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Quantitative Immunoelectrophoretic Analysis of Human Antibodies Against Herpes Simplex Virus Antigens

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By use of crossed immunoelectrophoresis with intermediate gel, antibody titers against six individual herpes simplex virus (HSV) glycoproteins and two non-glycosylated proteins were determined in 100 human sera. High antibody titers were found against two different HSV type-common glycoproteins designated Ag8 and Ag11 (containing glycosylated polypeptides D and B, respectively). The anti-Ag8 and -Ag11 titers correlated with HSV neutralizing antibody titers. Most of the serological cross-reactivity between HSV type 1 and type 2 was probably caused by antibodies to Ag8 and Ag11. Human antibodies against one HSV type 1-specific glycoprotein (Ag6, containing glycosylated polypeptide C) and two HSV type 2 glycoproteins (Ag4 and Ag9) were also demonstrated, and the titers correlated better with neutralizing antibody titers of the homologous than of the heterologous virus type. The data presented can be directly applied to the further development of diagnostic reagents.

A number of distinct herpes simplex virus (HSV) glycoproteins are present in the plasma membrane of infected cells and on the outer surface of enveloped virions (9, 10, 15, 16). Little effort has so far been made to identify the individual HSV antigens of immunological importance in humans, except for two independent studies which showed that humans had higher antibody titers against HSV envelope antigens than against nucleocapsid antigens (6, 11).

Crossed immunoelectrophoresis of HSV antigens extracted from infected cells with nonionic detergents has shown that both HSV-infected and immunized rabbits develop antibodies to a number of individual HSV antigens (18). Recently, we have demonstrated that five monospecific rabbit antisera against five different HSV glycoproteins were all capable of neutralizing the virus particle (B. F. Vestergaard and B. Norrild, International Agency for Research on Cancer [IARC] publication, in press). Furthermore, we were able to rate the glycoproteins according to their ability to function as targets for neutralizing antibodies.

Clarification of the immunological importance of the different HSV glycoproteins in naturally infected humans is pertinent for the development of potent and well-defined serological reagents. The aim of this work was therefore to detect the number, specificities, and titers of human antibodies against individual HSV structural proteins.

MATERIALS AND METHODS

Virus, cell cultures, standard antigens, and standard antibodies. HSV type 1 (F) and HSV type 2 (G) were propagated in a rabbit cornea cell line (13). Solubilized viral antigens were prepared from cells infected with a multiplicity of 5 plaque-forming units of either HSV type 1 or type 2 per cell. Radioactive labeling of the antigens was done by adding ^{14}C-labeled protein hydrolysate (Amersham, England) to the maintenance medium in a concentration of 1 μCi/ml 3 h after infection. The cells were scraped off the glass surface 24 h after infection and solubilized in 5% Triton X-100, as described earlier (17, 18). These crude preparations of HSV type 1 and type 2 antigens had a protein content of about 10 mg/ml and are referred to as standard antigens.

Antibodies to HSV were produced in rabbits inoculated intracutaneously with HSV type 1- or type 2-infected rabbit cornea cells, as described earlier (17, 18). A purified immunoglobulin preparation was made from pools of whole sera by the method of Harboe and Ingild (8). This antibody preparation containing antibodies against both HSV types is referred to as standard antibodies (SAb).

Human sera. Blood was drawn from the cubital vein and clotted overnight at 4°C, and the sera were stored at −20°C. The donors were women of 30 to 60 years of age, and their status concerning herpetic infections was not known.

Crossed immunoelectrophoresis with intermediate gel. This technique has been described in detail elsewhere (1, 3, 4, 19; Vestergaard and Norrild, IARC, in press). Each of the 280 crossed immunoelectrophoreses in the present work were done in the same way, using glass plates (7 by 7 cm) covered with 1% (wt/vol) agarose (HSB, M_r = −0.10, Litex, Denmark) dissolved in 36 mM Tris-12 mM barbital (pH 8.6) containing 1% (vol/vol) Triton X-100. The first-dimensional electrophoresis was done with 15 μl of HSV type 1 or type 2 standard antigen subjected to electrophoresis in a 1.5-mm-thick gel at 10 V/cm for 90 min with the anode to the right. The intermediate gel (7 by 1.5 cm large and 1.25 mm thick) was placed between the

first- and second-dimensional gels and contained 15 μl of the human serum to be tested per cm^2, or, in the control system, varying amounts of SAb. The second-dimensional gel (4.5 by 7 cm large and 1 mm thick), containing 12.5 μl of SAb per cm^2, was subjected to electrophoresis at 1.5 V/cm for 16 h.

