Characteristics of Bovine Mammillitis Virus

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SUMMARY

A virus isolated from cattle in Scotland does not seem to have been described previously as existing in Europe. The virus has been provisionally designated bovine mammillitis virus (BMV). It grows in tissue cultures, particularly of bovine origin, giving rise to large multinucleate cells which have type A inclusions in many of their nuclei. Baby mice injected with it develop skin lesions, but only a limited number of passages can be made. The BMV particle has a nucleocapsid of about 80 m μ diameter which, in complete particles, is surrounded by a loose envelope. The virus is sensitive to ether and chloroform, and contains DNA with a base composition of 64% G+C and a band-width molecular weight of 34×10^6 . BMV shows antigenic similarity, in neutralization tests and double-diffusion tests in agar, to the group II viruses (prototype Allerton) of lumpy skin disease (Alexander, Plowright & Haig, 1957). All these features place it in the family of the herpesviruses.

INTRODUCTION

Milking cows on several farms in the West of Scotland were affected with ulcers on their teats during the autumn of 1963. From one a virus was isolated in tissue cultures. Infected culture fluid produced lesions similar to those of the naturally occurring disease when injected into cattle, and from these the virus was re-isolated (Martin, Martin & Lauder, 1964). The clinical syndrome did not appear to have been described previously but might have been confused with cowpox. The virus seemed a new agent with general features similar to those of the Herpesvirus group; and also to resemble in particular the group II viruses (prototype Allerton) described in association with one type of lumpy skin disease of cattle in Africa (Alexander, Plowright & Haig, 1957). These findings are reported in this paper.

The virus has been provisionally named bovine mammillitis virus (BMV).

METHODS

Viruses. BMV was isolated initially in bovine lymph node cells, but subsequent passages were made in a variety of cells including calf kidney (CK), bovine conjunctiva (DBC), lamb testis (LT) and the BHK 21/C13 line of baby hamster kidney cells.

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The Allerton strain of the group II lumpy skin disease viruses of Alexander *et al.* (1957) was received from the Kenya Veterinary Research Laboratories, Kabete, as the second calf kidney passage and was passaged further in CK or BHK 21/C13 cells.

All virus titrations were carried out in tubes or bottles, generally using four replicate cultures per ten-fold dilution. Titres are expressed as 50 % cytopathogenic doses (Cp.D 50) per ml.

Media. Eagle's basal medium with 2× concentration of vitamins and amino acids and supplemented with tryptose phosphate broth (10 %, v/v), or Hanks's balanced salt solution with lactalbumin hydrolysate (0.5 %, w/v) and yeast extract (0.1 %, w/v), were generally used for growing and maintaining cells. Unheated calf serum (10 %, v/v) was incorporated in all media, but was omitted when growing virus, to avoid any possible neutralization with different batches of serum. The diluent for viruses and sera also consisted of Eagle's medium plus tryptose phosphate, without calf serum.

Antisera. The antisera against BMV and Allerton virus used in the neutralization and precipitation tests were produced in guinea pigs. These were given two intradermal injections of virus, as tissue culture fluid of the 7th CK cell passage, at an interval of 1 month and bled 10 days after the second injection. Antisera against other viruses came from sources given in the Acknowledgements, were prepared in rabbits unless otherwise indicated, and were stated to have the following neutralizing titres (reciprocal of end-point dilution) against 100 Cp.D 50 of the homologous virus: pseudorabies antiserum, > 1000; equine herpes type 1 and type 2 antiserum, both > 1000; B virus antiserum (from monkey), 100; infectious bovine rhinotracheitis (IBR) antiserum, 512; malignant catarrhal fever (MCF) antiserum (from ox), 64; herpes simplex antiserum (from guinea pig), 32. The varicella human acute-phase serum had a complement-fixing titre of < 8; while the convalescent-phase serum had a titre of ≥ 512 .

