The Immunological Relationship between Canine Herpesvirus and Four Other Herpesviruses

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SUMMARY

Canine herpesvirus (CHV) was compared with four other herpesviruses by several serological techniques. Cross-neutralization was demonstrated between CHV and herpes simplex virus types 1 and 2 and pseudorabies virus. Non-neutralizing cross-reactions were found with these viruses and also with equine abortion virus and bovine mammillitis virus. The data suggest that CHV is immunologically more closely related to herpes simplex virus than to the other viruses used in this study.

INTRODUCTION

Canine herpesvirus (CHV) causes a fatal haemorrhagic disease in neonatal puppies and an upper respiratory infection in adult dogs. The virus was first characterized as a herpesvirus by Spertzel et al. (1965) and Carmichael et al. (1965) and it has been cultured only on cells of canine origin (Spertzel et al., 1965; Prydie et al., 1966; Cornwell et al., 1966; Karpas et al., 1967). Early workers found no cross-neutralization with other herpesviruses, namely herpes simplex virus (HSV), pseudorabies virus (PRV) (Spertzel et al., 1965; Carmichael et al., 1965; Prydie et al., 1966), infectious bovine rhinotracheitis virus (Spertzel et al., 1965; Carmichael et al., 1965), equine rhinopneumonitis virus (Carmichael et al., 1965; Prydie et al., 1966), feline rhinotracheitis virus (Prydie et al., 1966) and B virus (Spertzel et al., 1965). However, in 1968, CHV was reported to be immunologically related to HSV (strain MP) as demonstrated by a sensitive cross-neutralization test (Aurelian, 1968).

The objective of this study was to elucidate further the immunological relationship between CHV and four other herpesviruses i.e. HSV, PRV, bovine mammillitis virus (BMV) and equine abortion virus (EAV; equine herpesvirus type 1); these viruses and their corresponding antisera were compared by gel immunodiffusion, radioimmunoassay (RIA), neutralization of infectivity and Western blotting techniques.

METHODS

Cells and viruses. Baby hamster kidney cells (BHK-21) and Madin-Darby canine kidney cells were grown at 37 °C in Eagle's medium supplemented with 10% tryptose phosphate broth and 10% newborn calf serum (ETC).

The CHV strain was grown from an isolate obtained by Dr H. Thompson. The strains of HSV type 1 (HSV-1), HSV type 2 (HSV-2) and PRV were HFEM, 3345 and the Dekking strain, respectively. BMV (the Italian strain, isolated by G. Castrucci) and EAV (strain Rac-h) were obtained from Dr R. A. Killington, University of Leeds, Leeds, U.K.). Virus stocks were grown by infecting cells at an input multiplicity of 0.025 to 0.1 p.f.u./cell.

Preparation of immune sera. Antisera to CHV-infected dog kidney (DK) cells (prepared either in an adult dog or a rabbit) were tested against HSV-1, HSV-2, PRV, EAV and BMV. Hyperimmune rabbit antisera to HSV-1, HSV-2, PRV- and EAV-infected cells were also tested against CHV. In addition, rabbit antisera to HSV-1 glycoproteins B (gB) and D (gD) were included in some tests, and antiserum to HSV-2 major DNA-binding protein was used in Western blotting.

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Antiserum to HSV-1 gD (Band II) was prepared as described by Watson & Wildy (1969). Antiserum to HSV-2 major DNA-binding protein and to HSV-1 gB were obtained from Dr K. L. Powell (Wellcome, Beckenham, U.K.) and Dr I. W. Halliburton (University of Leeds).

