Comparative Analyses of the Proteins and Antigens of Five Herpesviruses

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SUMMARY

The proteins of five herpesviruses – herpes simplex types 1 and 2 (HSV-1 and HSV-2), bovine mammillitis virus (BMV), pseudorabies virus (PRV) and equine abortion virus (EAV) – have been compared by polyacrylamide gel electrophoresis of purified virus and by antigenic analysis using virus neutralization and agar-gel immunodiffusion tests. Although there were general similarities in the number, size range and overall complexity of polypeptides separated by polyacrylamide gel electrophoresis from purified particles of the five viruses, no single component of identical mobility was present in all viruses. The major capsid polypeptide from each of the five viruses comprised about 10% of virion protein mass but had distinctive mol. wt. of 158,000 to 160,000 (HSV-2 and BMV), 145,000 (EAV) and 140,000 (PRV) relative to 155,000 for HSV-1.

Despite differences of apparent mol. wt. there was a one-for-one correspondence for the majority of structural polypeptides of HSV-1 and HSV-2 and many polypeptides of BMV were recognizably analogous to those of HSV-1 and HSV-2. In contrast the polypeptides of PRV and, more particularly, EAV differed markedly from those of all the other viruses. The antigenic analysis complemented these results; HSV-1, HSV-2 and BMV all cross-neutralized, whereas PRV and EAV were only neutralized by homologous antisera. Agar gel immunodiffusion tests with extracts of infected cells demonstrated at least one antigen common to all five viruses and showed BMV to be more closely related to HSV-1 and HSV-2 than were PRV or EAV although less closely related than HSV-1 to HSV-2. PRV and EAV share more antigens with each other than with the other viruses.

INTRODUCTION

At present almost seventy viruses fulfil criteria for inclusion within the herpesvirus group, i.e. they possess double-stranded DNA genomes which are replicated in the nucleus and encapsidated within a 100 nm diam. icosahedral protein shell of 162 capsomeres which is enclosed within a lipoprotein (ether-sensitive) envelope (Wildy, 1972; Fenner, 1976). Viruses conforming with these criteria are diverse in their host and disease associations and in known biochemical properties. For example, the range of values for mean \( \% (G + C) \) of herpesvirus DNAs [i.e. 33 to 75 \( \% (G + C) \), Goodheart & Plummer, 1975] is comparable to
that encompassed by the Bacteriaceae (De Ley, 1969). Although it is clear that there are varying degrees of antigenic relatedness between certain members of the group, there is as yet no convincing systematic basis for subgrouping of the herpesviruses (Wildy, 1973). Thus herpes simplex viruses 1 and 2 show an estimated 50% homology between their nucleic acids (Kieff et al. 1972), are cross-neutralized by virtue of their common possession of the Band II antigenic site (Sim & Watson, 1973) and share multiple antigens as demonstrated by agar gel immunodiffusion (Honess et al. 1974). Herpes B-virus (Sabin, 1934), bovine mammillitis virus (Sterz, Ludwig & Rott, 1973/74), SA8 (Hampar & Martos, 1973) and herpesvirus tamarinus (Blue & Plummer, 1973) also cross neutralize with herpes simplex virus. In the case of B-virus three antigens in common with herpes simplex have been shown by gel immunodiffusion (Watson, et al. 1967) while Sterz et al. (1973/74) have shown significant homology between the DNA of bovine mammillitis virus and that of herpes simplex virus. Other viruses which do not cross-neutralize with herpes simplex virus have been shown to share antigens by immunodiffusion, immunofluorescence or immune agglutination of virus particles (e.g. pseudorabies virus and varicella-zoster – Watson et al. 1967; Honess et al. 1974; Marek’s disease virus – Ross, Frazier & Biggs, 1972; and equine abortion virus – Blue & Plummer, 1973).

Despite this evidence for a range of antigenic relationships amongst certain of the herpesviruses there is little indication of its structural basis. Detailed accounts have been given of the structural proteins of herpes simplex virus (Roizman & Furlong, 1974) and equine abortion virus (O’Callaghan & Randall, 1976) but there have been no direct comparisons of the two viruses. Studies of structural polypeptides of pseudorabies virus have also shown a superficially similar composition to HSV-1 but no reliable direct comparisons have been made and there are discrepancies between independent estimates of number and mol. wt. of structural proteins (Shimono, Ben-Porat & Kaplan, 1969; Stevely, 1975). There is therefore no reliable estimate of the degree of congruence between structural proteins of different herpesviruses.

