The Detection of the Herpesvirus of Bovine Malignant Catarrhal Fever in Rabbit Lymphocytes in vivo and in vitro

(Accepted 7 January 1980)

SUMMARY

The herpesvirus of bovine malignant catarrhal fever (MCFV, alcelaphine herpesvirus 1) causes an acute, fatal lymphoproliferative disorder in rabbits. In dying rabbits, virus antigen and infectivity were associated with medium sized lymphocytes and not with the proliferative lymphoblastoid cells. Virus antigen was found diffusely in the cytoplasm and as diffuse or particulate areas in the nucleus of infected cells. Fresh suspensions of the lymphoid tissues from rabbits killed 1 to 3 days after the onset of pyrexia contained 1 to 4 of both infective and fluorescent antigen positive cells per 10^6 cells. After 2 to 3 days of culture there was a 50- to 1000-fold increase in their concentration when a similar concentration of cells also contained herpesvirus type particles, both in the cytoplasm and the nucleus. About 40- to 100-fold more antigen-positive cells developed in cultures of lymphoid tissues from rabbits killed on the 2nd or 3rd day of pyrexia compared with the cultures from rabbits killed on the first day of pyrexia. Both cytosine arabinoside and 5-iododeoxyuridine inhibited the development of the infected cells upon culture. The findings are discussed in relation to lymphoproliferative disorders caused by other herpesviruses.

The herpesvirus of malignant catarrhal fever (MCFV, alcelaphine herpesvirus 1, Reid et al. 1975) causes a fatal disease in cattle and rabbits characterized by pyrexia, lymphoid proliferation, angitis, conjunctivitis, keratitis and inflammation of the nasal and tracheal mucosa (Daubney & Hudson, 1936; Plowright et al. 1960). Infectivity in the lymphoid tissues of cattle and rabbits is associated with intact cells although there may be small quantities of cell-free virus in rabbit plasma and washings of dispersed rabbit lymphoid tissues (Plowright, 1964, 1968). However, neither the virus antigen nor the virus particles were seen in such tissues on immunofluorescent and ultrastructural examination (Rossiter et al. 1977; Edington et al. 1979). The herpesvirus of Marek’s disease (MDV; Biggs, 1968), herpesvirus saimiri (HVS; Falk et al. 1972; Giddens, 1975), the Epstein-Barr virus (EBV) which is associated with Burkitt’s lymphoma (BL) and infectious mononucleosis (IM; see Epstein & Achong, 1977), herpesvirus ateles (HVA; Falk et al. 1974), herpesvirus sylvilagus (Wenger & Hinze, 1974) and MCFV (Plowright, 1964, 1968) are all herpesviruses which cause excessive lymphoid proliferation and are highly cell-associated in infected lymphoid tissues. The cell-association of MDV, HVS, EBV, HVA and herpesvirus sylvilagus in the infected host may be manifest latently in an unexpressed form and/or expressed in malignant transformation which may be activated to a productive cycle of virus replication upon explant and culture or by physical or chemical stresses (see review by Epstein, 1975). Here we describe the detection of malignant catarrhal fever virus in lymphocytes in cultured explants of lymphoid tissues from experimentally infected rabbits. Category 2 New Zealand white rabbits (Ranch Rabbits, Crawley Down, Sussex) of either sex and 3 to 6 months of age were infected intravenously (i.v.) with 100 median infective doses (ID_{50}) of the African strain of cell-associated MCFV (Plowright et al. 1960) and their temperature recorded daily. Infectivity titrations were by a quantal method in which serial tenfold dilutions of a sample in Eagle’s MEM containing 2% foetal bovine serum (FBS), 20 mM-bicarbonate and antibiotics were
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tested in three or four tubes of monolayer cultures of bovine testis (BT) cells (R. G. Watt et al. unpublished data). The characteristic c.p.e. (Plowright et al. 1960) was recorded and the ID₅₀ calculated by the method of Spearman-Kärber (Dougherty, 1964); the ID₅₀ were multiplied by 0.69 to obtain the concentration of infectious cells (Dougherty, 1964).