Staining and autoradiography. The plates were stained with Coomassie brilliant blue after the gel had been washed and dried. A Kodirex X-ray film was placed over the gel and developed after 1 week of exposure. Only the autoradiographic pictures were used for measurements of the precipitates, because many human sera contained large amounts of lipids which gave a strong background staining with Coomassie brilliant blue.

The scoring system. It is possible to semiquantitate the amounts of antibodies in the intermediate gel directed against any of the HSV antigens precipitated in the second-dimensional gel, because the area underneath each precipitate is inversely proportional to the amount of the corresponding antibodies (5, 12). The titers of the human antibodies were expressed in arbitrary units which were derived from control plates with varying amounts of SAb in the intermediate gel, as described by Axelsen and co-workers (1, 3, 4). Five sets of control plates were made for both HSV type 1 and type 2 during the study. Each control set consisted of eight plates with the following amounts of SAb in the intermediate gel: 0, 25, 50, 100, 150, 200, 300, and 400 μl. The human antibody titers against each HSV antigen were expressed as the amount of SAb in microliters giving the same or closest height of the immunoprecipitate in question, measured from the border line between the first-dimensional gel and the intermediate gel. A lowering of a precipitate to more than 400 was considered 400.

Neutralization. Plaque reduction multiplicity analysis was done as described previously (19). The neutralizing titer was calculated as the reciprocal of the serum dilution causing a 90% reduction of the plaque count.

Statistics. In the present study, 12 different antibody titers were obtained for each of the 100 human sera examined (HSV type 1 and type 2 neutralizing titer and 10 antibody titers against 10 individual HSV antigens). The correlation between antibody titers against the HSV antigens and the neutralizing titers was calculated by Spearman's rank correlation coefficient.

RESULTS

The control system. Figure 1 shows the crossed immunoelectrophoretic pattern of HSV type 1 antigens. The numbering of precipitates is in accordance with earlier publications (17–19; Vestergaard and Norrild, IARC, in press). Ag6A and Ag3A have not been identified previously. It is clearly seen that the increasing concentration of SAb from Fig. 1A to C causes a lowering of the heights of all immunoprecipitates. Figure 2 shows the precipitating pattern of HSV type 2 antigens. Again the lowering of the immunoprecipitates caused by increasing amounts of SAb in the intermediate gel is obvious.

Table 1 shows the actual difference of height in millimeters obtained in the control system. It can be seen that the variation in height was different for the various antigens and also different for antigenically related antigens from HSV type 1 and type 2 (Ag3 and Ag8).

The plate-to-plate variation of the heights of Ag3, Ag4, Ag6, Ag8, Ag9, and Ag11 in the control system was less than or equal to 10% of the total height variation range obtained with SAb (0 to 400 μl) in the intermediate gel.

The test system. In this system, human sera were incorporated in the intermediate gel instead of SAb, and antibody titers to Ag3, Ag4, Ag6, Ag8, Ag9, and Ag11 were calculated by measuring the heights of the corresponding immunoprecipitates. Four of the 200 test plates are shown to demonstrate the variation of the HSV immunoprecipitating pattern obtained with human sera in the intermediate gel.

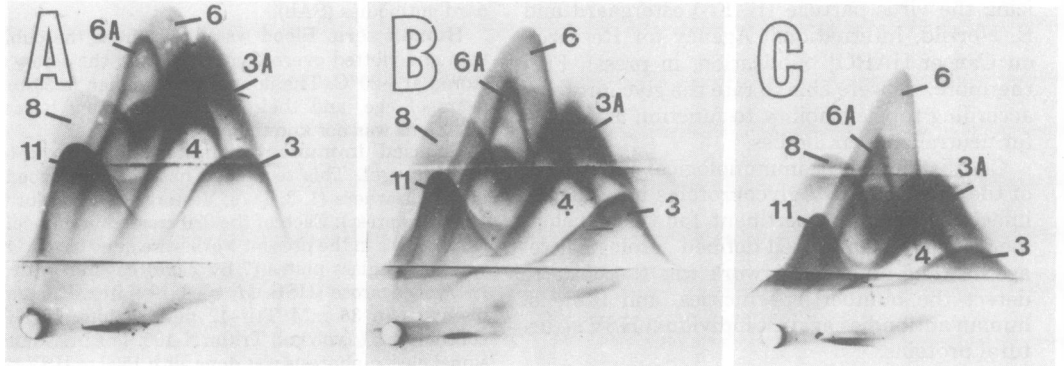

FIG. 1. *Crossed immunoelectrophoresis with intermediate gel; 15 μl of HSV type 1 standard antigen in the first-dimensional gel and 12.5 μl of SAb per cm^2 in the second-dimensional gel. The intermediate gel contained (A) 50, (B) 150, or (C) 300 μl of SAb.*