In this paper we present a study of five herpesviruses which exhibit a range of antigenic cross-reactivity; herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), bovine mammillitis virus (BMV), equine abortion virus (EAV) and pseudorabies virus (PRV). We confirm the cross-neutralization of HSV-1, HSV-2 and BMV, demonstrate an antigen common to all five viruses and present a direct comparison of the structural proteins of purified enveloped particles of the five viruses separated by high resolution polyacrylamide gel electrophoresis.

METHODS

Cells and viruses. BHK21, HEp-2, Chang human conjunctival and RK13 cells were all grown in 80 oz roller bottles at 37 °C in Eagle's medium (Glasgow modification) supplemented with 10% (v/v) tryptose phosphate broth and 5% (v/v) calf serum (BHK21, HEp-2 and Chang) or 5% (v/v) rabbit serum (RK13). HSV-1 was strain HFEM (Watson et al. 1966), HSV-2 was strain 3345 (Sim & Watson, 1973), BMV was the Italian isolate of Castrucci et al. (1972), kindly provided by Dr W. B. Martin. Some neutralization and gel diffusion experiments were also done with the Glasgow strain of BMV isolated by Martin et al. (1966). PRV was the strain isolated by Dr F. Dekking and EAV was the Polish isolate RAC-H (Mayr et al. 1965; Thomson et al. 1976).

Virus stocks were prepared by infection of BHK21 cells at low multiplicity (0-01 p.f.u./cell; Watson et al. 1966). Plaque titrations were performed by the suspension assay of Russell (1962) using an overlay medium containing carboxymethylcellulose. All five viruses
Proteins and antigens of herpesviruses

gave efficient (10 to 200 particles/p.f.u.) and reproducible plaque formation in BHK21 cells using this method.

**Immune sera and serum absorption.** The preparation and properties of rabbit hyperimmune antisera to RK13 cells infected with HSV-1, HSV-2 and PRV (general antisera) have been described in detail elsewhere (Watson et al. 1966, 1967; Honess et al. 1974). Antiserum to the Band II antigen of HSV-1 was prepared as described by Watson & Wildy (1969). Antisera to BMV were kindly provided by Dr Hanns Ludwig and were prepared by immunization of rabbits with partially purified BMV (Sterz et al. 1973/74). Antiserum to EAV was prepared in a gnotobiotic foal as described previously (Thomson et al. 1976).

Antisera were absorbed with heterologous antigen (see Results) by incubating 1 ml of general antisera with a homogenate of 10⁷ BHK21 cells infected with heterologous virus. Absorbed antisera were clarified by sedimentation at 40000 rev/min for 60 min and the supernatant fluids concentrated to 1 ml by vacuum dialysis (Sim & Watson, 1973).

**Preparation of infected cell antigen and agar gel immunodiffusion.** BHK21 cells infected with from 1 to 20 p.f.u./cell were harvested after incubation at 37 °C for 24 h (HSV-1, HSV-2 & PRV) or 48 h (BMV, EAV), washed in phosphate buffered saline, resuspended in distilled water at a concentration of 10⁸ cell/ml and disrupted by ultrasonic oscillation. Immunodiffusion tests were performed in 2 to 3 mm layers of 1% Ionagar (Oxoid Ltd.) using a hexagonal array of wells which allowed reactions between antigen at 1/1 dilution with antiserum at dilutions of 1/1 to 1/27 to be observed on the same plate as reactions of undiluted antiserum with dilutions of antigen varying from 1/1 to 1/27 (Honess et al. 1974).

**Neutralization tests.** Virus (4 × 10⁴ p.f.u./ml) was incubated for 3 h at 25 °C with an equal volume of antiserum, diluted where necessary. Surviving virus was assayed by the method of Russell (1962). Antisera were inactivated for 30 min at 56 °C before use. Results are expressed as neutralization rate constants, ‘k’ (Sim & Watson, 1973).

