

# Identification of a Common Antigen of Herpes Simplex Virus, Bovine Herpes Mammillitis Virus, and B Virus

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In immunoelectrophoretic analyses one common antigen was demonstrated in antigen preparations from herpes simplex virus types 1- and 2- (HSV-1 and HSV-2), bovine herpes mammillitis (BHM) virus-, and B virus-infected cells solubilized by Triton X-100. The antigen was also demonstrated in solubilized purified HSV-1 and BHM virus. The common antigen was identified as antigen 11 of HSV-1 or HSV-2. Differences were found in the polypeptide composition of the related antigens when isolated from the four different herpesviruses, but a glycopolypeptide with a molecular weight of 125,000 was present in each of the four different antigen preparations, indicating that this polypeptide carried the common antigenic determinants.

Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) are immunologically related to bovine herpes mammillitis (BHM) virus and B virus (8, 9, 11, 14; R. A. Killington, J. M. Yeo, R. W. Honess, I. W. Halliburton, and D. H. Watson, in *Third International Symposium on Oncogenesis and Herpesviruses*, in press). Cross-neutralization experiments indicated that common antigens are present and located in the envelope of the virus (10, 11).

In this report we have identified and further characterized one common antigen of the herpesviruses originating from three different host species. Our results suggest that the common antigen has a complex polypeptide structure in which the cross-reacting antigenic determinants are localized to one glycopolypeptide of antigen 11 (Ag-11) of HSV-1 and HSV-2 (7).

(Part of this study was presented at the Third Herpesvirus Workshop, Cold Spring Harbor, New York, August 1976, and at the Third International Symposium on Oncogenesis and Herpesviruses, Harvard University, Boston, 1977.)

## MATERIALS AND METHODS

**Cell cultures and viruses.** HSV-1 (strains F and KOS) and HSV-2 (strains G and 196) were grown in human epidermoid carcinoma no. 2 (HEp-2) cells or in primary rabbit kidney (RK) cells. The B virus (strain 130-65) was grown in RK cells; antigen preparations of this virus were kindly supplied by M. D. Daniel, Harvard Medical School, Boston, Mass. BHM virus (strain TVA) was passaged in bovine fetal lung, bovine brain, or bovine testes cells. The growth con-

ditions and maintenance of the different cells were as published previously (7, 8). All infections were performed by using a multiplicity of infection of 5 to 10 PFU/cell.

**Radioactive labeling of infected cells.** The cells were labeled from 4 to 20 h postinfection by addition to the maintenance medium of either 3  $\mu$ Ci of <sup>14</sup>C-labeled protein hydrolysate per ml (>45 mCi/matom), 10  $\mu$ Ci of L-[<sup>35</sup>S]methionine per ml (275 Ci/mmol), 0.5  $\mu$ Ci of D-[1-<sup>14</sup>C]glucosamine per ml (59 mCi/mmol), or 70  $\mu$ Ci of D-[1-<sup>3</sup>H]glucosamine per ml (15,000 to 25,000 mCi/mmol). The composition of the maintenance medium is specified elsewhere (8). All radioisotopes were purchased from Amersham (England).

**Preparation of antigens.** The infected cells were harvested at 20 h post-infection, and the proteins were solubilized in 4 times the volume of packed cells in 0.020 M glycine-0.0076 M Tris buffer (pH 8.6, 20°C) containing 5% (vol/vol) nonionic detergent Triton X-100 (Serva, Heidelberg, West Germany) (6). Cell-free HSV-1 or BHM virus was purified by ultracentrifugation in sucrose gradients (8) and solubilized as described above.

**Purification of Ag-11.** <sup>14</sup>C-amino acid-labeled Ag-11 was purified from the culture medium of HSV-1-infected HEp-2 cells. The medium was collected 20 h after infection, ultracentrifuged at 100,000  $\times$  g for 1 h at 4°C in a SW27 rotor, and concentrated by precipitation with an equal volume of saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (4°C). After dialysis against 0.05 M Tris-hydrochloride buffer (pH 7.5, 20°C) containing 15 mM NaN<sub>3</sub> and 1% (vol/vol) Triton X-100, the sample was fractionated by ion-exchange chromatography on a DEAE-cellulose column (Whatman DE-52) equilibrated in the buffer mentioned above. Elution was performed by using stepwise-increasing concentrations of NaCl to 0.3 M.