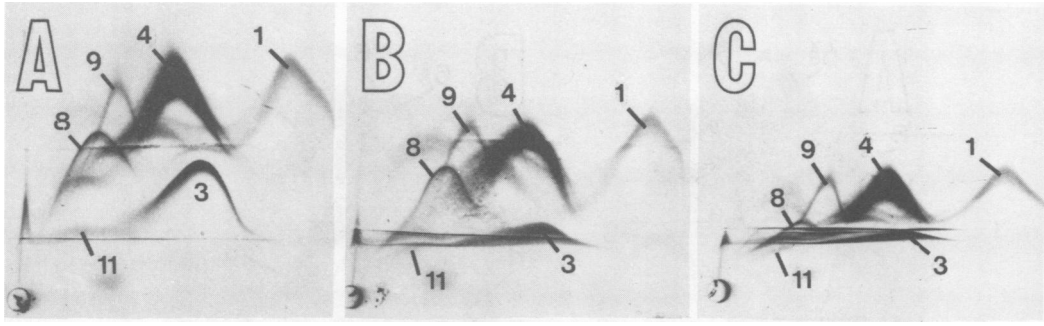

FIG. 2. *Identical to Fig. 1, but with 15 μl of HSV type 2 standard antigen in the first-dimensional electrophoresis.*

TABLE 1. *Height difference of HSV immunoprecipitates in the crossed immunoelectrophoretic control system*[a]

HSV type	Height (mm) at SAb concn in intermediate gel:		Total height difference (mm)
	None	400 μl	
Type 1			
Ag3	18	2	16
Ag6	40	28	12
Ag8	27	17	10
Ag11	20	7	13
Type 2			
Ag3	14	0	14
Ag4	32	8	24
Ag8	20	0	20
Ag9	25	7	18

[a] Numbers represent the arithmetic mean calculated from five different sets of gels.

Figure 3 shows the reactivity of two human sera with HSV type 1 antigens. Figure 3A shows that antibodies in the human serum reacted with Ag11 (titer 50), Ag3 (titer 100), and Ag3A, but not with Ag8 and Ag6. The neutralizing titer of the serum obtained with HSV type 1 was 30. The serum in Fig. 3B showed a very strong reaction against Ag11 (titer 400), Ag8 (titer 300), Ag6 (titer 200), and Ag3 (titer 200), but no reaction against Ag3A. This serum had an HSV type 1 neutralizing titer of 320.

Figure 4 shows the reactivity of two human sera with HSV type 2 antigens. The serum in Fig. 4A reacted with Ag8 (titer 200) and Ag9 (titer 100), but not with any other antigens except Ag11, which cannot be clearly seen. The neutralizing HSV type 2 titer of this serum was 35. The serum in Fig. 4B reacted with Ag8 (titer 400), Ag9 (titer 150), Ag4 (titer 40), and possibly Ag11. The serum had a neutralizing titer of 220.

Table 2 lists the HSV antigens in order of the corresponding antibody titers. It can be seen that highest titers were found against Ag8 derived from HSV type 2. Antibodies against Ag11 derived from HSV type 1 had the second highest titers.

Table 3 shows the degree of correlation between HSV neutralizing antibody titers and the antibody titers against the different HSV antigens. It can be seen that a positive correlation between the two kinds of antibody determination was found for all HSV antigens.

Antibody titers against Ag8 (derived from HSV type 2) and Ag3 (derived from HSV type 1 or type 2) gave almost similar correlation coefficient values with both HSV type 1 and type 2 neutralizing titers, whereas antibody titers against HSV type-specific antigens—Ag6 (type 1) and Ag4 and Ag9 (type 2)—correlated better with neutralizing antibody titers of the homologous HSV type. Antibody titers against the type-common Ag11 (derived from HSV type 1) correlated better with HSV type 1 neutralization than with HSV type 2 neutralization.

Among the 100 sera examined, 12 were without HSV neutralizing antibodies. None of these sera gave any reaction in the crossed immunoelectrophoretic assay.