**Purification of enveloped virus particles.** In preliminary experiments we found that although optimal conditions for virus growth and purification differed somewhat for the five viruses, the use of Chang conjunctival cells (HSV-1 and HSV-2) or BHK21 cells (PRV, EAV and BMV) allowed acceptable virus growth and provided suitable sources of virus for subsequent purification. Monolayer cultures of Chang conjunctival cells were infected with 5 p.f.u./cell of HSV-1 or HSV-2 and incubated at 37 °C for 24 or 48 h or at 32 °C for 48 or 72 h. Under these conditions HSV-1 infected cells gave a total virus yield of more than 200 p.f.u./cell with particle/infectivity ratios of 20 to 100 and HSV-2 infected cells gave from 10 to 100 p.f.u./cell with particle/infectivity ratios of 30 to 300. In each case about 50 % of the total yield of infectious virus was present in the cytoplasm and 50 % in the medium and virus was purified from both fractions (see below). BHK21 cells were infected with 1 to 5 p.f.u./cell of BMV and incubated at 37 °C for 48 h, and with 5 p.f.u./cell of EAV and PRV and incubated for 24 h at 37 °C. BMV infected cultures yielded ~ 10 to 20 p.f.u./cell with a particle infectivity ratio of 100 to 300 of which approx. 50 % was present in the medium. EAV- and PRV-infected cells each yielded more than 100 p.f.u./cell with particle/infectivity ratios of 10 to 50 but, whereas 95 % of infectious EAV was present in the medium and 5 % in the cytoplasm, the reverse situation was true for PRV. EAV was therefore purified from the medium and PRV from the cytoplasmic fraction of infected cultures. HSV-1 was also purified from the cytoplasmic fraction of BHK21 cells for comparative purposes and was similar in its growth behaviour to PRV. The purification procedures used were the previously described methods for the purification of enveloped particles of HSV-1 from the supernatant medium (Powell & Watson, 1975) or from the cytoplasmic fraction (Spear & Roizman, 1972; Heine et al. 1974) of infected cells. These purification procedures
were monitored and the final purity of the products was assessed by infectivity titrations, quantitative electron microscopy, measurements of total protein per physical particle and by analysing the loss of proteins characteristic of uninfected cells by polyacrylamide gel electrophoresis. In all cases average recoveries of total particles and infectivity were about 10% and by all the above criteria the purity of the final products was as good or better than the results of the same procedures applied to HSV-1, i.e. > 90% enveloped particles, < 3 x 10^-19 g of protein/particle (Heine et al. 1974; Powell & Watson, 1975).

Polyacrylamide gel electrophoresis. Methods for sample preparation, SDS-polyacrylamide gel electrophoresis on slabs of polyacrylamide cross-linked with N,N'-diallyltartardiamide, and procedures for fixation, staining and autoradiography were as described by Heine et al. (1974). The calibration of this gel system with standard proteins of known mol. wt. and the behaviour of polypeptides of HSV-1 have also been described in detail by Heine et al. (1974). The separation deviates from a strictly linear relationship of log mol. wt. versus migration at high mol. wt. and because of the absence of sufficient well characterized proteins of > 200000 mol. wt. we have not attempted to determine mol. wt. in excess of 200000.

Other methods. Protein concentrations were measured by the method of Lowry et al. (1951) with bovine serum albumin as calibrating standard. Particle counts were done by the oop drop method of Watson, Russell & Wildy (1963).

RESULTS
Neutralization of virus infectivity by homologous and heterologous antisera

The results of the neutralization tests are shown in Table 1. HSV-1, HSV-2 and BMV all cross-neutralized. Although the results in Table 1 for BMV were obtained with the Italian isolate, similar results were obtained with the Glasgow strain. The ratios of k-values against homologous and heterologous viruses were larger for anti-HSV-1 or anti-HSV-2 versus BMV (60 and 20) than for anti-BMV against HSV-1 (approx. 4) or anti-HSV-1 against HSV-2 (7) and anti-HSV-2 against HSV-1 (2). Neither EAV nor PRV were neutralized by heterologous antisera and antisera to PRV and EAV had no significant effect on heterologous viruses. It should be noted that the significance of the negative heterologous neutralization by anti-EAV is not equivalent to that of other antisera since the rate constant for the homologous neutralization was much lower than that observed with the other antisera.

Antiserum to the Band II antigen of HSV-1 which had a neutralization rate constant of 2.4 against HSV-1 and 0.55 against HSV-2 also neutralized BMV. However, prolonged incubation periods were necessary to show significant neutralization and k values for anti-Band II antiserum against BMV were in the range 0.01 to 0.02.

Gel immunodiffusion tests

In the first series of experiments dilutions of antisera were tested against a range of dilutions of homogenates of cells infected with homologous and heterologous viruses and the number of precipitin bands enumerated (Table 2). None of the antisera tested reacted with extracts of uninfected cells or components of the tissue culture medium and although different antisera gave from 4 (anti-BMV) to at least 13 (anti-PRV) precipitin bands against the homologous antigen, each serum also revealed precipitin bands in common with at least one heterologous virus. Antisera to HSV-1, HSV-2 and PRV gave at least one precipitin band with antigens of all heterologous viruses. We have previously noted that HSV-1 and HSV-2 share at least 5 antigens (Honess et al. 1974) and we have now shown that HSV-1 and HSV-2 have 4 to 6 in common with BMV, 2 to 3 with EAV and at least one
Table 1. Neutralization of virus infectivity by homologous and heterologous antisera