Neutralization and precipitation tests. Neutralization tests were performed in tubes. Serial tenfold dilutions of virus were made. Generally, four tubes were used for each dilution. A constant 1/5 or 1/10 dilution of antiserum, inactivated at 56° for 30 min., was mixed with an equal volume of each dilution of virus and allowed to interact either at 37° for 60 min. or at 4° overnight. Each tube was inoculated with 0.2 ml. of virus + serum mixture, given 1 ml. of medium without calf serum, and incubated at 37°. Tests were read after 5 days.

Double-diffusion tests were done in agar on glass slides, using 2 ml. of Difco 'Bacto' agar per 3 in. × 1 in. slide. The concentration of agar was 0.7 % (w/v) in 0.14 M-NaCl buffered with 0.1 M-phosphate, pH 7.0 (PBS). Cups 3 or 5 mm. in diameter were cut in the agar with cork-borers and filled to the brim with reactants. The edge-to-edge distance between cups was 4–6 mm. Slides were placed in humidified boxes at room temperature, and tests were usually read after 3 days. The antigen preparations for gel-diffusion tests were made from BMV or Allerton virus grown in BHK 21/C13 cells at 34° for 3 days. The cells were scraped off into the medium and spun down at 1500 rev./min. for 10 min. The deposit was resuspended in PBS at a concentration of $\sim 10^8$ cells/ml. and disrupted by ultrasonic vibration in a Dawe Soniclean bath. Concentrates were stored at –20°. Antisera were heated at 56° for 30 min. before use. The anti-BMV serum contained low levels of precipitating antibody against a calf serum component and was therefore mixed

before use with an equal volume of calf serum. The antiserum prepared against Allerton virus formed no precipitate with calf serum.

DNA extraction. The method used was based on that developed previously for herpes viruses (Russell & Crawford, 1964). Infected cell suspensions were treated with sodium dodecyl sulphate (1 %) for 2 min. at 25° and then made 1 N with sodium perchlorate and deproteinized by shaking with chloroform + isoamyl alcohol (24:1) as described by Marmur (1961). The DNA was then collected on a glass rod after addition of 2 volumes of ethanol and redissolved in standard saline citrate (Marmur, 1961).

Density gradient centrifugation. Equilibrium density gradient centrifugation was used to determine the base composition and molecular weight of the DNA (Meselson, Stahl & Vinograd, 1957). The DNA was centrifuged in CsCl (density 1.72 g./ml.) containing Tris buffer (0.05 M, pH 8.5) for 1 day at 44,770 rev./min. followed by 2 days at 31,410 rev./min. in a Spinco model E ultracentrifuge. Ultraviolet absorption photographs were taken and scanned with a Joyce-Loebl microdensitometer to determine the shape and positions of the bands. A derivative of the band width squared was plotted against relative DNA concentration and the molecular weight calculated from the slope of the plot (Thomas & Berns, 1961). The base composition was calculated from the position of the bands relative to marker DNA of known density (Sueoka, 1961).

Ether and chloroform sensitivity. Ether sensitivity tests were performed after the method of Andrewes & Horstmann (1949). Chloroform sensitivity was estimated by mixing 1 part of chloroform with 2 parts of infective tissue culture fluid. The mixture and the control were shaken for 10 min. at room temperature, centrifuged lightly and the supernatant inoculated on to monolayers.

Electron microscopy. Cultures of foetal bovine conjunctiva cells were infected with BMV and harvested 5 days later. Both the cells and the supernatant culture fluid were prepared for examination in the electron microscope. The tissue culture fluid was centrifuged at 800–1000 rev./min. to remove cell debris, then at 100,000g for 90 min. The pellet obtained was resuspended in a drop of fluid; a droplet was placed on a specimen grid, which was then washed with distilled water and inverted on to a solution of potassium phosphotungstate (PTA), 1 % (v/v), pH 7, the excess stain being drained off on filter paper before examination.