**Antibodies.** Polyspecific antibodies to HSV-1 and HSV-2 were obtained by infection of rabbits through four subsequent subcutaneous inoculations with in-

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fectured RK cells maintained in rabbit serum (B. F. Vestergaard and B. Norrild, in *Third International Symposium on Oncogenesis and Herpesviruses*, in press). Bovine antiserum to BHM virus was prepared as reported previously (11). In the present study, preimmune serum and serum taken after the fourth subcutaneous booster inoculation was used. Normal rhesus monkey serum and antiserum to B virus were kindly provided by M. D. Daniel, Boston, Mass.

The rabbit monospecific sera to the Ag-11 of HSV-1 was produced by immunization with the corresponding immunoprecipitate cut out from the agarose gel (Vestergaard and Norrild, in *Third International Symposium on Oncogenesis and Herpesviruses*, in press). In all experiments, purified immunoglobulin preparations with 15 mM Na<sub>2</sub>CO<sub>3</sub> were used (4).

**Fused rocket electrophoresis.** Fused rocket electrophoresis was performed in 1% (wt/vol) HSA agarose (Mr = -0.13, Litex A/S Glostrup, Denmark) in 0.18 M Tris-0.06 M barbital buffer (pH 8.6, 16°C) with 1% (vol/vol) Triton X-100. The antigens were allowed to diffuse from their wells for 1 to 2 h and were then subjected to electrophoresis into the antibody-containing, second-dimensional gel (12) at 2 V/cm for 18 h. The antibody concentrations are specified in the legends to the figures.

**Crossed immunoelectrophoresis.** For crossed immunoelectrophoresis, the intermediate gel technique (1) was used. The 1% (wt/vol) HSB agarose (Mr = -0.10, Litex, Glostrup, Denmark) was made in the same buffer as used for fused rocket electrophoresis.

The amount of solubilized antigen applied for the first-dimensional run is specified in the individual experiments. The first-dimensional electrophoresis was run at 10 V/cm for 1.5 h at 16°C.

The amount of antibodies used for the intermediate and the second-dimensional gels are specified in the legends to the figures. The second-dimensional run was performed at 2 V/cm for 18 h at 16°C.

**Isolation of immunoprecipitates from the antibody-containing gels.** Precipitates from 10 individual rockets were cut out of the unstained agarose gel and pooled. The polypeptides were eluted in 250  $\mu$ l of 0.06 M Tris-H<sub>2</sub>PO<sub>4</sub> buffer (pH 6.9), containing 2% (wt/vol) sodium dodecyl sulfate (SDS) and 2.5% (vol/vol) 2-mercaptoethanol (6).

Immunoprecipitates (using 15  $\mu$ l of the antigen preparation) were taken from the following antigen-antibody combinations: (i) <sup>14</sup>C-amino acid-labeled HSV-1 or HSV-2 antigens subjected to electrophoresis in gels containing antibodies to B virus (20  $\mu$ l/cm<sup>2</sup>); (ii) HSV-1 or HSV-2 antigens labeled as in (i) and subjected to electrophoresis in gels containing antibodies to BHM virus (10  $\mu$ l/cm<sup>2</sup>); and (iii) the following antigens were analyzed in monospecific antibodies to Ag-11: HSV-1 or HSV-2 antigens labeled as in (i) or labeled with [<sup>14</sup>C]glucosamine, L-[<sup>35</sup>S]methionine-labeled BHM antigen, and <sup>14</sup>C-amino acid- or [<sup>3</sup>H]glucosamine-labeled B antigen (antibody concentration, 15  $\mu$ l/cm<sup>2</sup>).

**Analytical SDS-polyacrylamide gel electrophoresis (SDS-PAGE).** Slab gels (14 by 28 by 0.15 cm) were run using the discontinuous polyacrylamide gel system of Ornstein as modified by Norrild et al. (6).

The acrylamide gel concentration was 7.5% (wt/vol)

in the separation gel and 3.5% (wt/vol) in the spacer gel. Both were cross-linked with 0.2% (wt/vol) N,N'-methylenebisacrylamide (purchased from Serva, Heidelberg, West Germany).

After staining in Coomassie brilliant blue, the gels were destained and dried. Autoradiography was done on Kodirex X-ray film using 2 weeks of exposure.

Gels containing <sup>3</sup>H-labeled samples were prepared for scintillation by the method of Bonner and Laskey (3). The dried gels were exposed at -70°C for 2 weeks.