Animals were killed 1 to 3 days after the onset of pyrexia (≥ 104°F) and their lymphoid tissues removed, dispersed as described by Ford & Hunt (1973) and cultured in RPMI 1640 medium (Gibco Bio-cult Ltd., Paisley, Renfrewshire, Scotland) containing 20% heat-inactivated (56 °C, 30 min) FBS, 2 mM-L-glutamine and antibiotics. Cultures of 3 x 10⁶ viable cells/ml and 4 ml per 1 oz glass bottles were gassed with 5% CO₂ and incubated at 37 °C. Samples of cells were removed daily, counted for cell viability by the nigrosine exclusion test (Hudson & Hay, 1976) and examined for virus antigen using an indirect immunofluorescence (IIF) test; for the presence of virus particles by electron microscopy (Morgan & Rose, 1967); and for infectivity in BT cells. Infectivity was examined in intact cells, culture medium and cells disrupted using an ultrasonic probe (soniprobe type 7530, Dawe Instruments Ltd., Western Avenue, London, W. 3) at maximum output for 15 s. About 1000 cells were examined per sample by electron microscopy.

In the IIF test acetone-fixed smears of the lymphoid cells, 2 x 10⁶ to 1 x 10⁹ cells per smear were reacted with a bovine antiseraum to MCFV followed by FITC labelled rabbit antiserum to bovine IgG (FITC R-B IgG) and fluorescent antigen-positive cells counted using a Vicker's transmitted light (M41 Photoplan) microscope fitted with a 200 W mercury vapour lamp, BG12, BG38 exciter and GG.9 and OG.4 barrier filters. Controls were: (1) cultured lymphoid cells from normal rabbits treated as above and (2) smears of cultured lymphoid cells from infective rabbits treated with pre-infection (normal) bovine serum followed by FITC R-B IgG. The bovine virus antiserum was from a terminal MCFV infected calf; it had a titre of 2048 in an indirect immunofluorescence antibody test (Rossiter et al. 1977). For use in the IIF test the antiserum was absorbed for 1 h at 37 °C and 14 h at 4 °C with disrupted and washed extracts of pooled lymph nodes, spleen, thymus and bone marrow from normal rabbits, using the equivalent of 10⁶ cells/ml of serum. The antiserum was similarly absorbed with acetone dried liver powder (Sigma Chemicals, Poole, Dorset) from (a) rabbits and (b) cattle using 100 mg powder/ml serum. FITC R-B IgG was prepared by the method of Nairn (1976); standard methods were used to prepare bovine IgG (Hudson & Hay, 1976) and R-B IgG (Herbert, 1973). To determine the specificity of the IIF test, γ-globulins in the bovine antiserum to virus were precipitated with (NH₄)₂SO₄ (Nairn, 1976) and 20 mg of the globulin in 1 ml hypotonic (1/25) phosphate buffered saline A, pH 7.3 (PBS A, Oxoid Ltd., Basingstoke, Hampshire), was mixed with an equal volume of an extract of either 10⁶ disrupted and washed MCFV infected (C-500 strain, Watt et al. unpublished data) or uninfected BT cells (control) each in hypotonic PBS A. The mixtures were stirred for 1 h at 37 °C and 14 h at 4 °C, clarified by centrifugation at 10000 g for 30 min and the supernatants tested undiluted with acetone-fixed smears of cultured lymphoid cells from infected rabbits. The cell sediments were washed with PBS A until no protein (A₄₅₀ nm = <0.01) was detected in supernatants. After this each sediment was eluted with 2 ml of 0-1 M-glycine-HCl buffer, pH 2.7, the eluates were neutralized with 1 N-NaOH, clarified and tested in the IIF test as above. Fluorescent cells in frozen sections (Nairn, 1976) were examined by IIF microscopy as for smears (see above). The sections were counterstained for 1 min with 0.1% Evans blue in PBS A prior to examination. An eyepiece graticule was used to measure 200 or more fluorescent antigen-positive cells in tissue sections. Stock solutions of cytosine arabinoside (araC) and 5-iododeoxyuridine (IdUrd; Sigma Chemicals, Poole, Dorset) at 1 mg/ml distilled water were stored at -20 °C.

Freshly dispersed lymph nodes, spleen and thymus from animals with pyrexia and
Table 1.* Infections cells,† positively fluorescing, † and viable cells§ in cultures of peripheral lymph nodes from MCFV infected rabbits

(a)  

<table>
<thead>
<tr>
<th>Rabbit no.</th>
<th>Day of culture</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td>1</td>
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<td>2</td>
<td>28, 20, 74</td>
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<tr>
<td>3</td>
<td>50, 34, 56</td>
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<tr>
<td>4</td>
<td>617, 700, 53</td>
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(b)  