For the preparation of canine antisera, DK cells were infected at an input multiplicity of 1 p.f.u./cell in Eagle's medium containing 2% calf serum. Cells were harvested after 24 h, washed twice in phosphate-buffered saline (PBS) and resuspended in water at a concentration of $1.4 \times 10^8$ cells/ml. Formaldehyde was added to a final concentration of 0.04%. Infected cells were incubated at 37 °C overnight, then assayed for residual infectivity. Injections of $2.8 \times 10^8$ inactivated infected cells and 1 ml alum adjuvant were carried out every month and blood was taken 10 days after each injection. For the preparation of rabbit antisera, DK cells were starved of serum for 24 h, infected with CHV (at an input multiplicity of 1 p.f.u./cell) in Eagle's medium containing 10% rabbit serum, and harvested after 24 h. The cells were washed in PBS and resuspended in water at a concentration of $1.4 \times 10^8$ cells/ml. Injections of $2 \times 10^8$ cells in 1.5 ml Freund's incomplete adjuvant were administered subcutaneously at monthly intervals and the rabbits were bledd 10 days after each injection.

Hyperimmune antisera to HSV-1 (anti-HSV-1) HSV-2 (anti-HSV-2) PRV (anti-PRV) and EAV (anti-EAV) were prepared by a modification of the method described by Watson et al. (1966). Rabbit kidney cells were infected at low input multiplicity (0.05 p.f.u./cell) and incubated for 2 to 3 days. The infected cells were washed in PBS, resuspended in small volume of water and inactivated in 0.04% formaldehyde. Subcutaneous injections, given at monthly intervals, consisted of approximately $6 \times 10^8$ infected cells and 1 ml Freund's incomplete adjuvant. Rabbits were bled 10 days after the fifth injection. Subsequent injections were given every 3 months.

Absorption of antisera to CHV-infected cells. Immune rabbit serum and immune dog serum were absorbed with DK cells at a concentration of $10^6$ cells per ml of serum. DK cells were washed three times in PBS and disrupted by sonicatin. Serum and cells were shaken for 1 h at 37 °C; then overnight at 4 °C. The serum was spun for 10 min at 3000 r.p.m., and ultracentrifuged for 1 h at 100000 g (Buchan et al., 1970).

Ager gel immunodiffusion. Virus antigens were prepared by infection of BHK cells with HSV-1, HSV-2, PRV, EAV or BMV and DK cells with CHV at input multiplicities between 1 and 10 p.f.u./cell, followed by incubation for 24 h at 37 °C. Cells were harvested, washed and cultured in PBS, resuspended in water at a concentration of $10^8$ cells/ml and disrupted by sonicatin. Uninfected DK and BHK cell antigens were prepared similarly. Purified agar (1%; Oxoid) in 0.85% NaCl, 0.1% sodium azide was heated to 100 °C and allowed to set for 2 h at room temperature. Wells were cut using a template of six wells (8 mm diameter × 2.5 mm depth) arranged around a centre well of similar dimensions. Serum and antigens were loaded at approximate volumes of 0.12 ml per well and left for 72 h at room temperature in a humidified box.

RIA. Virus antigens were prepared by infection of BHK cells with HSV-1, HSV-2, PRV, EAV or BMV and DK cells with CHV at a low input multiplicity of 0.025 p.f.u./cell, followed by incubation for 48 h at 37 °C. Cells were harvested, washed in PBS, resuspended in PBS at a concentration of $5 \times 10^7$ cells/ml and disrupted by sonicatin. Uninfected BHK and DK cell antigens were prepared similarly. Antigens were diluted further prior to use. Diluted virus antigen (50 μl) was added to each test well in flexible round-bottomed microtitre trays and left overnight at room temperature in a humid atmosphere. The antigen was removed and the trays were washed five times in PBS containing 0.05% Tween 20 (PBS-0.05% Tween 20). They were then incubated with PBS-0.01% Tween 20 at 37 °C for 1 h and, finally, washed in PBS-0.05% Tween 20.

Antisera were centrifuged at 3000 r.p.m. for 10 min, then diluted in PBS-0.05% Tween 20 containing ovalbumin at 200 μg/ml. Antiserum dilution (50 μl) was added per well and incubated for 1 h at room temperature. Antisera were removed and trays were washed five times in PBS-0.05%, Tween 20.

Radioiodinated Protein A ($^{125}$I-Protein A) was diluted in PBS-0.05% Tween 20 containing ovalbumin at 200 μg/ml. Antiserum dilution (50 μl) was added per well and incubated for 1 h at room temperature. Antisera were removed and trays were washed five times in PBS-0.05%, Tween 20.