## RESULTS

### Demonstration of a common antigen.

Rocket immunoelectrophoresis of antigens prepared by Triton X-100 solubilization of cells infected with either HSV-1, HSV-2, BHM, or B virus showed several precipitation arcs when analyzed in the homologous antibodies. Fused rocket immunoelectrophoresis of the antigens performed in heterologous antibodies showed one continuous precipitation line. This was formed independently of the tissue culture cells used for multiplication of the virus (Fig. 1A, B, and C). Purified virus of HSV-1 or BHM was solubilized in Triton X-100-containing buffer, and the same common antigen was present in the virions (Fig. 1B).

Spur formation as formed in the common precipitate between antigens from HSV, B, and BHM virus-infected cells (Fig. 1A, 1C) indicated partial identity between these antigens. Besides this major antigen, additional faint rockets could be demonstrated by using high concentrations of antibodies. In Fig. 1 these weak reactions were observed for BHM antigen analyzed in HSV-1 polyspecific antibodies or HSV antigens analyzed in B virus-specific antibodies (Fig. 1C). Control uninfected cell preparations gave no rockets when analyzed with the different antisera (Fig. 1B).

In what follows, our investigations were concentrated only on the major common antigen.

### Identification of the common antigen.

The cross-reacting precipitating antigen was identified by using the crossed immunoelectrophoretic technique with intermediate gels. A HSV-1 reference antigen was analyzed in polyspecific antibodies to HSV-1 present in the second dimension. The intermediate gel contained normal monkey antibodies (Fig. 2A). Five viral immunoprecipitates were present, numbered according to their electrophoretic mobility (13). When antibodies to either B virus, BHM, or monospecific antibodies to Ag-11 were included in the intermediate gel, Ag-11 was the sole antigen that reacted with the heterologous antibodies (Fig. 2B, C, and D). Identification of Ag-11 as the common antigen was further supported by fused rocket immunoelectrophoresis in antibodies to Ag-11 (Fig. 1D). One continuous im-

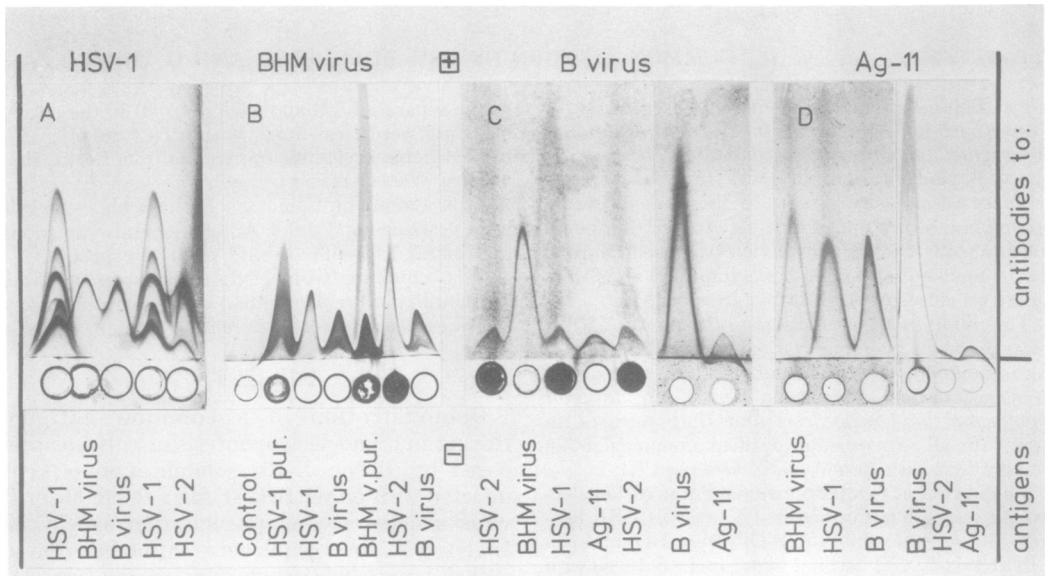


FIG. 1. Fused rocket immunoelectrophoresis. Coomassie brilliant blue staining. The antigens analyzed were cellular extracts of HSV-1, HSV-2, BHM-, and B virus-infected tissue culture cells or solubilized purified virus preparations of HSV-1 (HSV-1 pur.) or BHM (BHM V pur.). Ag-11 purified as described in Materials and Methods was also used. Control preparations of uninfected HEp-2 and RK cells were analyzed. A 15- $\mu$ l volume of each antigen was applied. The antibodies in the gel were: (A) polyspecific HSV-1 (8  $\mu$ l/cm<sup>2</sup>); (B) bovine BHM virus antibodies (10  $\mu$ l/cm<sup>2</sup>); (C) monkey B virus antibodies (20  $\mu$ l/cm<sup>2</sup>); (D) monospecific antibodies to Ag-11 (15  $\mu$ l/cm<sup>2</sup>). Electrophoresis was performed at 2 V/cm for 18 h.

