Bovine Cytomegaloviruses: Identification and Differential Properties

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SUMMARY

Biological properties and restriction enzyme patterns of the slowly replicating herpesviruses isolated from cattle affected with different diseases in North America and Europe were analysed. These virus isolates induced identical plaques that developed within 7 to 9 days in bovine foetal spleen cells and within 5 days in actively growing Georgia bovine kidney cells. These virus isolates were found to be antigenically related when tested in the indirect immunofluorescence test, and antigenic relationships with bovine herpesvirus 1 (BHV-1), BHV-2, BHV-3 or BHV-6 were not detected. The genomes of these strains were shown to have virtually identical cleavage sites when treated with restriction enzymes EcoRI, BamHI, SstII, SphI and HindIII. The resulting restriction enzyme patterns differed strikingly from those of BHV-1, BHV-2, BHV-3 and BHV-6. Because the herpesviruses tested become enveloped on the nuclear as well as on endoplasmic membranes, a process through which they induce cytoplasmic vesicles filled with enveloped viral particles, and because of the unique cytoplasmic inclusions that are induced, we classify them tentatively as bovine cytomegaloviruses.

INTRODUCTION

Herpesviruses are important causes of disease and latent infections in bovid animals (Ludwig, 1983). Bovid herpesvirus 1 (BHV-1) is recognized as the cause of infectious bovine rhinotracheitis (IBR), and other clinical syndromes (Madin et al., 1956; McKercher et al., 1957). A specific biotype of this virus is associated with genital infections in cows and bulls (Kendrick et al., 1958; Studdert et al., 1964). Mammillitis and skin disease are induced by BHV-2 (Martin et al., 1966). Antelope-associated bovine malignant catarrhal fever (MCF) in Africa was proven to be caused by BHV-3 (Plowright et al., 1960, 1965). Herpesviruses were isolated from lungs of sheep (Malmquist et al., 1972; De Villiers et al., 1975), and these isolates were later categorized as BHV-5 by De Villiers (1979). The herpesvirus associated with enteritis and death of newborn goats was differentiated as BHV-6 by Engels et al. (1983).

Other herpesviruses which differed from these were isolated from cattle with conjunctivitis and respiratory distress (Bartha et al., 1966; Mohanty et al., 1971; Smith et al., 1972), from lymphatic elements of cattle affected with MCF in Germany and the United States (Liebermann et al., 1967; Storz, 1968), from the uterine exudate of a cow with metritis (Parks & Kendrick, 1973), from leukocytes of cows with lymphosarcoma (Van Der Maaten & Boothe, 1972), and from cell cultures derived from kidneys of apparently normal calves (Luther et al., 1971). We isolated in 1975 a herpesvirus (75-P-2756) from an American bison (Bison bison) affected with MCF and found the morphology and ultrastructural features of morphogenesis compatible with those of human and murine cytomegaloviruses (Todd & Storz, 1983).

Comparative biological and genetic properties of this last group of bovine herpesviruses, also referred to as BHV-4, are described in this report. All strains of BHV-4 that were studied had identical cytopathogenic as well as plaquing properties, and the restriction enzyme pattern of their DNA was virtually identical to that of a previously described bovine cytomegalovirus.
METHODS

Cell cultures. Primary cultures of bovine foetal spleen cells (BFS) were prepared according to Malmquist et al. (1969). After three passages in Eagle’s minimum essential medium (MEM) containing 10% lamb serum, 500 µg streptomycin per ml and 500 units penicillin per ml, the MEM was replaced with lactalbumin vitamin medium. BFS cells were used in the 4th to 30th subpassages. In addition, the mycoplasma-free Georgia bovine kidney (GBK) cell line was used in growth-curve experiments and to propagate the viral strains for DNA extraction. These cells were grown and maintained in Dulbecco’s modified Eagle’s medium.

Virus strains. The reference strains were the bison herpesvirus 75-P-2756 (Todd & Storz, 1983), the Cooper strain of IBR (IBR-C) for BHV-1 (Chow et al., 1955) and the bovine herpes mammillitis strain TVA for BHV-2 (Sterz et al., 1973). Bovine herpesvirus strains that differed from these and which represented BHV-4 were Movar 33/63 (Bartha et al., 1966), 66-P-347 (Storz, 1968), DN-599 (Mohanty, 1975), DDV-71 (Parks & Kendrick, 1973) and UT (Ludwig, 1983).