The $^{125}$I-Protein A was removed and the trays were washed five times in PBS-0.05%, Tween 20. The trays were dried, wells were cut out and radioactivity was counted in a Multigamma counter (LKB).

RIA titre was defined as the reciprocal serum dilution at which the radioactivity when using infected cell antigen was twice the value obtained with uninfected cell antigen.

Neutralization tests. Neutralization of virus infectivity was measured by both conventional and augmented tests (Holmes et al., 1985). Anti-rabbit IgG and anti-dog serum (whole serum), for augmented neutralization tests were obtained from BDS, Birmingham, U.K. All sera were heat-inactivated at 56 °C for 30 min and diluted in PBS. Virus dilution (50 μl) ($5 \times 10^7$ p.f.u./ml) and serum dilution (50 μl) were incubated at 37 °C. After incubation for 4 h, either 100 μl ETC was added (for a conventional assay) or 100 μl anti-IgG of the appropriate animal species was added at a dilution of 1:1 in PBS (for an augmented assay). Incubation was continued for 20 min. Surviving virus was assayed in either BHK cells or DK cells. Preimmune canine or rabbit serum was included in every test.

Significant neutralization was judged by comparison of total plaque counts with preimmune serum, and plaque counts with the serum under test, which distribute in Poissonian fashion. The term 'significant' indicates a probability level of $P < 0.05$. 

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Western blotting. BHK cells (4 x 10⁶; for HSV-1, HSV-2, PRV, BMV or EAV) or DK cells (3 x 10⁶; for CHV) were infected at an input multiplicity of 10 p.f.u./cell in 50 mm Petri dishes and incubated for 20 h at 37 °C. Cells were harvested, washed twice in PBS and resuspended in 1 ml of disruption mixture (0.05 M-Tris–HCl pH 7, 1% SDS, 5% v/v 2-mercaptoethanol and 5% w/v sucrose, with bromophenol blue as the marker dye). [³⁵S]Methionine-labelled HSV-1 marker was prepared as above except that, 3 h after infection, the cells were washed twice in Eagle’s medium without amino acids and containing 2% calf serum (AAF EC2). Cells were then incubated in AAF EC2 containing 30 μCi [³⁵S]methionine and harvested at 20 h post-infection. The antigens were heated at 45 °C for 10 min and disrupted by sonication before loading onto polyacrylamide gels (Laemmli, 1970). Electrophoresis was carried out at 150 V, the gels were sliced into appropriate sections and the proteins were transferred to nitrocellulose using the method described by Burnette (1981).

Incubation of nitrocellulose with antibody and ¹²⁵I-Protein A was carried out essentially according to the method of Burnette (1981). Nitrocellulose strips (approx. 5 cm wide) were blocked in 5% bovine serum albumin in PBS for 2 h at room temperature and 1 h at 37 °C. They were then incubated in antisera diluted in PBS containing ovalbumin at 200 μg/ml for 90 min. The strips were washed for 10 min in PBS, then twice for 20 min in PBS–0.05% NP40 and finally for 10 min in PBS. They were then incubated with ¹²⁵I-Protein A diluted in PBS containing ovalbumin at 200 μg/ml to give approximately 3 x 10⁴ c.p.m./cm², for 30 min at room temperature. The strips were washed as above, stained for 1 min in 0.1% amido black, 45% methanol, 10% acetic acid, and destained for 1 min in 90% methanol, 2% acetic acid. The nitrocellulose was dried overnight and autoradiographed.

RESULTS

Immunological reactions as detected by agar gel diffusion and RIA

The results of the agar gel diffusion and RIA tests are shown in Table 1. In the gel diffusion tests CHV antigen reacted with antisera to HSV-1 and HSV-2, giving one common line. A positive result was also obtained in the RIA. Anti-HSV-1 and anti-HSV-2 gave RIA titres of 40 and 110 respectively (each of these values is a mean obtained from three tests using different antisera). No immunoprecipitin lines were seen when anti-HSV-1 gD, anti-PRV and anti-EAV were tested against CHV antigen. In the RIA, anti-EAV gave a titre of 630 while anti-HSV-1 gD, anti-HSV-1 gB and anti-PRV were negative.