<table>
<thead>
<tr>
<th>Day of pyrexia</th>
<th>Day of culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1st</td>
<td>8 ± 1.6, 91 ± 9</td>
</tr>
<tr>
<td>2nd</td>
<td>122.4 ± 132, 53 ± 7</td>
</tr>
<tr>
<td>3rd</td>
<td>238.6 ± 516, 55 ± 6</td>
</tr>
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* In group (a) rabbits were killed on the 2nd or 3rd day of pyrexia. Figures in part (b) represent mean of counts in cultures from 5 rabbits ± standard deviation.
† No. of infectious cells/10⁶ cells.
‡ No. of fluorescent antigen positive cells/10⁶ cells.
§ % viable cells.
Fig. 1. (a) Acetone fixed lymph node cells from a 48 h culture reacted with bovine antiserum to MCFV followed by FITC R-B IgG; infected cells contain diffuse granular fluorescence in both cytoplasm and nucleus with the exception of two cells (arrows) in which diffuse granular fluorescence is restricted to the nucleus. (b) Acetone-fixed 8μm thick frozen sections of spleen from MCFV-infected rabbit. The section was reacted with bovine antiserum to MCFV followed by FITC R-B IgG, and contains an infected cell with diffuse granular fluorescence in both the cytoplasm and nucleus. (c) Electron micrograph showing a herpesvirus type nucleocapsid (N) with a central core in the nucleoplasm of a lymphocyte. Unenveloped particles (P) are clustered in the cytoplasm, while one nucleocapsid is budding into a cytoplasmic vesicle (CV).
lymphadenitis contained 1 to 4 fluorescent virus antigen-bearing cells/10^6 cells. Upon culture of the suspensions, the concentration of the positive cells increased 50- to 1000-fold by 2 to 3 days (Table 1). The cell viability in cultures decreased to about 10% by 3 to 4 days of culture (Table 1). The greatest number of positive cells developed in cultures of pooled peripheral lymph nodes whereas the bone marrow preparations were usually devoid of the positive cells. About 40- to 100-fold more positive cells were seen in cultures of the lymphoid tissues from rabbits killed on the 2nd or 3rd day of pyrexia compared with those from rabbits killed on the first day of pyrexia (Table 1 b). In the fluorescent cells, virus antigen was found both in the nucleus and the cytoplasm, diffusely in the cytoplasm and as diffuse or particulate areas in the nucleus (Fig. 1 a); a few cells contained diffuse or particulate antigen in the nucleus alone. The pattern of fluorescence did not change during the 2 to 3 days of culture but at the end of this period a large proportion of the cells were dead, making such determinations more difficult.

In frozen sections of lymph nodes, spleen and thymus the fluorescent antigen positive cells were usually seen in paracortical, red pulp and cortical regions respectively. In all three tissues the positive cells had a diameter of 11 ± 0.6 μm being distributed normally within one standard deviation about the mean. The cellular distribution of fluorescent antigen was as observed in cultured cells (Fig. 1 b). Fluorescence was not observed in cultured lymphoid cells either from normal rabbits treated with the bovine antiserum to virus or from infected rabbits treated with the pre-infection bovine serum. In smears of cultured lymphoid cells with 1000 to 5000 positive cells in the standard test, 60 to 180 weakly staining cells were seen when tested with the bovine antiserum to virus first absorbed with disrupted MCFV infected BT cells; similar absorption of the bovine antiserum with disrupted uninfected BT cells had no effect. Furthermore the absorbed antibody to virus was recovered upon acid elution only from the extract of infected BT cells. This antibody-containing eluate gave counts of fluorescent cells similar to the standard test; no staining occurred with the eluate from the extract of uninfected BT cells.

Both araC and IdUrd in concentrations of 0.2 to 20 μg/ml and 1 to 100 mg/ml of culture respectively, inhibited the development of fluorescent antigen positive cells by more than 99% whether the drugs were left in the cultures for the duration of the experiment or removed 24 h after their addition. The pattern of fluorescence in the few positive cells in cultures with the drugs resembled that in untreated preparations; no MCFV early antigens were seen in the cultures with the drugs as described in MCFV infected bovine kidney cells

Infectivity in monolayers of BT cells was detected only when intact lymphoid cells were used, both supernatant fluid and disrupted cells being negative. In four rabbits, the infectivity of the peripheral lymph nodes closely correlated with the number of positively fluorescing cells (Table 1 a). On culturing, the concentration of both the infective and the antigen positive cells increased in parallel up to 48 h but by 72 h three of the four rabbits had 5- to 60-fold more antigen positive cells than infective cells (Table 1 a).

Herpesvirus-like nucleocapsids, 70 to 80 nm in diam., were seen in 0.1 to 0.3% of cells in cultures with similar concentrations of fluorescent antigen positive cells. The nucleocapsids occurred in low numbers both in the nucleus and cytoplasm and were either empty or contained a central core. Occasional aggregates were seen in the cytoplasm (Fig. 1 c). Enveloped particles were rarely recorded and then only in the cytoplasm.