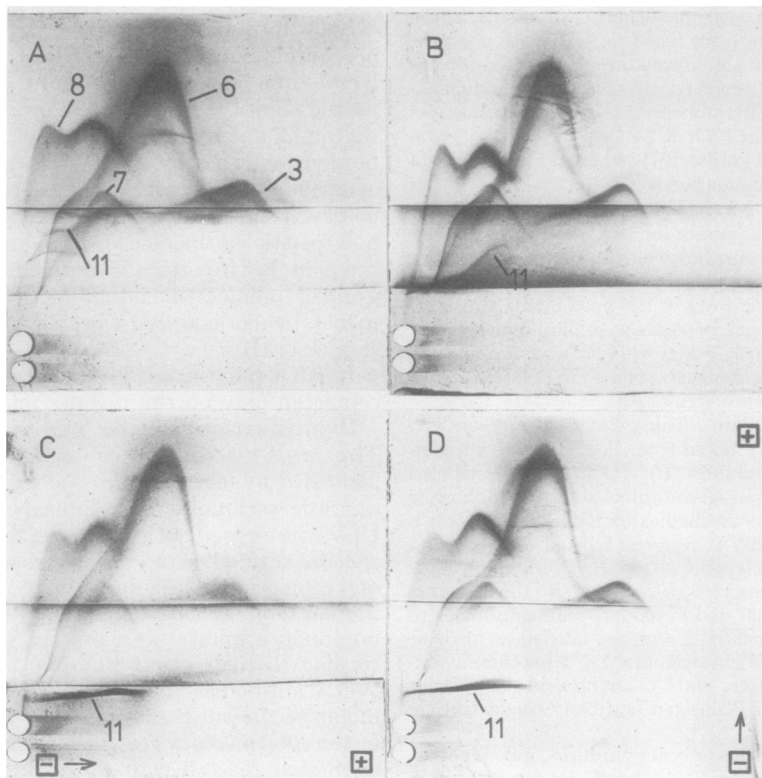


FIG. 2. Crossed immunoelectrophoresis. Coomassie brilliant blue staining. To all four plates was applied 30  $\mu$ l of HSV-1 cellular antigen for the first-dimensional electrophoresis, and all second-dimensional gels contained 15  $\mu$ l of polyspecific HSV-1 antibodies per cm<sup>2</sup>. The antibodies present in the intermediate gels were: (A) 20  $\mu$ l of normal monkey antibodies per cm<sup>2</sup>; (B) 20  $\mu$ l of monkey antibodies to B virus per cm<sup>2</sup>; (C) 10  $\mu$ l of bovine antibodies to BHM virus per cm<sup>2</sup>; (D) 15  $\mu$ l of rabbit monospecific antibodies to Ag-11 per cm<sup>2</sup>. Electrophoresis was performed as described in Materials and Methods.

munoprecipitation line was found, and spur formation was seen between HSV-1 and BHM. Purified Ag-11 (isolated from the maintenance medium of HSV-1-infected cells and giving only one precipitation line when analyzed in poly-specific HSV-1 antibodies) was included in the fused rocket immunoelectrophoresis in antibodies to B virus (Fig. 1C), in monospecific antibodies to Ag-11 (Fig. 1D), and in antibodies to BHM (data not shown).

