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The Nature of the Acute Lymphoid Proliferation in Rabbits Infected with the Herpes Virus of Bovine Malignant Catarrhal Fever

N. EDINGTON, J. PATEL, P. H. RUSSELL and W. PLOWRIGHT

Department of Virology, Royal Veterinary College, Royal College Street, London NW1 0TU, United Kingdom

Abstract—*The virus of malignant catarrhal fever of cattle (alcelaphine herpesvirus 1) induced an acute fatal lymphoproliferative disorder in rabbits. The lymphoblastoid cells were detected initially in the paracortical zone of lymph nodes and around the Malphigian corpuscles of the spleen, but subsequently invaded and divided in many tissues, particularly the lung and also the kidney, liver, peripheral nerves and retina. The focal necrosis of lymphocytes present in lymph nodes and spleen, was most extensive in the thymus. Bovine testis cultures yielded virus from the tissues in which the cell proliferation occurred, but virus antigens could not be detected by immunofluorescence or virions by electron-microscopy in the primitive cells. The detection of infectivity 4 days after primary inoculation by subinoculation into rabbits and the development of immunofluorescent antibody during the course of the terminal syndrome were considered as evidence that a primary cycle of replication had occurred prior to the lymphoproliferative changes. These acute lymphoproliferative changes were compared and contrasted with those induced by herpesviruses in other species.*

INTRODUCTION

THE VIRUS of malignant catarrhal fever (MCFV or alcelaphine virus 1 [1]) produced a fatal syndrome in cattle and experimental rabbits in which the cardinal features were a lymphadenopathy, with the lymphoid cell proliferation widespread in many organ systems, a severe angiitis and a rhinotracheitis and a keratoconjunctivitis [2–5]. The herpesvirus of Marek's disease, *H. saimiri*, *H. sylvilagus*, and the Epstein–Barr virus have been associated with lymphoproliferative disorders in the domestic fowl, marmoset, cottontailed rabbit and man respectively, [6]. It therefore seemed relevant to re-examine the pathogenesis of MCFV infection to see whether the pattern of changes resembled those associated with these herpesvirus infections in other species. Particular attention was given to the nature and sequence of the changes in lymphoid tissue and the correlation of these with the detection of virus antigen.

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MATERIALS AND METHODS

Experimental procedure

The pathogenesis was studied in two experiments.

(1) Each of 14 rabbits was inoculated intraperitoneally (i.p.) with 2.5 ml aliquots of a 10% suspension of peripheral lymph nodes and spleen from an MCFV-infected rabbit killed on the second day of pyrexia. The inoculum had a titre of $10^{4.5}$ ID_{50}/ml in bovine testis (BT) cells. These rabbits were killed in pairs at 2 day intervals, the last two being observed until they developed pyrexia and lymph node enlargement on day 15. The examination of tissues is detailed below.

(2) Ten further rabbits were similarly inoculated and also examined at 2-day intervals. In this experiment rabbit to rabbit transmission was used to detect virus infectivity. Five ml of a pool of 10% suspensions of tissues from rabbits killed at 2, 4, 6 and 8 days of incubation were given i.p. to receptor animals. Pool A consisted of spleen, peritoneal and lung macrophages, while pool B con-

tained nasal mucosa, retropharyngeal and popliteal lymph node.

The remaining two rabbits were left as controls to develop the pathognomonic lesions of MCFV.

Detection of virus

By recovery in cell cultures. Suspensions (10% w/v) of tissues (see Table 1) were prepared in maintenance medium and stored in 10% dimethylsulfoxide at −70°C. After rapid thawing at 37°C, 0.2 ml quantities were adsorbed onto tube cultures of bovine testis cells which had been passaged 14 or 15 times [7]. After 2 hr incubation at 37°C cultures were rinsed 3 times with phosphate-buffered saline (PBS) and incubated for 21–26 days in L15 medium [8] supplemented with 2% foetal bovine serum (FBS). Viral replication was identified by the development of the characteristic cytopathic effect [9].

By rabbit inoculation of tissue. Rabbits were given 5.0 ml of pool A or pool B as detailed above.