<table>
<thead>
<tr>
<th>Antiserum against:</th>
<th>HSV-1</th>
<th>HSV-2</th>
<th>BMV</th>
<th>PRV</th>
<th>EAV</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1</td>
<td>18.0</td>
<td>2.4</td>
<td>0.3</td>
<td>&lt; 0.005*</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>(1:0)†</td>
<td>[6:0]</td>
<td>[60]</td>
<td>[&gt; 3600]</td>
<td>[&gt; 3600]</td>
<td></td>
</tr>
<tr>
<td>HSV-2</td>
<td>1.4</td>
<td>3.0</td>
<td>0.14</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>(2:1)</td>
<td>[1:0]</td>
<td>[2:1:4]</td>
<td>[&gt; 600]</td>
<td>[&gt; 600]</td>
<td></td>
</tr>
<tr>
<td>BMV</td>
<td>0.35</td>
<td>N.D.</td>
<td>1.46</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>(3:8)</td>
<td>[1:0]</td>
<td></td>
<td>[&gt; 270]</td>
<td>[&gt; 270]</td>
<td></td>
</tr>
<tr>
<td>PRV</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
<td>4.4</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>(≥ 880)</td>
<td>[≥ 880]</td>
<td>[≥ 880]</td>
<td>[1:0]</td>
<td>[≥ 880]</td>
<td></td>
</tr>
<tr>
<td>EAV</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
<td>0.07</td>
</tr>
<tr>
<td>(≥ 14)</td>
<td>[≥ 14]</td>
<td>[≥ 14]</td>
<td>[≥ 14]</td>
<td>[≥ 14]</td>
<td>[1:0]</td>
</tr>
</tbody>
</table>

* k = 0.005 was the limit of sensitivity in these experiments.
† Ratio = k_homologous virus/k_heterologous virus.
‡ N.D., not done.

Table 2. The number of precipitin bands formed in agar gel immunodiffusion reactions of antisera to herpesvirus against antigens of cells infected with homologous and heterologous viruses

<table>
<thead>
<tr>
<th>Antiserum prepared against</th>
<th>HSV-1</th>
<th>HSV-2</th>
<th>BMV</th>
<th>EAV</th>
<th>PRV</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1</td>
<td>≥ 12</td>
<td>≥ 6</td>
<td>6</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>HSV-2</td>
<td>≥ 5</td>
<td>≥ 7</td>
<td>4</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>BMV</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EAV</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>PRV</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>≥ 13</td>
</tr>
</tbody>
</table>

With PRV. Results quoted here are for the Italian BMV isolate but similar results were obtained with the Glasgow strain. Antisera to PRV identified at least one antigen shared between PRV and each of the other viruses tested and gave three precipitin bands with EAV. Antiserum to BMV only gave 4 precipitin bands against the homologous antigen and did not show any cross reaction with PRV or EAV but nevertheless gave two precipitin bands with HSV-1 and HSV-2.

In a second set of experiments those dilutions of extracts of cells infected with each of the five viruses which had been shown to give optimal reactions in tests with a range of dilutions of heterologous sera were tested together against each of the antisera in turn. Samples of virus antigens were placed in wells arrayed hexagonally around a central well containing antiserum. With antisera to HSV-1 or HSV-2 it was shown that the precipitin band observed with the PRV infected cell extract was identical to one of the precipitin bands observed against EAV and BMV. Also, at least two of the lines formed against EAV were a subset of those formed against BMV or HSV-2 antigens by antisera to HSV-1. Similarly, at least three of the precipitin bands formed between antiserum to HSV-1 and BMV were also common to HSV-1 and HSV-2. Experiments in which multiple antisera were tested together against a single antigen also showed at least one reaction of identity between EAV infected cell extracts and antisera to HSV-1, HSV-2 and PRV.

Since different combinations of serum and antigen dilutions were necessary to demonstrate certain of the precipitin lines it was not possible to test the identity or non-identity of
Fig. 1. Polypeptides from purified enveloped particles of equine abortion virus (E), bovine mastommaillitis virus (B), pseudorabies virus (P), herpes simplex virus-type 1 (H1) and type 2 (H2) separated by electrophoresis on 6%, 9%, and 15% polyacrylamide gel slabs in the presence of SDS. The 6% and 9% gels and the right-hand portion of the 15% gel are photographs of Coomassie brilliant blue stained gel films. The left-hand portion of the 15% gel is a corresponding autoradiogram of $^{35}$S-methionine radioactivity. Filled and open arrows indicate examples of polypeptides of equine abortion virus (E) and pseudorabies virus (P) which have unusually low ratios of $^{35}$S-methionine radioactivity to total protein estimated by Coomassie brilliant blue staining. Positions of selected HSV-1 polypeptides are shown to the right of the 9% gel and the left of the 15% gels.

all combinations of heterologous reactions by the simple comparisons described above. Some further information was obtained by analysing the ability of the various heterologous antigens to remove antibody populations reacting with homologous and other heterologous viruses. With all antisera, prior absorption with the homologous antigen removed all reactivity of antisera in subsequent gel diffusion tests against homologous and heterologous antigen samples. However, anti-HSV-1 absorbed with an excess of EAV-infected cells failed to react with EAV- or PRV-infected cells but retained multiple precipitating antibody activities against extracts of cells infected with BMV, HSV-1 or HSV-2. Anti-HSV-1 absorbed with PRV also retained multiple reactivities against HSV-1, HSV-2 and BMV infected cell extracts. Finally antisera to HSV-1 absorbed with BMV retained multiple precipitating antibody activities against both HSV-1 ($\geq 6$) and HSV-2 ($\geq 2$).