DISCUSSION

Crossed immunoelectrophoresis with intermediate gel (1) is particularly suited for studies of human antibodies in infectious diseases, as it is possible by this method to obtain information about the number, specificities, and titers of antibodies directed against individual antigens of the infectious agent in terms of a reference system and without purification of the individual antigens (1, 3, 4). The reproducibility of quantitation by the crossed immunoelectrophoretic procedure depends solely on the use of a well-defined reference system (2). In the present study, the SAb was purified rabbit immunoglobulin prepared from a large pool of sera from

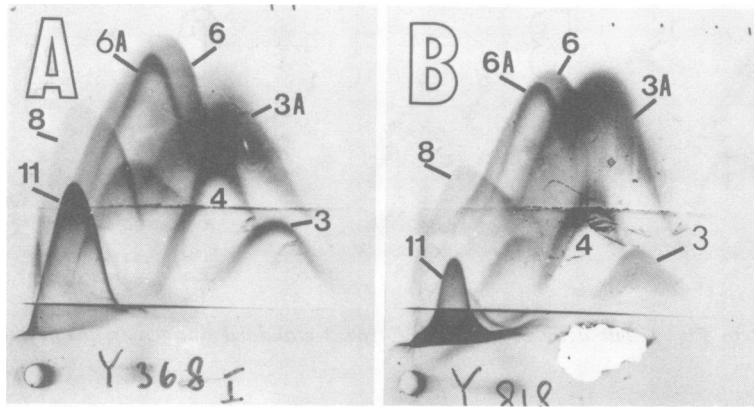

FIG. 3. *Identical to Fig. 1, but with human sera in the intermediate gels at a concentration of 15 μl/cm². (A and B) Precipitating patterns with two different sera.*

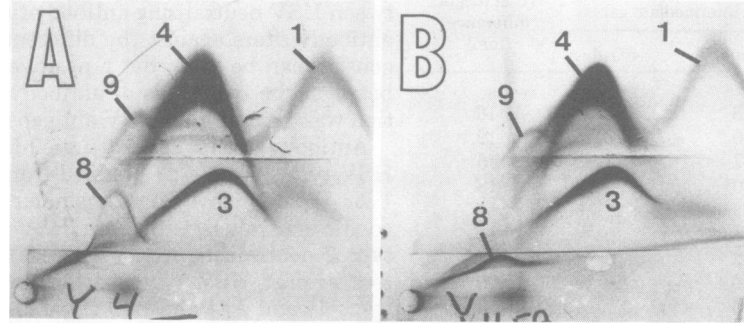

FIG. 4. *Identical to Fig. 2, but with human sera in the intermediate gels at a concentration of 15 μl/cm². (A and B) Precipitating patterns with two different sera.*

TABLE 2. *Mean and range of antibody titers in 100 human sera against eight individual HSV antigens*

Antigen (HSV type)	Mean	Range
Ag8 (2)	182	0–400
Ag11 (1)	100	0–400
Ag8 (1)	86	0–400
Ag6 (1)	80	0–400
Ag9 (2)	65	0–300
Ag4 (2)	35	0–150
Ag3 (1)	35	0–200
Ag3 (2)	30	0–150

TABLE 3. *Correlation between HSV neutralizing antibody titers and antibody titers to eight individual HSV antigens calculated from analysis of 100 human sera*

Antigen (HSV type)	HSV type 1 neutralization		HSV type 2 neutralization	
	Rs[a]	*P*	Rs[a]	*P*
Ag8 (2)	0.56	<0.001	0.58	<0.001
Ag11 (1)	0.57	<0.001	0.45	<0.001
Ag8 (1)	0.59	<0.001	0.44	<0.001
Ag6 (1)	0.52	<0.001	0.33	0.01 > *P* > 0.001
Ag9 (2)	0.34	0.01 > *P* > 0.001	0.53	<0.001
Ag4 (2)	0.31	0.02 > *P* > 0.01	0.51	<0.001
Ag3 (1)	0.57	<0.001	0.52	<0.001
Ag3 (2)	0.51	<0.001	0.58	<0.001

[a] Rs, Correlation coefficient.

rabbits immunized with HSV type 1 and type 2. This preparation was stable at 4°C, and it has been used in a previous study (19).

HSV antigen preparations made from different host cell systems infected with different strains of virus do not show any qualitative differences (17–19; Vestergaard and Norrild, IARC, in press), and the eight antigens listed in the present study are always found. The quantity of the different HSV antigens, however, varies considerably from batch to batch, because factors influencing the general state of the cell cultures used are difficult to standardize. It is therefore necessary to use the same batch of viral antigen throughout a study. The antigen

preparation was stable at −70°C but should be thawed once only.