By immunofluorescence. Tissues were snap frozen in liquid nitrogen and stored at −70°C. Subsequently sections, 8 μm thick, were cut on a cryostat from the following tissues: nasal and tracheal mucosa, lung, submandibular salivary gland, liver, heart, kidney, testicle, brain, eye, spleen, femoral bone marrow; submandibular, retropharyngeal, popliteal lymph nodes, caecal tonsil and appendix, thymus, adrenal, and Harderian and lachrymal glands. They were fixed in acetone for 10 min at 4°C. Rabbit lung macrophages washed from the tracheo-bronchial tree into PBS [10], were resuspended at a concentration of 5.0×10^5/ml and deposited on to slides by spinning at 1500 rev/min in a cytospin for 10 min prior to acetone fixation at 4°C. Bovine hyperimmune serum conjugated with fluorescein isothiocyanate (FITC) was used on all preparations, using an FITC-labelled normal bovine globulin as a control. Sections were incubated for 30 min at 37°C with a 1:5 dilution of the conjugated sera, washed for 20 min at room temperature in constantly stirred PBS and then mounted in a 50% glycerol: PBS solution. Parallel titrations were made with the conjugated hyperimmune serum on MCFV-infected bovine kidney (BK) cells grown in microwell slides [11]. This procedure usually gave a titre of 1:80–160. Examination of all preparations was made by ultraviolet transillumination using a Zeiss standard RA microscope and an HBO 200 W lamp.

Detection and assay of antibody

Antibodies detected by immunofluorescence (IIF) and virus neutralisation (VN) were assayed as described previously [11].

Pathology

Portions of all the tissues examined for virus by direct fluorescence (*vide supra*) together with tongue, oesophagus, stomach, aorta, bladder, spinal cord and sciatic nerve were fixed in 10% buffered formalin sectioned and stained by haematoxylin and eosin. Fragments of retropharyngeal and popliteal lymph nodes, bone marrow and spleen were fixed in 2% glutaraldehyde and then for 30 min in 1.0% osmium tetroxide prior to processing and embedding in araldite. Washed lung macrophages were similarly fixed but spun in microhaematocrit tubes and pre-embedded in 2% agar before processing and araldite embedding.

RESULTS

Isolation of virus in cell cultures

The detection of virus infectivity by inoculation of BT cells is summarised in Table 1. No recovery was made from rabbits killed at 2, 4, 6 and 8 days p.i. One rabbit killed 10 days after inoculation was the first to yield virus, from the submandibular and popliteal lymph nodes, spleen and lung. Virus was also isolated from the lung and popliteal node of one rabbit at 12 days and from all lymphoid tissues and a wide range of parenchymatous organs of the other. This latter pattern was repeated in the two reacting rabbits, killed on day 15.

Recovery of virus in rabbits

Pooled tissue suspensions (A and B) harvested from animals killed at 2 and 4 days p.i. produced pathognomonic lesions of MCF in receptor rabbits within 15–32 days. Pools from rabbits killed at 6 and 8 days p.i. did not induce clinical disease in receptor animals, which were susceptible to i.v. challenge with cell-free MCFV at 60 days p.i.

Immunofluorescence

No evidence of viral antigens was seen in any of the tissues examined at any stage of infection.

Antibody

The results of antibody tests are summa-

Table 1. *Virus recovery and macroscopic lesions in rabbits inoculated i.p. with MCFV*

Days after inoculation	2		4		6		8		10		12			15
Animal No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Tonsil												+	+	+
Submandibular node			C	C		C				+		he+	he+	he+
Retropharyngeal node		C	C									he+	he+	he+
Popliteal node										+	he+	he+	he+	he+
Peyer's patch												+	+	+
Spleen										+		e+	e+	e+
Thymus		p	p									p+	p+	p+
Bone marrow												+	+	+
Turbinate												f+	f+	f+
Trachea														
Lung										+	+	i+	i+	i+
Eye												+	+	
Liver												i+	i+	i+
Kidney												i+	+	i+
Adrenal												+	+	+
Testis														+
Buffy coat												+	+	+

+—Virus isolation; C—congestion; p—petechiae; h—haemorrhage; e—enlargement and oedema; i—infiltration of lymphoblasts; f—exudate.

rised in Table 2. Titres of IIF antibody were >1:64 in the three rabbits showing pyrexia and lymphadenitis (nos. 12–14) with rabbits 13 and 14 having neutralising antibody titres of 1:16 and 1:4 respectively. In all earlier rabbits the IIF antibody levels were <1:4 and no neutralising activity was detected.

Pathology

The macroscopic changes have been summarised in Table 1. Within 6 days of inoculation 4 of the 6 rabbits had congested lymph nodes in the pharyngeal region while a fifth animal showed similar changes in the popliteal node. The thymus of one of the two rabbits killed on both days 2 and 4 had diffusely distributed petechiae. A cloudy peritoneal exudate, varying in volume from 5–25 ml, was collected from both rabbits on day 4 and from one rabbit at day 6.

No abnormalities were detected in the rabbits killed at 8 and 10 days but at 12 days the popliteal node in one (No. 11) was oedematous and mottled with haemorrhages, while in the other animal (No. 12) all the peripheral lymph nodes, excluding those of the alimentary tract, were grossly enlarged and showed extensive haemorrhage. The liver and lung in this case showed a lobular pattern of pale cellular infiltration, and the kidneys had multiple discrete foci of invasion: there was gross enlargement of the spleen and haemorrhages in the thymus. The lesions were very similar to those seen in both rabbits killed at 15 days.