In principle these absorption tests should allow resolution of the experimental difficulties described for tests of identity or non-identity. However, by absorbing antisera with, e.g., uninfected cell extracts it was possible to show that non-specific losses occurred in precipitating activity of antisera during the experimental procedures. Thus antiserum to HSV-1 gave 6 precipitin bands with BMV infected cell extracts and 3 with EAV infected cell extracts in the original tests. After absorption of anti-HSV-1 with uninfected BHK21 cells
only 4 precipitin bands were observed with BMV infected cell extracts, of which 2 were also observed in tests of the same antigen with antiserum to HSV-1 after absorption with EAV. Thus we may conclude that 2 of the 3 precipitin bands obtained in reactions of anti-HSV-1 with EAV are a subset of the precipitin bands given by anti-HSV-1 with BMV. We cannot however conclude that the third precipitin band represents an EAV antigen shared with HSV-1 but not also shared with BMV. In proceeding across the series HSV-2, BMV, EAV, PRV we have been able to show that at least some of the antigens shared by each member of the series and HSV-1 are a subset of those shared with 'earlier' members of the group and we have no positive evidence for any antigen shared with a 'later' member which is not also shared with an 'earlier' member.

Polypeptides of purified enveloped virus particles

Preparations of all five viruses free of visible impurity, consisting of $> 90\%$ of enveloped virus particles with particle/infectivity ratios of from 10 to 300 and containing less than $3 \times 10^{-15}$ g of protein per particle were examined by polyacrylamide gel electrophoresis. Comparisons of HSV-1 purified from the medium or cytoplasmic fractions of Chang cells or the cytoplasmic fraction of BHK21 cells revealed no major differences in the number or relative molarity of polypeptides labelled with $^{35}$S-methionine or stained with Coomassie brilliant blue. A representative set of comparisons of the electrophoretically separated polypeptides from purified samples of the five viruses is illustrated in Fig. 1.

For the purpose of the present paper we have not considered it helpful to devise independent systems of nomenclature for all five viruses. We have chosen instead to show the calibration for apparent mol. wt. and the relative migration of the major polypeptides of HSV-1 and we shall use these scales to index polypeptides of the other viruses.

The five viruses showed at least a superficial resemblance in number and size distribution of structural polypeptides. HSV-1 has been shown to contain more than 30 electrophoretically distinct components, only 20 of which individually represent more than 1 % of the particle protein mass (Spear & Roizman, 1972; Heine et al. 1974; Powell & Watson, 1975). We find from 15 (BMV) to 20 (EAV and PRV) major components each comprising more than 1 to 2 % of the virus protein mass. In the case of BMV and EAV very few additional components present at lower molarities were seen. Samples of PRV did contain a number of additional minor polypeptides notably in the mol. wt. range from 8 to $9 \times 10^4$ (most clearly seen on the 15 % gel shown in Fig. 1, where they are compressed within a small range of relative mobilities).

Each of the five viruses contained a very high mol. wt. (> 200 000) polypeptide apparently analogous to VP1-2 of HSV-1 (Spear & Roizman, 1972; Heine et al. 1974). The BMV and HSV-2 analogues of VP1-2 co-migrated but those of all other viruses had distinctive electrophoretic mobilities. Similarly a major non-glycosylated polypeptide constituting $\geq 10\%$ of the protein mass of virus particle and apparently analogous to the major capsid polypeptide (VP5) of HSV-1 was seen with all five viruses. The mol. wt. of this component differed significantly between HSV-1, HSV-2, PRV and EAV. However, there was no significant difference between the mol. wt. of BMV and HSV-2 capsid polypeptides. The approximate mol. wt. for the capsid proteins of HSV-1, HSV-2 and BMV, EAV and PRV were 155 000, 158 to 160 000, 145 000 and 140 000 respectively.