**Analysis of the common antigen by SDS-PAGE.** The results presented so far suggest that the herpesviruses analyzed contain a common antigen with antigenic determinants corresponding to Ag-11 of HSV-1 or HSV-2. To analyze whether the common antigen complex was identical in all four herpesviruses, polypeptide analyses of the different "Ag-11" precipitates was performed by SDS-PAGE. The immunoprecipitates obtained by rocket electrophoresis of radioactively labeled HSV-1, HSV-2, BHM, and B virus antigen preparations in agarose gels containing either heterologous antibodies or monospecific antibodies to Ag-11 were cut out and solubilized for SDS-PAGE. From Fig. 3 and 4 and from Table 1, it can be seen that the four different immunoprecipitates carrying the common antigen determinants were complexes of several polypeptides. Only the polypeptide of molecular weight 125,000 is in common and is a glycosylated protein as shown by [<sup>3</sup>H]- or [<sup>14</sup>C]-glucosamine labeling (Fig. 3C and Fig. 4C). This finding suggests that this glycopolypeptide is the carrier of the common antigenic determinant in the "Ag-11" complex isolated from all four viral antigen preparations. Of the additional polypeptides present in the individual antigens (Table 1), the polypeptide of molecular weight 97,000 is in common between HSV-1, HSV-2, and B virus antigen.

## DISCUSSION

HSV, BHM virus, and B virus, herpesviruses of three different natural host species, are genetically independent viruses, as based upon the differences in their DNA composition (5, 11; H. Ludwig, G. Pauli, B. Norrild, and M. D. Daniel, submitted for publication). They are, however, antigenically related as already found earlier (8, 9, 11, 14; Killington et al., in *Third International Symposium on Oncogenesis and Herpesviruses*, in press). The cross-reactivity has been confirmed, and in this report the major common antigen has been identified by immunoelectrophoretic analysis in combination with SDS-PAGE.

The immunochemical analysis revealed that the Ag-11 of HSV-1 or HSV-2 is responsible for the cross-reactivity, which is based on investi-

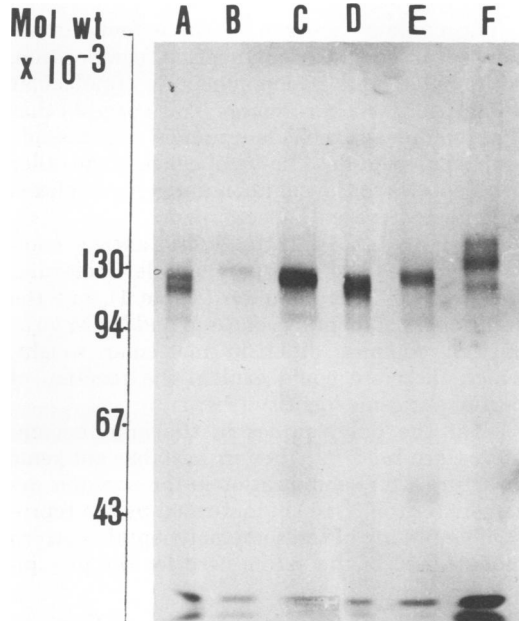


FIG. 3. Autoradiogram of SDS-PAGE. The polypeptides present in individual viral antigens were analyzed after rocket immunoelectrophoresis and solubilization for SDS-PAGE. The following combinations were analyzed: (A) 15  $\mu$ l of <sup>14</sup>C-amino acid-labeled HSV-1 antigen precipitated in gel containing 20  $\mu$ l of antibodies to B virus per  $\text{cm}^2$ ; (B) 15  $\mu$ l of HSV-2 antigen labeled and subjected to electrophoresis as in (A); (C) 15  $\mu$ l of [<sup>14</sup>C]glucosamine-labeled HSV-2 antigen precipitated in gel containing 15  $\mu$ l of monospecific antibodies to Ag-11 per  $\text{cm}^2$ ; (D) 15  $\mu$ l of HSV-1 antigen labeled as in (A) but subjected to electrophoresis in gel containing 10  $\mu$ l of antibodies to BHM virus per  $\text{cm}^2$ ; (E) 15  $\mu$ l of [<sup>35</sup>S]methionine-labeled HSV-2 antigen precipitated in gel containing 10  $\mu$ l of antibodies to BHM virus per  $\text{cm}^2$ ; (F) 15  $\mu$ l of [<sup>35</sup>S]methionine-labeled BHM antigen subjected to electrophoresis in 15  $\mu$ l of monospecific antibodies to Ag-11 per  $\text{cm}^2$ . The SDS-PAGE was performed in 7.5% (wt/vol) polyacrylamide gels subjected to electrophoresis at a constant current of 50 mA for 9 h at 4°C.

gations with the crossed immunoelectrophoretic technique using intermediate gels (Fig. 2), with rocket electrophoresis in monospecific antibodies to Ag-11 (Fig. 1D), and with cross-reactivity with purified Ag-11 (Fig. 1C and D). Since cross-neutralization of the four viruses can be demonstrated, the common antigen must be located on the surface of the viral envelope. It has recently been shown by <sup>125</sup>I-labeling that the Ag-11 complex is exposed on the plasma membrane of infected cells, but only the glycopolypeptide (molecular weight, 125,000) was available for iodination (B. Norrild, O. J. Bjerrum, H. Ludwig, and B. V. Vestergaard, *Virology*, in press). Similar analysis is in progress with purified virus.