Cell pellets were obtained by scraping infected monolayers from bottles into a small volume of medium and centrifuging at 800–1000 rev./min. for 5 min. After washing in buffered saline, the cell pellet was fixed in buffered osmium tetroxide (1 % (v/v), pH 7.4) for 45 min. at 4°. The fixed cells were then washed quickly in water, dehydrated with increasing concentrations of ethanol, and embedded in prepolymerized methacrylate (9:1 mixture of *n*-butyl and methyl methacrylate). Thin sections were cut with a Huxley-type microtome and stained with lead hydroxide (Millonig, 1961). All examinations were made in a Siemens Elmiskop 1 at magnifications of $\times 20,000$ and $\times 40,000$.

RESULTS

Tissue culture

A small volume of fluid aspirated from the lesions on one cow produced morphological changes when inoculated on to monolayers of calf lymph node, DBC, or BHK 21/C13 cells. The appearance of control and infected DBC cultures is shown in Pl. 1, figs. 1, 2. After 24–48 hr incubation at 37°, discrete foci of cytopathic change appeared which became confluent as they enlarged and increased in number. They consisted of large cell masses of bizarre shape, often with long cytoplasmic processes, apparently formed by the fusion of many cells. Large, refractile, globular cells could be seen, loosely attached to the remaining cell sheet or floating freely in the medium. The end process, which followed in 2–4 days from the onset, was complete disruption and detachment of the cell monolayer.

Coverslip preparations of uninfected and BMV-infected DBC cells were fixed with methanol and stained with haematoxylin and eosin (Pl. 1, figs. 3, 4). Infected cultures showed large syncytia with varying numbers of nuclei, sometimes as many as 30–40 in one giant cell. Many nuclei contained single, homogeneous, eosinophilic inclusions separated from the marginated chromatin by a clear zone. The virus grew and produced large syncytia in several different cell types, such as CK, DBC and LT cells, reaching levels of 10^4 – 10^5 Cp.D50/ml. in the culture fluid. Such fluids, mixed with an equal volume of buffered glycerol (50%, v/v) and stored for 4 months at 4°, showed a loss of $10^{0.5}$ Cp.D50/ml., whereas samples stored without glycerol lost $10^{1.0}$ Cp.D50/ml. Storage at –20° was satisfactory for at least 4 months with or without added glycerol.

BMV was sensitive to ether and chloroform. With control virus titres of $10^{4.0}$ – $10^{5.5}$ Cp.D50 per ml. of culture fluid, no infectivity could be detected in the treated preparations, even when tested undiluted.

Animal inoculation

Experimentally produced lesions in cattle resembled those of the natural disease (Martin *et al.* 1964; Martin, Martin, Hay & Lauder, 1966). Skin lesions were produced in guinea pigs and baby mice. In the former, on inoculation of BMV culture fluid into the skin of the back or of the hind-footpads, an inflamed area developed at the site of inoculation, followed by superficial exfoliation. White mice 1–3 days old could be infected readily with BMV, whereas those more than about 7 days old failed to develop lesions. On subcutaneous or intraperitoneal inoculation of infective fluid of the fifth to the tenth culture passage into newborn mice, inflamed patches appeared on the skin of the head and trunk after 4–7 days. The lesions were particularly severe around the eyes, nose and on the ears of some mice, presenting red or bluish areas of alopecia. Similar spots were sometimes also seen on the toes, legs or tail. At the first passage most of the mice developed skin lesions, lost weight and died within 10 days. In the next few passages, made with 10% mouse carcass suspension, deaths generally occurred by the fifth day. It has so far not been possible, however, to continue mouse-to-mouse transmission of the virus beyond about the 5th passage.

Chick embryos inoculated by either the intravenous, yolk sac, or chorioallantoic route did not die; nor were obvious lesions produced in the embryos.