The smallest significant structural components of HSV-1, HSV-2, BMV and PRV were in the range 29 000 (PRV) to 36 000 HSV-1, (VP23) whereas EAV had two prominent components of 11 500 and 17 000 mol. wt. with no obvious counterparts amongst the polypeptides of the other viruses. It is relevant to note that HSV-1 and HSV-2 do produce polypeptides
in this size range in the infected cell but they are arginine-rich, methionine-poor nuclear proteins which are not significantly represented in purified virus particles. In contrast the low mol. wt. structural components of EAV were not anomalously arginine rich nor methionine poor (see e.g. comparison of autoradiogram of $^{35}$S-methionine radioactivity and corresponding Coomassie brilliant blue stained gel illustrated for the 15% gel of Fig. 1). Both EAV and PRV contained some polypeptides which were markedly under-represented in the autoradiogram of $^{35}$S-methionine radioactivity relative to their contribution to the profile of stained components (annotated to right of Fig. 1).

Comparisons of structural polypeptides stained with Coomassie brilliant blue or labelled with $^{35}$S-methionine with the profile of components labelled with $^{14}$C-glucosamine showed multiple electrophoretically heterogeneous glycosylated polypeptides in each of the five viruses. As previously noted (Spear & Roizman, 1972; Heine et al. 1974) the most prominent glycoproteins of HSV-1 are in the mol. wt. range 120 to 130,000 (VP7-8 and 8-5) and 55 to 65,000 (VP17-19e) and HSV-2 has two similar but non-identical groups of components in these mol. wt. ranges but, in addition, a prominent glycosylated protein migrating somewhat more slowly than VP13 and with an apparent mol. wt. of 85,000 (see also Cassai, Sarmiento & Spear, 1975; Cassai et al. 1976). BMV had three major regions of glycosylated polypeptides, one with a mol. wt. of about 120,000 migrating slightly faster than VP8-5 of HSV-1, and two regions with modal mol. wt. of 62,000 and 51,000. PRV had four major regions of glycosylated polypeptides, a complex region of 110 to 120,000 mol. wt. and one of 72,000 accounting for the majority of glucosamine label but with two other regions at 60,000 and at 45,000 mol. wt. EAV differed from the other viruses in having a prominent glucosamine-labelled component of > 200,000 mol. wt. in the region of its VP1-2 analogue. However, the glucosamine label in this region did not co-migrate with the VP1-2 analogue, did not correspond with any other $^{35}$S-methionine or $^{14}$C-arginine labelled component and stained poorly with Coomassie brilliant blue. It seems likely that this material is mucopolysaccharide similar to that previously noted in studies of EAV (Perdue et al. 1974) and EBV (Dolyniuk, Wolff & Kieff, 1976b). In addition, EAV had prominent glycosylated polypeptides which corresponded to electrophoretically heterogeneous $^{35}$S-methionine labelled polypeptides with modal mol. wt. of 82,000, 56,000 and 46,000.

**DISCUSSION**

In this paper we have used serum neutralization, immunodiffusion and a comparison of the number and size of structural polypeptides to examine common and divergent features herpesvirus proteins.

We have confirmed the observation of Sterz et al. (1973/74) and shown that HSV-1, HSV-2 and BMV were cross-neutralized, whereas PRV and EAV were only neutralized by homologous antisera. Although it seems plausible to propose that the relationship between homologous and heterologous neutralization constants should show some correspondence with differences in protein sequence and structure this correspondence is obviously not a simple proportionality. In particular, detailed analysis is complicated by the common observation of non-reciprocal or asymmetric measures of the ratio of homologous and heterologous rate constants for a given pair of viruses dependent upon which virus serves as the immunizing agent. For example, homologous antisera neutralize isolates of HSV-1 and HSV-2 by virtue of cross-reacting (i.e. Band II) and multiple type-specific antigenic sites. However, rabbit antisera to HSV-1 infected cells typically give $k_1/k_2$ ratios of > 3 to < 10, whereas antisera to HSV-2 infected cells give $k_2/k_1$ ratios of > 0.5 to < 3. Moreover,
Proteins and antigens of herpesviruses

Fig. 2. Diagrammatic comparisons of the number, apparent mol. wt. and relative contribution of major polypeptides from purified enveloped particles of eight herpesviruses. Data in (a) for murine cytomegalovirus (MCMV) are taken from Kim et al. (1976a), for human cytomegalovirus isolate AD169 (HCMV) from Fiala et al. (1976) and for Epstein–Barr virus (EBV) from Dolyniuk, Pritchett & Kieff (1976a) and Dolyniuk, Wolff & Kieff (1976b). Samples of HCMV and EBV were each co-electrophoresed with HSV-1 and should therefore be directly comparable with data shown in (b). The MCMV polypeptides are located solely on the basis of the published values of their apparent mol. wt. Data shown in (b) are based on the results of the present paper with the additional annotation of major capsid components (Gibson & Roizman, 1972; Perdue et al. 1974; Stevely, 1975; Dolyniuk et al. 1976b). Only polypeptides which constitute more than 1% of protein mass are shown. Solid bands are used to represent components which are electrophoretically homogeneous. Electrophoretically heterogeneous components are indicated by the use of cross-hatched (prominent components) and stippled (less prominent components) symbols. Most of the electrophoretically heterogeneous components are glycosylated.
antisera specifically directed against shared determinants do not give \( k_{\text{homologous}}/k_{\text{heterologous}} \) ratios of unity. In the present paper this asymmetry is marked in the cross-neutralization of HSV and BMV (Table 1) and examples of the same phenomenon are familiar from studies on the cross-neutralization of HSV-1 and B-virus and HSV and SA 8 (see e.g. Hampar & Martos, 1973).