Quantification of infectivity, plaque characteristics and neutralization. The plaque test with BFS cells was used to quantify infectivity and to compare the kinetics of formation and morphology of plaques induced by different strains, which all replicated well in BFS cells. The overlay contained 0.8% Noble agar in lactalbumin vitamin medium and 5% heat-inactivated bovine foetal serum. Six-well cluster plates with confluent monolayers of BFS cells were used. Aliquots of 0.25 ml of virus dilutions were adsorbed for 1 h; the overlay was added thereafter. A second plaque method employed GBK cells to which virus dilutions were added before seeding 1 × 10^5 to 2 × 10^5 cells/well in 24-well cluster plates. These actively growing cell cultures were overlaid with 1-6% carboxymethyl-cellulose in maintenance medium. The plates were incubated in CO2 incubators at 37°C. Cell cultures inoculated with IBR virus were stained with crystal violet 4 days after infection, while cultures with BHV-4 test strains were stained 9 to 12 days after infection of BFS cells and 5 to 6 days after infection of GBK cells. Plaque tests with BHV-2 were done with 1% carboxymethylcellulose in the overlay, and they were read after 3 to 4 days.

Serial tenfold dilutions of virus were mixed in 0.25 ml amounts with equal volumes of 1:5 dilutions of selected bovine sera, incubated for 1 h at room temperature and plated on BFS cells in 0.2 ml amounts. After adsorption for 1 h the overlay was added.

Indirect immunofluorescence. Sera used were from bison 75-P-2756 and from two calves (1244 and 1271) that had been inoculated with strain 66-P-347, 3 weeks prior to collection, and IBR antiserum from a calf, 883. Antigen consisted of BFS cells treated with 4% paraformaldehyde 3 days after inoculation with strains 75-P-2756, 66-P-347 or IBR-C. Uninoculated BFS cells similarly fixed served as controls. Serum samples, diluted from 8 to 256 in twofold steps, were added to the slides which were incubated in a humidity chamber at 37°C for 1 h. After rinsing, fluorescein isothiocyanate-conjugated anti-bovine IgG diluted 1:16 with a 1:35 solution of rhodamine-labelled bovine serum albumin was added and incubated for 30 min. The slides were then prepared for evaluation with a Leitz Orthomat microscope.

Electron microscopic analysis. Monolayers infected with each of the different strains used were scraped from the substrate and centrifuged at 800 g for 20 min. Small drops of the supernatant fluid were placed on Formvar- and carbon-coated grids, stained with 2% phosphotungstic acid and dried under vacuum prior to examination with a Hitachi HU-12 electron microscope. The critical features of the envelopment process of strain 75-P-2756 in BFS cells were analysed in specimens prepared as described by Todd & Storz (1983).

One-step replication curve experiments. Duplicate GBK monolayers (1.0 × 10^6 cells) were inoculated at a multiplicity of infection of 2. At given intervals the cells and supernatant were harvested independently, frozen and thawed three times, centrifuged for 10 min at 3000 rev/min, and the supernatants of these preparations were used for infectivity titration by plaque assay in GBK cells. Five days after infection the cells were fixed in 4% formalin and stained by the Giemsa method.

Preparation of viral DNA from nucleocapsids. Ten cultures (diam. 15 cm) of GBK cells infected at a multiplicity of infection of 0.1 were used per isolate for each DNA preparation. Supernatants (approx. 200 ml/isolate) were harvested 100 to 120 h after infection, and centrifuged (10 min; 3000 rev/min). The resulting supernatant was further centrifuged for 60 min at 25000 rev/min in a Beckman SW27 rotor. To remove the remaining cellular debris the pellet was resuspended in TE buffer (0.01 M-Tris-HCl pH 7.4, 0.001 M-EDTA) with 0.1% Nonidet P40 (NP40), placed on a sucrose cushion (30% w/v in TE), and centrifuged for 90 min at 35000 rev/min in a Beckman SW41 rotor. For lysis of the nucleocapsids the pellet was resuspended in 5 ml TE with 1% SDS, and proteinase K (Sigma) was added to a final concentration of 0.1 mg/ml. The suspension was incubated at 40°C overnight to assure complete lysis of the nucleocapsids. The DNA was extracted twice with water-saturated phenol/creosol/hydroxyquinoline (90:10:0.01, by v, v and w), the DNA was further extracted with isoamyl alcohol/chloroform (1:24, v/v). Two to 3 vol. absolute ethanol was added to the resulting aqueous phase, which was kept overnight at -20°C. The DNA precipitate was sedimented by centrifugation for 30 min at 5000 g (− 20°C). The precipitate was then dissolved in tenfold-diluted sterile standard saline citrate and stored at 4°C. Following this protocol, a yield of 20 to 50 µg of total viral DNA was usually obtained. For this estimation, the staining intensities of lambda HindIII fragments in agarose gels served as standards.
Restriction enzyme analysis. The following endonucleases (Bethesda Research Laboratories) were used: EcoRI, BamHI, SacI, SphI and HindIII. Digestion conditions were those recommended by the supplier.