The common antigen of these four viruses showed a complex polypeptide composition, with only one glycopolypeptide (molecular weight, 125,000) in common. This suggests that the common antigenic determinant might reside in this polypeptide. The significance of the other polypeptides in the common antigen complex is unknown at present.

The HSV-1, HSV-2, and B virus antigen complex contains a second polypeptide (molecular weight, 97,000) in common (Table 1), but the additional polypeptides of the B and BHM virus antigen complex differ in molecular weight, which therefore could explain the reaction of partial antigenic identity (Fig. 1).

That the polypeptides in the immunocomplexes are held together in a stable antigenic structure after solubilization in the nonionic detergent Triton X-100 is confirmed by the reproducible finding of the same polypeptide pattern independent of the serum used for the precipi-

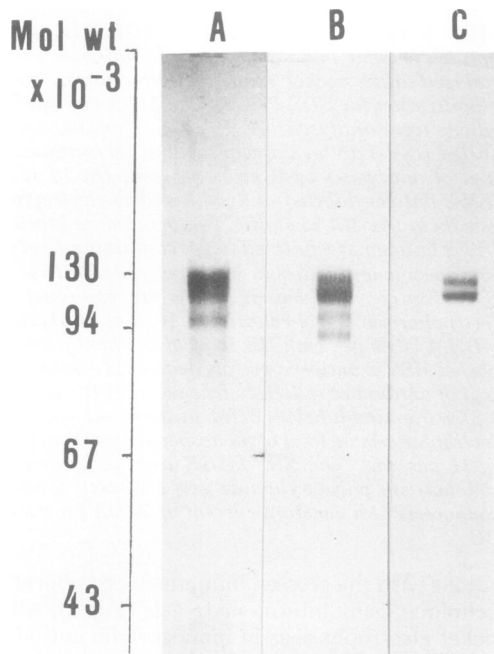


FIG. 4. Autoradiogram of SDS-PAGE. The polypeptide pattern of individual viral antigens was analyzed after rocket immunoelectrophoresis in monospecific antibodies to Ag-11 and solubilization for SDS-PAGE. (A)  $^{14}\text{C}$ -amino acid-labeled HSV-1 antigen; (B)  $^{14}\text{C}$ -amino acid-labeled B virus antigen; (C) [ $^3\text{H}$ ]glucosamine-labeled B virus antigen. Electrophoresis was performed as described for Fig. 3. The dried gel was scintillated and exposed for 2 weeks at  $-70^\circ\text{C}$  as described in Materials and Methods. All samples were run on the same gel, but only three slots are shown.

TABLE 1. Molecular weight of the viral polypeptides<sup>a</sup>

Mol wt ( $\times 10^{-3}$ ) <sup>b</sup>			
HSV-1 <sup>c</sup>	HSV-2	B	BHM
			155
			133
125 <sup>d</sup>	125 <sup>d</sup>	125 <sup>d</sup>	125 <sup>d</sup>
113		113 <sup>d</sup>	
97	97 <sup>e</sup>	97	
		91	

<sup>a</sup> Polypeptides were extracted from immunoprecipitates formed from cellular antigen preparations which had been subjected to electrophoresis in agarose gel containing monospecific antibodies to Ag-11.

<sup>b</sup> Molecular weights are mean values of five experiments.

<sup>c</sup> Antigen preparation.

<sup>d</sup> Polypeptides labeled when using glucosamine radioisotope.

<sup>e</sup> Very weak band.

tation. Stable antigen complexes have also been described by the solubilization of membranes in other systems (2).

The rather similar polypeptide composition in the common antigen of the three primate herpesviruses compared to that of the bovine herpesvirus antigen strengthens the idea that the common antigen determinant of the Ag-11 complex has been well preserved during evolution, even though additional polypeptides participate in the antigen structure. One might speculate about Ag-11 serving an important biological function, which is unknown at present. Further analysis of the appearance of Ag-11 on the surface of cells infected with the different herpesviruses is in progress.

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