Serology

BMV was allowed to react, in a neutralization test, with antisera against various members of the Herpesvirus group. No neutralization was obtained with any antiserum other than the homologous serum and that against Allerton virus (Table 1). In a further test, with equine herpes, types 1 and 2, malignant catarrhal fever (MCF), B virus antisera, and varicella acute and convalescent sera, there was no neutralization of BMV. In cross-neutralization tests with BMV and Allerton virus and their respective antisera, neutralization occurred in the homologous and in both heterologous virus + serum mixtures (Table 2).

Table 1. *Neutralization tests with BMV and various antisera*

*Control (normal rabbit serum)	Antisera						
	Herpes simplex	‡Herpes simplex	MCF	IBR	Pseudo- rabies	Allerton	BMV
5.8†	6.0	5.5	6.0	5.6	6.0	4.0	3.0

* All sera diluted 1/10, except ‡ diluted 1/5.

† Titre of BMV ($\log_{10}$ Cp.D 50/ml.).

Table 2. *Cross-neutralization of BMV and Allerton virus*

Virus	Rabbit normal serum	Rabbit Allerton antiserum	Guinea-pig Allerton antiserum	Guinea-pig BMV antiserum
Allerton	4*	1.5	≤ 0.5	1.6
BMV	≥ 6	2.3	≤ 0.5	2.3

* $\log_{10}$ Cp.D 50/ml.

In double-diffusion tests in agar, BMV and Allerton virus concentrates gave two bands of precipitate with both the homologous and the heterologous antiserum. No such bands were given by these antisera with a herpes simplex (HFEM strain) virus concentrate similarly prepared from infected BHK21/C13 cells and shown to react with a rabbit antiserum to herpes simplex. The BMV and Allerton virus concentrates did not react with this herpes antiserum. There was complete fusion of the respective bands formed by BMV and Allerton antiserum with a given virus preparation, indicating the similarity of the antigen-antibody systems concerned. Each virus completely absorbed the precipitins from both antisera.

Electron microscopy

Negatively stained PTA preparations of fluid from infected cultures showed particles which appeared circular in profile and had hollow capsomeres arranged in cubic symmetry. These particles measured about 80 m μ in diameter. Some were surrounded by a membrane which was readily distorted and even ruptured during preparation (Pl. 1, fig. 5). The diameter of completely enveloped particles varied widely and some measured up to 250 m μ .

In sections of infected cells, virus particles were seen both in the cytoplasm and in the swollen nuclei. The organization of the cytoplasm appeared to be disrupted. The complete particles resembled those of herpes simplex virus in having a dense

nucleoid surrounded by a double ring, and were approximately 100 m μ in diameter. Intranuclear virus particles were bounded by a single electron-opaque ring, in contrast to cytoplasmic particles which possessed two such rings (Pl. 1, fig. 6).

Virus DNA

The DNA from cells infected with BMV or Allerton virus was compared with respect to base composition and molecular weight (Table 3). Within the precision of the methods used, the DNA's of the two viruses were identical. The base composition of 64–65 % guanine plus cytosine is lower than the figure of 71 % G + C previously found for another bovine herpesvirus, IBR virus (Russell & Crawford, 1964). The molecular weight, as estimated from band width in equilibrium density gradients, is similar to that found for other viruses of the herpes group ($\sim 32 \times 10^6$). This value is a minimum estimate and other methods of molecular weight determination might be expected to give higher values.

Table 3. *Base composition and molecular weight of BMV and Allerton virus DNA*

DNA	Base composition (% G + C)	Molecular weight (NaDNA)
BMV	64	34×10^6
Allerton virus	65	32×10^6