Gel electrophoresis and scanning. Horizontal gels (11 × 14 cm) made of 0.8% or 1.5% agarose in electrophoresis buffer (38 mM-Tris base, 26 mM-NaH2PO4, 1 mM-EDTA) were used. The gels were run for 1 or 10 h at 100 V or 50 V, respectively, and stained for 30 min in ethidium bromide (2 μg/ml) to visualize the DNA fragments. The stained gels were photographed under u.v. illumination (305 nm) using Polaroid-Land film type 57. The negatives of the DNA photographs were scanned using a laser densitometer (LKB).

RESULTS

Virus morphology

All BHV-4 strains studied had non-enveloped and enveloped viral structures. The nucleocapsids had a diameter of 90 to 100 nm and consisted of ordered arrangements of short tubular capsomeres, which appeared as small rings when viewed face-on towards the centre of the virion. Many capsids in preparations of the BHV-4 strains tested were centrally filled with stain, which indicated that they were empty. An integument zone of moderate density was observed between capsid and viral envelope. The envelope was pleomorphic and had small projections. Some virions from IBR-C preparations had completely intact envelopes, into which stain did not penetrate. These appeared then as fringed round structures with a diameter of 150 to 180 nm. Such forms were not seen in the BHV-4 virus preparations. We did not see other differences among the negatively stained preparations of the strains studied. Importantly, evidence for the presence of other viruses in these preparations was not found.

Cytomegalic features of reference strain 75-P-2756

The capsomeres formed in the nucleus, often in highly ordered linear arrays. The envelope was rarely observed to be formed by budding of the nucleocapsid on the inner nuclear membrane. Often the nucleocapsids were found free in the cytoplasm of infected cells, and they obtained an envelope by budding on membranes of extended vesicles of the Golgi body elements or the smooth endoplasmic reticulum (Fig. 1). Large membrane-bound accumulations of virions formed in the cell cytoplasm through this process (Fig. 1). Another striking feature characteristic of cytomegaloviruses of man and mice was the development of electron-dense inclusions in the cytoplasm at 48 to 72 h after infection of BFS cells with strain 75-P-2756. These inclusions were always found outside the infected nucleus, but their shape was variable (Fig. 2). They consisted of an amorphous electron-dense substance, granular fibrils and viral capsids with and without cores. Enveloped viruses were also associated with these cytoplasmic inclusions. Similar ultrastructural morphogenetic features were found in cells infected with 66-P-347, DN-599 or ÚT. Giemsa-stained BFS cells infected with 75-P-2756 or 66-P-347 had Cowdry type A nuclear inclusions, but unequivocal cytoplasmic inclusions were not detected by light microscopic evaluation.

Kinetics of plaque formation and morphology in BFS cells

Plaques in BFS monolayers infected with 75-P-2756 were not discernible for almost 7 days after infection when pinpoint, clear plaques became detectable (Fig. 3). These increased slowly in size between 8 and 13 days after infection, when they reached a diameter of 0.5 mm. Accordingly, the plaque assay plates were routinely stained 9 or 10 days after plating. The plaques induced in BFS cells by the strains Movar 33/63, 66-P-347, DN-599 and DDV-71 were indistinguishable from 75-P-2756 plaques. The comparisons were made with limiting tenfold dilutions of the viral strains. These strains induced plaques within 5 days when plated in actively growing GBK cells in the presence of carboxymethylcellulose. In contrast, the plaques induced by IBR-C virus in BFS cells reached a diameter of 2 to 3 mm 4 days after plating. They were clear and uniformly round when Noble agar was used as solidifying medium, and they were of the same diameter and round with fuzzy margins when carboxymethylcellulose was used. Plaques were induced by BHV-2 with carboxymethylcellulose but not with Noble agar in the overlay. These plaques had a diameter of 1 to 2 mm by the 3rd day after plating, and they were turbid and had fuzzy edges. Disintegrating polykaryons were present in the turbid area of these plaques. The plaque morphology of the different bovine herpesviruses is given in Table 1.
Fig. 1. Morphogenesis of the 75-P-2756 strain of bovine cytomegalovirus 48 h after infection of BFS cells. Viral capsids in nucleus (N) (arrow), envelopment of virions on cytoplasmic membranes (arrowhead), and membrane-bound cytoplasmic compartments containing enveloped virions (CV) are shown. Bar marker represents 500 nm.