When canine anti-CHV serum was tested against the various antigens by agar gel diffusion, one immunoprecipitin line was seen against EAV antigen, while HSV-1, HSV-2, PRV, and BMV antigens did not produce any lines. However, by RIA, HSV-1, HSV-2, PRV and EAV reacted with anti-CHV serum, giving titres of 690, 350, 1260 and 2000, respectively. No reaction between anti-CHV serum and BMV antigen was observed.

The homologous reaction between anti-CHV serum and CHV antigen gave three immunoprecipitin lines and an RIA dilution endpoint of 8910.

Table 1. Cross-reactions of CHV antigen with various sera and anti-CHV sera with various virus antigens

<table>
<thead>
<tr>
<th>CHV antigen and various sera</th>
<th>No. of immunoprecipitin lines</th>
<th>RIA titre</th>
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</thead>
<tbody>
<tr>
<td>Serum</td>
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<tr>
<td>Anti-HSV-1</td>
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</tr>
<tr>
<td>Anti-HSV-2</td>
<td>1</td>
<td>110</td>
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<tr>
<td>Anti-PRV</td>
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<td>&lt;5</td>
</tr>
<tr>
<td>Anti-EAV</td>
<td>0</td>
<td>630</td>
</tr>
<tr>
<td>Anti-BMV</td>
<td>ND*</td>
<td>ND</td>
</tr>
<tr>
<td>Anti-HSV-1 gD</td>
<td>0</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Anti-HSV-1 gB</td>
<td>ND*</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

<table>
<thead>
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<th>Anti-CHV serum and various virus antigens</th>
<th>No. of immunoprecipitin lines</th>
<th>RIA titre</th>
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<td>Virus antigen</td>
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<tr>
<td>HSV-1</td>
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<tr>
<td>HSV-2</td>
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<td>PRV</td>
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<td>1260</td>
</tr>
<tr>
<td>EAV</td>
<td>1</td>
<td>2000</td>
</tr>
<tr>
<td>BMV</td>
<td>0</td>
<td>&lt;4</td>
</tr>
<tr>
<td>CHV</td>
<td>3</td>
<td>8910</td>
</tr>
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* ND, Not done.
Table 2. Neutralization of infectivity of CHV by various antisera and of various viruses by anti-CHV serum

<table>
<thead>
<tr>
<th>CHV neutralization (%)</th>
<th>Virus neutralization (%)</th>
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</thead>
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<tr>
<td>Anti-HSV-1</td>
<td>37</td>
</tr>
<tr>
<td>Anti-HSV-2</td>
<td>21</td>
</tr>
<tr>
<td>Anti-PRV</td>
<td>36</td>
</tr>
<tr>
<td>Anti-EAV</td>
<td>0</td>
</tr>
<tr>
<td>Anti-HSV-1 gD</td>
<td>48</td>
</tr>
<tr>
<td>Anti-HSV-1 gB</td>
<td>0</td>
</tr>
</tbody>
</table>

* In the homologous reaction, serum was used at a final concentration of 1:1000. In all other reactions the final serum concentration was 1:1.

Neutralization of virus infectivity

The results of virus neutralization measured by a conventional test are shown in Table 2. Antisera to HSV-1, HSV-2 and PRV gave a reduction in CHV plaques of 37%, 21% and 36% respectively. Antiserum to HSV-1 gD gave a 48% reduction of CHV. Antisera to EAV and HSV-1 gB did not produce a significant reduction in CHV titre.