DISCUSSION

Among mammalian viruses, the herpesviruses are characterized by having DNA as their genetic material and emerging from the cell nucleus with their capsid enclosed in an envelope. BMV possesses these two properties and this suggests that it is a member of the herpesvirus family. This classification is supported by several attributes in which this virus resembles the other family members: it multiplies in a variety of cultured cells, giving rise to syncytia in which many nuclei contain type A inclusions (Andrewes *et al.* 1961); it is readily inactivated by ether and chloroform; its DNA has a molecular weight similar to that of other herpesviruses; and its virion has the typical herpesvirus structure in negatively stained preparations and in sections of infected cells. BMV is therefore identified as another bovine member of the family of herpesviruses. Like other bovine herpesviruses, it produces syncytia and intranuclear inclusions in cultured cells. It resembles IBR virus (Armstrong, Pereira & Andrewes, 1961), but differs from most lines of MCF virus (Plowright, Macadam & Armstrong, 1965) in its relative ease of cultivation and its release in significant amount from infected cells into the culture fluid. The serological reactions of BMV separate it clearly from these and other herpesviruses, with the exception of the Allerton prototype strain of the group II viruses of lumpy skin disease (Alexander *et al.* 1957), from which it cannot be distinguished in standard neutralization and agar-precipitation tests.

The viruses of group II, of which BMV appears to be an errant member, have hitherto been found only in Africa, in cattle with lumpy skin disease. They are represented by 11 isolates from four different outbreaks in the Republic of South Africa, including the Elsie's River, Pentrich Grange, and Allerton strains (Alexander *et al.*

1957; Weiss, 1960); by one isolate from Ruanda-Urundi (Huygelen, 1960); and by one, the Kiel-Hansen strain, from Kenya. Now BMV, this antigenically identical, or closely related, virus, similar in cultural and other characteristics, has been isolated in Scotland, but from cattle with a different clinical syndrome. The virus does not appear to have been described before in Europe, and the question of how it came to be on this island so far from its relatives in Africa is a matter for conjecture. It may have been present in Britain for a long time, either as a relatively avirulent strain or associated with a clinical syndrome mistaken for cowpox. Alternatively, it may have derived from one of the causative agents of lumpy skin disease, introduced in the recent past from Africa, but for some reason provoking a different host response. It is possible, although there is no proof at present, that these group II viruses can exist as harmless commensals which occasionally produce virulent mutants of varying pathogenic capacity.

Until now, the group II viruses have been thought to be associated only with clinical cases of lumpy skin disease, though Huygelen and his colleagues (1960) recorded udder lesions in cows in Ruanda-Urundi. It is now clear that they can be associated with at least one other syndrome, that of bovine mammillitis (Martin *et al.* 1966). Whether these viruses may also be present in unaffected and perhaps relatively resistant cattle populations has yet to be determined. It may happen that viruses of BMV or of Allerton type will be discovered in other European countries in which neither kind of disease has been reported. With the aim of distinguishing, within group II, between BMV and lumpy skin disease viruses and so perhaps coming closer to answering some of these questions, it is hoped to make a more detailed comparison of BMV and Allerton virus, particularly with respect to the disease which they cause in laboratory animals, to any differences in their histopathology, and to the possibility of serological differentiation within the group.

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EXPLANATION OF PLATE

Figs. 1-4 are photomicrographs of 5-day monolayer cultures of DBC cells: Figs. 1 and 2 are unfixed and unstained; figs. 3 and 4 are methanol-fixed and stained with haematoxylin and eosin. The mark in figs. 5 and 6 measures 100 μ .

Fig. 1. Uninoculated culture ($\times 65$).

Fig. 2. Culture infected 24 hr. previously with BMV, showing formation of syncytia ($\times 65$).

Fig. 3. Uninfected DBC cells ($\times 250$).

Fig. 4. Syncytia of BMV-infected DBC cells, showing type-A intranuclear inclusions in varying stages of development ($\times 250$).

Fig. 5. Complete BMV particle in concentrate of infective culture fluid, negatively stained with PTA, showing capsid and large, loose envelope ($\times 200,000$).

Fig. 6. Two BMV particles within cytoplasmic vesicles in 2-day-infected cell. Note nucleoid and double ring. ($\times 100,000$.)