Fig. 2. Cytoplasmic inclusions (CI) with electron-dense amorphous material, nucleocapsids and enveloped virions in BFS cells infected with bovine cytomegalovirus strain 75-P-2756. G, Golgi body. Bar marker represents 500 nm.

One-step replication curves
The ÜT and 66-P-347 and 75-P-2756 isolates remained highly cell-associated for 48 h. At this time the gradually increasing release of virus from infected cells reached but did not exceed the infectivity of the cellular fraction, as shown in Fig. 4 representing strain ÜT.
Fig. 3. Plaques induced in BFS cells 4 days after infection (a) with BHV-1 (Cooper strain) and 9 days after infection (b) with bovine cytomegalovirus 66-P-347.

Fig. 4. One-step replication curve of bovine cytomegalovirus strain UT assayed as cell-associated virus (●) and extracellular virus (○).

Table 1. Comparison of plaque properties of various bovid herpesvirus strains

<table>
<thead>
<tr>
<th>Virus</th>
<th>Titre (p.f.u./ml)</th>
<th>Diameter</th>
<th>Morphology and overlay*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHV-1 (IBR-C)</td>
<td>$20 \times 10^6$</td>
<td>4+ 2</td>
<td>Clear, round (NA)</td>
</tr>
<tr>
<td>BHV-2 (BHM-TVA)</td>
<td>$15 \times 10^6$</td>
<td>3+ 1-2</td>
<td>Clear, fuzzy (MC)</td>
</tr>
<tr>
<td>BHV-4 (75-P-2756)</td>
<td>$60 \times 10^6$</td>
<td>9+ 0-5</td>
<td>Clear, round (NA)</td>
</tr>
<tr>
<td>(Movar 33/63)</td>
<td>$28 \times 10^6$</td>
<td>9+ 0-5</td>
<td>Clear, round (NA)</td>
</tr>
<tr>
<td>(66-P-347)</td>
<td>$34 \times 10^6$</td>
<td>9+ 0-5</td>
<td>Clear, round (NA)</td>
</tr>
<tr>
<td>(DN-599)</td>
<td>$13 \times 10^6$</td>
<td>9+ 0-5</td>
<td>Clear, round (NA)</td>
</tr>
<tr>
<td>(DDV-71)</td>
<td>$14 \times 10^6$</td>
<td>9+ 0-5</td>
<td>Clear, round (NA)</td>
</tr>
</tbody>
</table>

* NA, Noble agar overlay; MC, carboxymethylcellulose overlay.
Table 2. Antigenic relationship between strains 75-P-2756, 66-P-347 and IBR-C

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>75-P-2756</th>
<th>66-P-347</th>
<th>IBR-C</th>
<th>BFS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PNI*</td>
<td>N</td>
<td>C</td>
<td>PNI</td>
</tr>
<tr>
<td>75-P-2756</td>
<td>&lt;1 8†</td>
<td>32</td>
<td>&lt;1 64</td>
<td>&lt;1 8</td>
</tr>
<tr>
<td>1244 (pre)</td>
<td>&lt;1 8</td>
<td>&lt;8</td>
<td>&lt;1 8</td>
<td>&lt;1 8</td>
</tr>
<tr>
<td>1244 (post)</td>
<td>&lt;1 8</td>
<td>64</td>
<td>&lt;1 8</td>
<td>&lt;1 8</td>
</tr>
<tr>
<td>1271 (pre)</td>
<td>&lt;1 8</td>
<td>&lt;8</td>
<td>&lt;1 8</td>
<td>&lt;1 8</td>
</tr>
<tr>
<td>1271 (post)</td>
<td>&lt;1 16</td>
<td>64</td>
<td>&lt;1 16</td>
<td>&lt;1 8</td>
</tr>
<tr>
<td>IBR-883</td>
<td>&lt;1 8</td>
<td>&lt;8</td>
<td>&lt;1 8</td>
<td>&lt;1 8</td>
</tr>
</tbody>
</table>

* PNI, Plaque neutralizing index of 1:5 serum dilution; N, nuclear fluorescence; C, cytoplasmic and juxtanuclear fluorescence.
† Highest dilution of serum giving specific fluorescence.