Antiserum to CHV reduced HSV-1 by 67%, HSV-2 by 75% and PRV by 52%. There was no significant neutralization by antiserum to CHV with EAV and BMV. Similar results were obtained by an augmented neutralization test (data not presented). The homologous reaction with anti-CHV serum (at a final dilution of 1:1000) reduced the proportion of CHV by 37% in the conventional and 74% in the augmented test.
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(a) Reaction with antiserum to EAV. Lane 1, [35S]methionine-labelled HSV-1 marker; lane 2, CHV-infected cell antigen; lane 3, DK cell antigen; lane 4, EAV-infected cell antigen; lane 5, [35S]methionine-labelled EAV. (b) Reaction with antiserum to PRV. Lane 1, [35S]methionine-labelled HSV-1 marker; lane 2, PRV-infected cell antigen; lane 3, BHK cell antigen; lane 4, CHV-infected cell antigen; lane 5, DK cell antigen.

Fig. 2. Western blotting of CHV-infected cells using antiserum to EAV and PRV. (a) Reaction with antiserum to EAV. Lane 1, [35S]methionine-labelled HSV-1 marker; lane 2, CHV-infected cell antigen; lane 3, DK cell antigen; lane 4, EAV-infected cell antigen; lane 5, [35S]methionine-labelled EAV. (b) Reaction with antiserum to PRV. Lane 1, [35S]methionine-labelled HSV-1 marker; lane 2, PRV-infected cell antigen; lane 3, BHK cell antigen; lane 4, CHV-infected cell antigen; lane 5, DK cell antigen.

Fig. 3. Western blotting of EAV- and PRV-infected cells using antiserum to CHV. (a) Reaction with absorbed antiserum to CHV. Lane 1, [35S]methionine-labelled HSV-1 marker; lane 2, BHK cell antigen; lane 3, HSV-1-infected cell antigen; lane 4, PRV-infected cell antigen; lane 5, EAV-infected cell antigen. (b) Reaction with preimmune serum. Lanes as in (a).
Fig. 4. Western blotting of BMV-infected cells using antiserum to CHV. (a) Reaction with absorbed antiserum to CHV. Lane 1, [35S]methionine-labelled HSV-1 marker; lane 2, BHK cell antigen; lane 3, BMV-infected cell antigen. (b) Reaction with preimmune serum. Lanes as in (a).

Fig. 5. Western blotting of CHV-infected cells using absorbed antiserum to CHV. Lane 1, [35S]methionine-labelled HSV-1 marker; lane 2, CHV-infected cell antigen; lane 3, DK cell antigen.

**Western blotting**

For these tests, anti-CHV sera were absorbed with DK cells. Immune and preimmune sera were incubated with homologous infected cell antigens, heterologous infected cell antigens and appropriate uninfected cell antigens.

Both antisera to HSV-1 and to HSV-2 reacted with a polypeptide of Mr, 129K in CHV-infected cells (Fig. 1). To investigate whether this was due to a cross-reaction of the major DNA-binding protein (Yeo et al., 1981) CHV-infected cells were incubated with antiserum to HSV-2 DNA-binding protein. This serum reacted with a polypeptide of Mr, 129K in HSV-1-, HSV-2-, and CHV-infected cells. In addition, antiserum to HSV-1 reacted with a polypeptide of Mr, 32K.

Antiserum to EAV also reacted with a polypeptide of Mr, 129K as well as polypeptides of Mr, 100K and 57K in CHV-infected cells (Fig. 2a). Antiserum to PRV reacted with a polypeptide of Mr, 151K, the major capsid protein of CHV (Fig. 2b).

The reaction of rabbit antiserum to CHV with PRV- and EAV-infected cells is shown in Fig. 3. In each case the serum identified a polypeptide of approximate Mr, 129K, together with bands
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of slightly higher $M_r$ (132K for PRV and 145K for EAV). A faint reaction was also seen with a polypeptide of $M_r$ 129K in HSV-1-infected cells. Tests with canine anti-CHV sera indicated that the major capsid proteins of PRV and EAV may also be involved in cross-reactivity (not shown).

The reaction of rabbit antiserum to CHV with BMV is shown in Fig. 4. Again, there was a predominant reaction with a polypeptide of approximately 129K $M_r$, and three other polypeptides of $M_r$ 112K, 95K, and 93K were also detected.

