinants.

As seen in Figs. 1A and B, 2, and 3, Ag11 is a complex of several antigens produced in large amounts in HSV type 1-infected cells, but only in minute amounts in HSV type 2-infected cells. Recently, we have shown that monospecific antisera against Ag8 and Ag11 have a strong neutralizing effect on both HSV types (Vestergaard, in preparation). It

is therefore reasonable, despite the heterogeneity of Ag8 and the quantitative type difference concerning Ag11, to assume that most of the cross neutralization between general antisera against HSV types 1 and 2 is caused by antibodies against these two major glycoproteins.

Immunoelectrophoretic analysis of the HSV antigens present in pool 1 showed that Ag6 and Ag7 were type 1 specific and Ag9 was type 2 specific (Fig. 2). This result was confirmed by analysis of the antibodies produced in HSV type 1- and type 2-infected guinea pigs (Fig. 3). This latter finding is important because antigens in amounts too small to be detected by the immunoelectrophoretic technique (i.e., from 10 to 100 ng of protein) (15, 16) still might elicit an antibody response during natural infection. In our first paper describing the crossed immunoelectrophoretic analysis of HSV antigens, Ag11 had not been detected in HSV type 2 preparations (4). However, animals experimentally infected with HSV type 2 consistently developed antibodies against Ag11, thus giving evidence that Ag11 indeed was an HSV type common antigen.

Nonionic detergents are capable of solubilizing membrane proteins without destroying the macromolecular structure and antigenicity (17), which is supported by our finding that rabbits immunized with pool 1 developed viral-neutralizing antibodies.

Rabbits immunized with the HSV type 1 preparation, containing Ag6, Ag7, Ag8, and Ag11, developed a stronger serum-neutralizing potency against HSV type 1 than type 2, and this could be explained by the presence of Ag6 and Ag7 in that preparation. Our recent finding that a monospecific antiserum against Ag6 neutralized HSV type 1, but not HSV type 2, supports such an interpretation (Vestergaard and Norrild, in preparation).

Rabbits immunized with the HSV type 2 preparation developed the strongest serum-neutralizing potency against the homologous virus, and this result might partly be due to the presence of Ag9 in that preparation.

We have recently shown that each HSV protein forming a well-defined precipitate by immunoelectrophoresis in Triton X-100 containing agarose gel is composed of several different polypeptides (18). The combination of immunoelectrophoretic analysis and neutralization data can identify the native viral protein entities that have immunological importance. Separation of the individual HSV type specific antigens present in pool 1, for use in human serology is now in progess.

Summary. HSV glycoprotein antigens obtained from Triton X-100-solubilized type 1- and type 2-infected cells were partially purified by ion-exchange chromatography and analyzed by crossed immunoelectrophoresis in Triton X-100 containing agarose gel. Two HSV type common, two HSV type 1 specific, and one HSV type 2 specific antigens were identified. Rabbits immunized with the HSV glycoprotein preparations developed strong viral-neutralizing antibodies.

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