Despite these obstacles to a detailed interpretation of cross-neutralization it is nonetheless true that the viruses which cross-neutralize share more common antigenic sites and have more similar structural polypeptide patterns than those which do not. Thus, estimates of the minimum number of shared antigenic determinants obtained by gel diffusion supported the view that BMV was only a little less closely related to HSV-1 and HSV-2 than these two viruses were to one another. These experiments also showed that many of the antigens common to HSV-1 and BMV were a subset of those common to HSV-1 and HSV-2. In addition we were able to show at least one antigen common to all five viruses and more antigens in common between PRV and EAV than between these viruses and HSV-1, HSV-2 or BMV. There have been a number of previous reports of antigens common to different herpesviruses which are not cross-neutralized (see e.g. Blue & Plummer, 1973; Hampar & Martos, 1973). Whilst it seems premature to assume the presence of an antigen common to all members of the group (Kirkwood, Geering & Old, 1972) the results reported here provide an indication for at least some conserved features of otherwise rather different members of the group. Thus the viruses examined here have nucleic acids with widely different nucleotide compositions; from 56% (G+C) for EAV through 64%, 66.5% and 68.7% for BMV, HSV-1 and HSV-2 to 73% (G+C) for PRV (see, e.g., Goodheart & Plummer, 1975). It also seems worthy of re-emphasis that although there are significant measures of DNA-DNA homology between HSV-1 and HSV-2 and between HSV-1 and BMV (Kieff et al. 1972; Sterz et al. 1973/74), studies of cross-hybridization with the other herpesviruses we have examined here have shown little (HSV-1 and PRV ≤ 8%) or no (HSV-1 and EAV) significant homology (Ludwig, Biswal & Benyesh-Melnick, 1972).

Our comparative study of the structural polypeptide composition of the five herpesviruses provided general support for the above pattern of relationships but in addition clearly illustrated diversity of both detail and design. To assist and abbreviate the comparisons we wish to make we have summarized the results from the present paper together with published information on these and other herpesviruses in Fig. 2(a, b). We have preferred this diagrammatic summary to a tabular presentation since it permits a ready appreciation of overall complexity and patterns of structural relationship as well as conveying detailed information on relative molarity and mol. wt. It also avoids introducing additional nomenclature to a field already overburdened with numbered ‘bands’.

A superficial inspection of Fig. 2(b) provides good general support for considering BMV to be more closely similar to HSV-1 and HSV-2 than to other viruses. Thus, although the mol. wt. of almost every analogous component differs between HSV-1 and HSV-2, an extensive one-for-one correspondence is obvious. Similarly, despite differences of mol. wt., likely correspondences with many of the components of HSV-1 and HSV-2 can be recognized in the polypeptides of BMV. In contrast, excepting the major capsid proteins, VP1-2 analogues and the common possession of multiple glycoproteins in the range 45000 to 60000, PRV and EAV differ sufficiently from each other and from HSV-1, HSV-2 and BMV to make the recognition of analogous components uncertain. From Fig. 2 we can conclude that each of the herpesviruses so far analysed contains a major high mol. wt. protein analogous to the capsid polypeptide of HSV-1 and of similar but non-identical mol. wt. (from 140000 – MCMV and PRV, to 160000 to 165000 – EBV, HSV-2 and BMV)
and comprising more than 10% of virion protein mass. In addition, more than two polypeptides with an aggregate mol. wt. > 90000 are also represented in the herpesvirus capsid structure. However, despite the demonstration of an antigen common to these viruses, and evidence that this involves a reaction with a capsid component in the case of HSV-1 and HSV-2 as well as of PRV and HSV-1 (Honess et al. 1974), there is no single co-migrating protein present in all five viruses. We already have clear evidence that antigens common to HSV-1 and HSV-2 are carried on analogous but non-identical polypeptides. Thus, antiserum to VP5 of HSV-1 reacts with HSV-2 (Powell & Watson, 1975), although, as shown here, and elsewhere (Cassai et al. 1975), the major capsid polypeptide of HSV-2 has a different mobility from VP5 of HSV-1. Further, the immune precipitates made from HSV-1 and HSV-2 infected cells with heterologous antisera contain many polypeptides with type characteristic mobility. Whilst there remain interesting results to be obtained from general structural comparisons it will be of more interest to relate conserved and divergent structural features of different herpesviruses with the identification of those polypeptides which bear conserved and non-conserved antigenic determinants.