In the homologous reaction, anti-CHV serum reacted with more than 20 polypeptides in CHV-infected cells (Fig. 5). The $M_r$ of these ranged from 25K to 185K. Prominent reacting polypeptides were 151K $M_r$ (the major capsid protein), 129K $M_r$ (the putative major DNA-binding protein), and polypeptides of $M_r$ 101K, 89K, 74K, 60K, 50K, 40K, 32K and 27K.

**DISCUSSION**

This study has demonstrated an immunological relationship between CHV and four other herpesviruses. There was reciprocal cross-neutralization between CHV and HSV-1, HSV-2 and PRV. Antiserum to HSV-1 gD also neutralized CHV, suggesting that CHV shares epitopes with this glycoprotein. This protein has been shown to have a common antigenic site(s) with HSV-2 gD, and heterologous antiserum to this epitope(s) is able to neutralize virus (Sim & Watson, 1973). However, the immunoprecipitin line formed between CHV and antiserum to HSV-1 and HSV-2 was not observed with antiserum to HSV-1 gD, as would be expected if the same antigen was involved, unless the reaction was too weak to be observed under these conditions. Earlier studies showed no cross-neutralization between HSV-1 and PRV, and it appears that CHV spans previously proposed serological groups (Killington et al., 1977; Honess & Watson, 1977). Previous cross-neutralization data grouped HSV-1 and HSV-2 with BMV (Stertz et al., 1973–1974; Killington et al., 1977), whereas in this study no neutralization of BMV by antiserum to CHV was observed. This may be explained in part by the lack of neutralization of CHV by antiserum to HSV-1 gB, as glycoprotein B is involved in cross-neutralization between HSV-1 and BMV (Ludwig et al., 1978). However, it should be noted that antiserum to BMV, with which the reciprocal assay would be carried out, was not available. There was no significant neutralization between CHV and EAV, suggesting that EAV is more distantly related to CHV than are PRV, HSV-1 and HSV-2. As immune serum prepared against EAV gave low levels of neutralization against homologous virus in our own and other laboratories (Killington et al., 1977), this may require further exploration.

In Western blotting, antisera to HSV-1 and HSV-2 reacted with a polypeptide of $M_r$ 129K in CHV-infected cells. It appeared that this was due to a cross-reaction with the major DNA-binding protein, as in these cells antiserum to the major DNA-binding protein of HSV-2 reacted with a polypeptide of $M_r$ 129K. In addition, as antiserum to EAV also reacted with a polypeptide of $M_r$ 129K in CHV-infected cells and antiserum to CHV reacted with a polypeptide of $M_r$ 129K in PRV-, EAV- and BMV-infected cells, it is possible that these reactions were also due to the major DNA-binding protein. As Yeo et al. (1981) have shown that this protein is conserved among several herpesviruses and may have a role in virus assembly, it would not be surprising if this relationship encompassed CHV. Other cross-reacting polypeptides were observed with PRV, EAV and BMV and, indeed, antiserum to PRV reacted with the major capsid protein of CHV, suggesting that there may be antigenic similarities involving the structural proteins of these viruses.

CHV has been reported to contain 32.7% (G + C) (Plummer et al., 1969) and, in this laboratory, we have estimated a (G + C) content of 38% (unpublished results). These values differ markedly from those reported for HSV-1 (66.3%), HSV-2 (68.4%), PRV (74%), EAV (56 to 57%) and BMV (64%) (reviewed by Goodheart & Plummer, 1975). It has been suggested that a wide variation of percentage (G + C) between herpesviruses need not necessarily reflect distance of antigenic relationship (Honess & Watson, 1977). However, preliminary results in our laboratory have shown that, under conditions of low stringency, there is no DNA homology between CHV and HSV-1. The antigenic relationships between these viruses may therefore be due to conservation of small regions of DNA coding for sites essential for the conformation or activity of specific proteins.
This study has demonstrated that CHV is immunologically related to HSV-1, HSV-2, PRV, EAV and BMV. The closest relationship appears to be with HSV-1 and HSV-2, as a cross-reaction was demonstrated with these viruses in each type of test. It is our intention to explore the relationship between CHV and HSV-1 in cross-protection studies.

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