Table 3. Comparison of DNA cleavage patterns of strains representing bovid herpesvirus 4

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>Strains with similar patterns</th>
<th>Strains with identical patterns</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI</td>
<td>All six strains*</td>
<td>66-P-347 = DN-599</td>
</tr>
<tr>
<td>BamHI</td>
<td>All six strains</td>
<td>66-P-347 = DN-599</td>
</tr>
<tr>
<td>SstII</td>
<td>All six strains</td>
<td>66-P-347 = DN-599</td>
</tr>
<tr>
<td>SphI</td>
<td>All six strains</td>
<td>66-P-347 = 75-P-2756 = ÜT;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DN-599 = DDV-71</td>
</tr>
<tr>
<td>HindIII</td>
<td>All six strains</td>
<td>All strains except Movar 33/63</td>
</tr>
</tbody>
</table>

* The strains are ÜT, DN-599, DDV-71, 75-P-2756, 66-P-347 and Movar 33/63 (see Methods).

Table 4. Estimated number of cleavage fragments

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>Movar 33/63</th>
<th>66-P-347</th>
<th>DN-599</th>
<th>DDV-71</th>
<th>75-P-2756</th>
<th>ÜT</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI</td>
<td>17+(1)*</td>
<td>17+(1)</td>
<td>17+(1)</td>
<td>17+(1)</td>
<td>17+(1)</td>
<td>17+(1)</td>
</tr>
<tr>
<td>BamHI</td>
<td>19+(1)</td>
<td>20+(2)</td>
<td>20+(2)</td>
<td>20+(2)</td>
<td>22+(2)</td>
<td>21+(2)</td>
</tr>
<tr>
<td>SstII</td>
<td>4+(3)</td>
<td>3+(5)</td>
<td>3+(5)</td>
<td>3+(5)</td>
<td>4+(5)</td>
<td>4+(5)</td>
</tr>
<tr>
<td>SphI</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>HindIII</td>
<td>18</td>
<td>19</td>
<td>19</td>
<td>19</td>
<td>19</td>
<td>19</td>
</tr>
</tbody>
</table>

* The numbers of cleavage fragments that appear in a 20- to 30-fold higher ratio are given in parentheses.

Antigenic comparison

Different bovine sera from herds with MCF-affected cattle and the two calves that had been inoculated experimentally with strain 66-P-347 were tested in the plaque neutralization test as fresh serum, following inactivation at 56 °C for 30 min, as well as after addition of guinea-pig complement. Infectivity-neutralizing activity against 75-P-2756 or 66-P-347 was not detected. An antigenic comparison was therefore made by indirect immunofluorescence. The results are given in Table 2. The serum of bison 75-P-2756 reacted with cells infected with the virus isolates 75-P-2756 and 66-P-347 but not with cells infected with IBR-C virus. Nuclear and diffuse cytoplasmic as well as strong juxtanuclear fluorescence was seen. The titres of antibodies reacting with nuclear herpesvirus antigens were always lower than those reacting with cytoplasmic antigens. Antibodies in sera of the two calves exposed to 66-P-347 reacted similarly with 75-P-2756-infected cells but not with cells infected with IBR-C virus.

Comparison of restriction enzyme patterns

The genomes of the different bovine herpesvirus isolates were digested with EcoRI, BamHI, SstII, SphI and HindIII. Each of the endonucleases induced a cleavage pattern, which was either identical or very similar to those of other BHV-4 strains (Fig. 5 a, b). The patterns induced by the
Bovine cytomegaloviruses

Fig. 5. Restriction enzyme pattern after treatment with (a) endonuclease SstII and (b) BamHI. Lanes 1, UT; lanes 2, DN-599; lanes 3, DDV-71; lanes 4, 75-P-2756; lanes 5, 66-P-347; lanes 6, Movar 33/63. Lanes 7 contain marker DNA: bacteriophage lambda HindIII fragments of 23.1 kb, 9.4 kb, 6.6 kb, 4.4 kb and 2.3 kb.

different endonucleases are compared in Table 3. The estimated number of cleavage fragments is given in Table 4. Scanning analysis revealed one or more fragments in the range of 200 to 2800 base pairs that had a 20- to 30-fold molar ratio. These fragments of high molar ratio emerged with EcoRI, BamHI, SstII but not with SphI or HindIII. These restriction enzyme patterns of the bovine viral isolates representing BHV-4 clearly differ from those of BHV-1, BHV-2, BHV-3 and BHV-6.