In considering our own and other studies of herpesvirus proteins we wish to make some additional comments and reservations. Firstly, not all polypeptides migrate ideally in our own or other gel systems and therefore changes in the relative migration of similar sized polypeptides can occur in gels of different strength or with different cross-linking agents (e.g. bisacrylamide, diallyltartardiamide or diacrylate). This problem is most acute with glycosylated versus non-glycosylated polypeptides but also occurs with non-glycosylated polypeptides. One example worthy of comment is the phosphorylated polypeptide VP22 of HSV-1 (Gibson & Roizman, 1974; annotated c within a lozenge, in Fig. 2) and the analogous component of HSV-2. The relative migration of VP22 and 23 from both HSV-1 and HSV-2 is highly dependent on gel strength (Spear & Roizman, 1972 and Heine et al. 1974) and the band order of VP22 and 23 from HSV-2 inverts with changes in gel strength in our gel system. This makes it particularly important to perform direct comparisons and to include well characterized viruses in these comparisons rather than rely on independent estimates of mol. wt. Secondly, estimates of polypeptide molarity based on the binding of Coomassie brilliant blue or on labelling with different amino acids can give values which may differ by many-fold, e.g., components annotated in Fig. 1. This is particularly true when a relatively rare amino acid such as methionine is used for labelling. Indeed, some estimates based on the incorporation of radioactivity from a labelled mixture of common amino acids (leucine, isoleucine and valine or a protein hydrolysate) were up to twofold different from those based on the binding of Coomassie brilliant blue stain (Spear & Roizman, 1972; Gibson & Roizman, 1974; Heine et al. 1974). Thus, whilst we have tried to encode information on real differences in polypeptide molarity in a semi-quantitative form (e.g. major capsid proteins of viruses in Fig. 2b each represent 10 to 15% of virus protein mass whereas the CMV polypeptide(s) of 150000 constitutes at least 23% of virus protein mass, Fiala et al. 1976) small differences should be interpreted with caution. Thirdly, we have only obtained data on polypeptides from one strain of EAV, PRV and BMV and whilst we have some idea of the degree of variation in the extant populations of HSV-1 and HSV-2 isolates (Cassai et al. 1976; Pereira et al. 1976) we do not know in what respects independent isolates of the other viruses may differ. In view of the lack of a uniform definition of virus strain, species or type it cannot be assumed that the variability between representatives of the other viruses will be of the same order as that observed between HSV-1 isolates (Pereira et al. 1976). Finally, we emphasize what should be self evident, that worthwhile comparisons can only be made between viruses which are not degraded nor significantly contaminated with non-structural...
virus proteins or components of the host cell. We have selected only one of four published accounts of the polypeptide composition of human cytomegalovirus (Fiala et al. 1976) for comparison with the present results. Whilst there are significant quantitative discrepancies in the proportional contribution of claimed structural proteins in the reports of Sarov & Abady (1975), Fiala et al. (1976) and Kim et al. (1976b), they do show some congruence in the size and number of major components. We are not able to reconcile the results of Gupta, St Jeor & Rapp (1977) with these preceding reports.

A study of five members of a group of over 70 members can only hint at the diversity to be anticipated but at least shows why the International Committee on Taxonomy of Viruses has encountered problems in the sub-division of the so-called Herpetoviridae into genera (Fenner, 1976). The Committee has defined only one genus – Herpesvirus (or herpes simplex group) consisting of ‘herpetoviruses of mammals that show a considerable degree of serological cross-reactivity (including neutralization)’. Plainly definition of ‘considerable’ will cause difficulty and the parenthetically proposed cross-neutralization may be a more useful genus limiting criterion, particularly in view of our demonstration of co-variation of this with the number of common antigenic components. On this basis pseudorabies virus (listed by I.C.T.V. as a possible member of the genus Herpesvirus and equine abortion virus would not be members of a genus including HSV-1 and HSV-2 together with bovine mammillitis. However, it should be noted that the exclusion of pseudorabies and equine abortion viruses may prove somewhat arbitrary if other members of the family prove to be less closely related serologically to HSV-1 and 2. We consider these problems elsewhere (Honess & Watson, 1977).

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REFERENCES


Proteins and antigens of herpesviruses


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