This is the first report to describe MCFV antigens and particles in lymphoid tissues from infected animals. The significance of the work rests on the specificity and the sensitivity of tests used. Previously, direct fluorescence (IF) tests failed to detect virus antigen in lymphoid tissues containing a few hundred infected cells per 10^9 cells (Rossiter et al. 1977; Edington et al. 1979); FITC conjugates of antisera to virus, however, stained virus antigen in infected
BT or bovine kidney (BK) cells up to dilutions of 1/60 (unpublished data). The in vitro detection system was probably a reflection of the higher levels of virus antigen in infected BT or BK cells compared with that synthesized in vivo in lymphoid cells. The lower sensitivity of IF tests compared to IIF tests, and the low number of infected cells involved, together with non-specific staining, may have contributed to the previous inability to detect MCFV antigen in lymphoid cells. Control tests for the specificity of the IIF test using preimmune serum or cultured lymphoid cells from uninfected rabbits were negative. Furthermore, antibody to MCFV in the bovine antiserum was specifically removed by immunoadsorption with virus-infected BT cells and followed by its recovery upon acid elution from extracts of the infected BT cells. Herpesvirus-like capsids were seen in the nucleus and cytoplasm of lymphocytes, the number of infected cells corresponding with the results by IIF and by infectivity titrations in BT cells. The low number of particles in cells suggests that unassembled virus antigen must be present to give rise to positive immunofluorescence. While immunoelectron microscopy would confirm the relationship, the analogy may be drawn with Marek's disease, where a similar sparse distribution of virus particles has been recorded (Frazier, 1974).

The major finding in the present experiments was that the lymphoblastoid cells (Edington et al. 1979) unlike those in disorders caused by MDV (Campbell & Woode, 1970; Lee et al. 1975; Powell et al. 1975), HVS (Falk et al. 1972; Giddens, 1975), EBV (Epstein et al. 1964; Epstein & Achong, 1977) and HVA (Falk et al. 1974) contained neither the virus antigen nor the virus particles. The infected cells were medium sized lymphocytes whose concentration increased 50- to 1000-fold during 48 to 72 h of culture (Table 1). While infectivity generally ran in parallel to the number of positively fluorescent cells, 5- to 60-fold fewer infected cells were found at 72 h in 3/4 rabbits (Table 1 a). As the viability is less than 20% at this stage the discrepancy suggested that only viable cells transfer infectivity to BT cells (Plowright, 1968) although the virus antigen may still be detected by the IIF test in both living and dead cells. As in the disorders cited above the increase in the concentration of MCFV infected lymphocytes upon explant and culture might be a result of an activation of latently infected cells (see Epstein, 1975). Alternatively the increase might represent (1) infected cells in an early eclipse phase at the time of explant and (2) a secondary cycle of virus replication in co-resident uninfected lymphocytes in the culture. The latter would seem most likely to be cell to cell transfer since we could not detect intracellular or extracellular infectivity in the cultures. The rapid loss of cell viability has made it difficult to delineate which of these situations or which combinations of events occurs. A surprising result was that in our tests with araC and IdUrd we failed to detect early virus antigens (EVA). While EVA can be detected in the nuclei of infected BK cells (Rossiter et al. 1978; unpublished data), in infected lymphocytes the antigen may require a more sensitive test such as the anticomplement fluorescence test used for EBV nuclear antigen (Reedman & Klein, 1973). By analogy with MDV (Ross et al. 1977) and EBV (Gergely et al. 1971) we also considered that a brief treatment with IdUrd might activate latently infected cells which otherwise might remain undetected. IdUrd almost completely inhibited the development of fluorescent antigen positive cells. This and the absence of transformation in MCFV infected rabbits (Edington et al. 1979) together with the failure to obtain continuously replicating lines of lymphoblastoid cells carrying MCFV, suggest a different virus-lymphocyte interaction from those induced by MDV, EBV, HVS and HVA; however, MCFV does closely parallel herpesvirus sylvilagus (Wenger & Hinze, 1974). The role of the MCFV-induced lymphoblastoid cells in the pathogenesis of the disease needs clarification.

This work establishes the target cell for MCFV replication in rabbits. The determination of the T or B cell specificity of these cells and a comparative investigation of the patterns of
virus expression in infected cattle will be a further contribution to the role of herpesviruses in proliferative disorders of the lymphoid system.

This work was supported by a grant (R2092) from the Ministry of Overseas Development. We thank Mrs N. Bevan, Mr. S. C. Broad and Mr M. Robins for technical assistance and the animal staff for assistance with animals.

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(Received 5 November 1979)