DISCUSSION

Maturation and envelopment of representative strains of BHV-4 by budding on nuclear or smooth cytoplasmic membranes of Golgi body elements as well as the formation of unique cytoplasmic inclusions characterize these strains as cytomegaloviruses (Todd & Storz, 1983; Craighead et al., 1972; Sarov & Alsady, 1975; Fong et al., 1980). Similar ultrastructural features were shown in figures of previous reports of BHV-4 isolates, but their comparative significance was not emphasized (Liebermann et al., 1967; Schulze et al., 1967).

The kinetics of plaque formation and their morphology in BFS cells of the bison herpesvirus strain are identical to plaques induced by the strains Movar 33/63, 66-P-347, DN-599 and DDV-71. The difference in the speed of plaque induction in BFS and actively growing GBK cells may
depend on the stage of the cells in the cycle of multiplication. These isolates replicate relatively slowly, which is characteristic of cytomegaloviruses isolated from other animal species. Furthermore, there is an antigenic relationship between the bovine isolate 66-P-347 and the prototype 75-P-2756 in the indirect fluorescent antibody test, but the neutralizing activity of the sera involved was minimal. Antigenic relations were established previously among the isolates DN-599, FTC and V11 (Potgieter & Mare, 1974). The DNA genomes of the viral strains 75-P-2756, 66-P-347, DN-599, DDV-71, Movar and ÚT have virtually identical restriction enzyme patterns, which differ from those of BHV-1 (IBR and IPV), BHV-2, BHV-3 (Ludwig, 1983), and BHV-6 (Engels et al., 1983). Consequently, we propose to classify the BHV-4 collection of viral isolates as bovine cytomegaloviruses on a tentative basis, pending further comparisons with other herpesviruses.

The relationship of this class of bovine cytomegaloviruses to cytomegaloviruses of man and other animal species needs further clarification. Preliminary data indicate that the genome of bovine cytomegalovirus is approximately 95 × 10⁶ mol. wt. (B. Ehlers & H. Gelderblom, unpublished results), and it is thus considerably smaller than the size reported for human, murine and equine cytomegaloviruses (Lakeman & Osborn, 1979). The appearance of DNA fragments with high molar ratio in patterns derived with different restriction enzymes suggests that a high proportion of the genome has tandem reiterations of the same sequence. This could indicate a genome structure similar to that of *Herpesvirus saimiri* (Fleckenstein et al., 1975).

The BHV-3 of the antelope-associated form of MCF did not induce plaques in BFS and GBK cells in our system, but Hazlett (1980) obtained plaques in rabbit kidney (RK13) cells with a cell-free MCF isolate. Specific antisera (kindly supplied by N. Edington, Royal Veterinary College, London) did not cross-react with BHV-4-infected cells (T. Leiskau & H. Ludwig, unpublished results). Furthermore, the restriction enzyme pattern of BHV-3 strains WC and C500 differed from those of the BHV-4 strains which we investigated (Ludwig, 1983). A recent report by Herring et al. (1983) characterized the DNA of BHV-3 as resembling that of *H. saimiri* and *H. ateles*. The fragmentary knowledge so far accumulated about the DNA structure of BHV-3 and BHV-4 implies that these represent different bovid herpesviruses. A herpesvirus isolated from lymphosarcomatous cattle appears to differ from other BHV-4 strains in its cytopathic and cell fusion activities (Van Der Maaten & Boothe, 1972).

The bovine cytomegaloviruses have been recovered from cattle affected with a variety of disease conditions as well as from apparently normal subjects. By recognizing the true nature of these virus isolates more enlightened approaches for clarifying the pathogenic roles are possible. It is apparent that the bovine cytomegaloviruses are present as a persistent infection and are expressed by hosts that are compromised (Osoño & Reed, 1983). The low levels of infectivity-neutralizing antibodies in cattle with bovine cytomegalovirus infections are also striking and are in accordance with the nature of cytomegaloviruses. There would appear to be multiple herpesvirus infections in cattle affected with MCF in Europe and North America if this condition is indeed caused by a virus more elusive than, but otherwise similar to, BHV-3.

These viruses evidently have a wide distribution in cattle of Europe and North America. This fact suggests that determined efforts be made to investigate bovine cytomegaloviruses as a potential pathogen for cattle, because these viral agents are important causes of disease in man and other animal species (Weller, 1971).

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