By these precautions and the use of the same agarose gel batch in the entire study it was possible to minimize the plate-to-plate variation and obtain precise information about the amount of antibodies in human sera reacting with individual HSV antigens.

As shown in previous studies, Ag8 and Ag11 represent the two major HSV type-common membrane-bound glycoproteins (18, 19). Both proteins are composed of several polypeptides, of which at least one is glycosylated (14). According to a common nomenclature of HSV type 1 glycosylated polypeptides agreed upon at the Herpesvirus Workshop, Cambridge, 1978 (P. G. Spear, personal communication), Ag8 and Ag11 contain polypeptides D and B, respectively. The high immunogenic activity of Ag8 and Ag11 in humans could be expected for several reasons. (i) High antibody titers against HSV envelope antigens have been found in humans (6, 11). (ii) Monospecific rabbit antisera against Ag8 and Ag11 have been found to neutralize both HSV type 1 and type 2, indicating both of these antigens to be strongly represented on the outer viral envelope (Vestergaard and Norrild, IARC, in press). (iii) Quantitative calculations have shown that Ag8 and Ag11 are produced in large amounts in HSV-infected cells (18), and recent immunoelectron microscopic observations on HSV-infected cells using monospecific anti-Ag8 antibodies show that this antigen is localized not only at the nuclear membrane but also at the plasma membrane (Hansen et al., manuscript in preparation).

Ag8 derived from HSV type 2-infected cells was the HSV antigen found most responsive to human antibodies. Ag8 from HSV type 1 and HSV type 2 shows the reaction of antigenic identity by immunoelectrophoresis (17). However, recent results suggest that Ag8 from HSV type 1 might possess some type 1-specific determinants besides the strong common ones, whereas Ag8 from HSV type 2 has common antigenic determinants only (19). This could explain why human anti-Ag8 (type 2) titers are higher than the anti-Ag8 (type 1) titers.

Monospecific antibodies against Ag11 derived from HSV type 1-infected cells have a strong neutralizing effect on both virus types (Vestergaard and Norrild, IARC, in press), but Ag11 is only produced in very small amounts in HSV type 2-infected cells (19) and the precipitate was too small to be useful in the present study. It is most likely, then, that the considerations mentioned above concerning Ag8 apply also in the case of Ag11.

We have previously found monospecific antibodies against Ag8 and Ag11 to have a stronger HSV-neutralizing potency than monospecific antibodies against Ag6, Ag9, and Ag4 when all five antibody preparations were equalized with respect to immunochemical activity (Vestergaard and Norrild, IARC, in press). This result, combined with the present finding that humans have higher antibody titers against Ag8 and Ag11 than against any other HSV antigen, may allow the conclusion that most of the serological cross-reactivity between HSV types 1 and 2 found in human sera is caused by antibodies to Ag8 and Ag11.

Ag6 represents the HSV type 1-specific virion envelope glycoprotein (14) now designated C in the common nomenclature agreed upon at the Herpesvirus Workshop, Cambridge, 1978 (Spear, personal communication). Monospecific antibodies against Ag6 neutralize HSV type 1 but not HSV type 2 (21). Our present results show that humans have antibodies against Ag6 (Table 2), and it is justified to conclude that this HSV type 1-specific antigen together with type 1-specific antigenic sites on Ag8 and Ag11 must be credited with eliciting the HSV type 1-specific antibodies in human sera.

Immunoelectrophoretically, both Ag9 and Ag4 behave like HSV type 2-specific antigens (18), but monospecific antibodies against these two antigens not only neutralize HSV type 2 but, to a lesser degree, also HSV type 1 (Vestergaard and Norrild, IARC, in press). This indicates that Ag9 and Ag4 also contain HSV common antigenic sites, but these two antigens are the only ones so far identified in the crossed immunoelectrophoretic profile that positively are bearers of HSV type 2-specific antigenic sites (18, 19). We have now shown that antibodies against Ag9 and Ag4 are present in human sera (Table 2), and it is reasonable to assume that the major part of antibodies against these two antigens represent the HSV type 2-specific antibodies.

Ag3 is a water-soluble non-glycosylated HSV type-common antigen with a complex peptide composition (14, 18). It might represent a major capsid antigen but its true nature is not known. Our present finding that humans have antibodies against Ag3 (Table 2) is in agreement with the earlier observation that HSV-infected rabbits rapidly produced antibodies against this antigen (18).

A detailed knowledge of the immunological function of the different antigenic structures of HSV in naturally infected humans is of the utmost importance for the development of diagnostic reagents. The results presented in this

paper can be applied directly for this purpose (7, 20).

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