Histology

The hyperaemia observed in the pharyngeal lymph nodes of rabbits killed within 6 days of inoculation tended to be focal and cortical. In all rabbits at 2 and 4 days there were ecchymoses in either the submandibular or retropharyngeal lymph nodes as well as follicular necrosis and hyperplasia. The

Table 2. *The occurrence of IIF and neutralising antibody in rabbits inoculated i.p. with MCFV*

Days after inoculation	2		4		6		8		10		12		15	
Animal No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
IIFA	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	32	256	64
Neutralising antibody	—	—	—	—	—	—	—	—	—	—	—	—	16	4
Virus recovery										+	+	+	+	+
Pyrexia >40.0												+	+	+

congestion and petechiae in the thymus were most obvious on the surface and in the cortex, being accompanied by a pericapsular accumulation of macrophages and neutrophils (Fig. 1). Intranuclear inclusions were not detected at this or any other stage of the development of the disease.

No significant changes were seen between days 6–12 of infection, although there was an increase in lymphocytes in the cortex of the submandibular and the popliteal glands of rabbit No. 10 at 10 days p.i., with an influx of lymphoblasts into the sinuses. A paracortical infiltration of lymphoblastoid cells, spreading throughout the cortex and the sinuses, into the medulla was observed in rabbit No. 12 at 12 days p.i. and in both rabbits killed at 15 days p.i. The submucosa of the nasal cavity and trachea, the periphery of the conjunctiva, the entire retina, the perineural space of peripheral nerves, and the bone marrow all contained accumulations of these primitive cells, while in the spleen, lung, liver, kidney and brain the invasion was predominantly perivascular. Mitotic figures were common at all sites and vessels were often engorged with cells (Fig. 2). These invading lymphoblastoid cells were 10–15 μm in diameter, possessing a large vesicular nucleus, with a prominent, often reticulated nucleolus, and had a high nucleus to cytoplasm ratio (Fig. 3). The cytoplasm contained few mitochondria and sparse endoplasmic reticulum.

The occurrence of these lymphoblast-like cells in lymph nodes was associated with concurrent focal areas of necrosis and haemorrhage (Fig. 4). Ultrastructural examination failed to reveal any evidence of herpes-like virions. The thymus was not invaded by lymphoblastoid cells but a progressive necrosis of lymphocytes around the large supporting reticular cells in the cortex was observed in reacting rabbits (Fig. 5). Whilst initially focal the lesion rapidly extended until the areas coalesced and the majority of the thymic cortex was oblated.

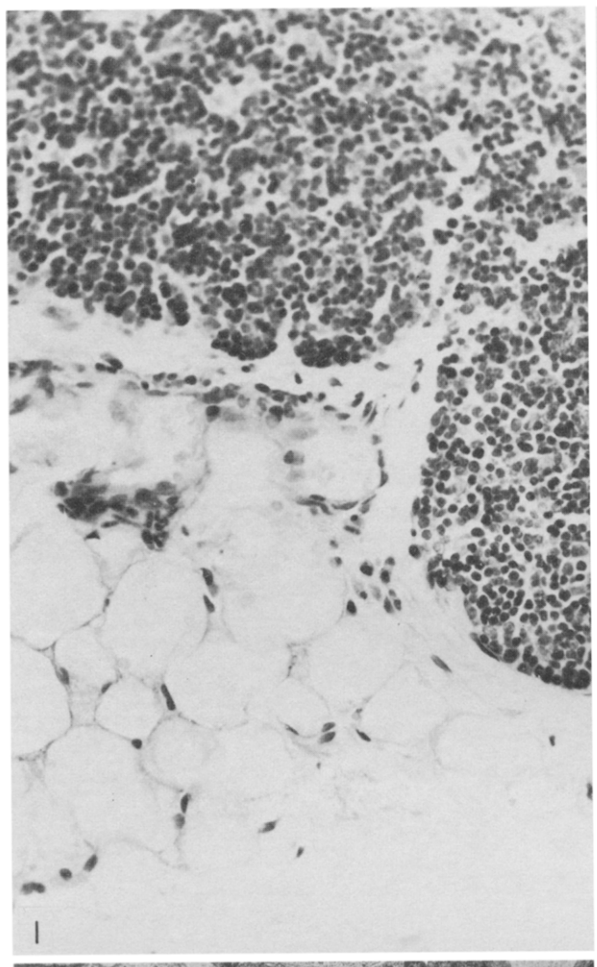
DISCUSSION

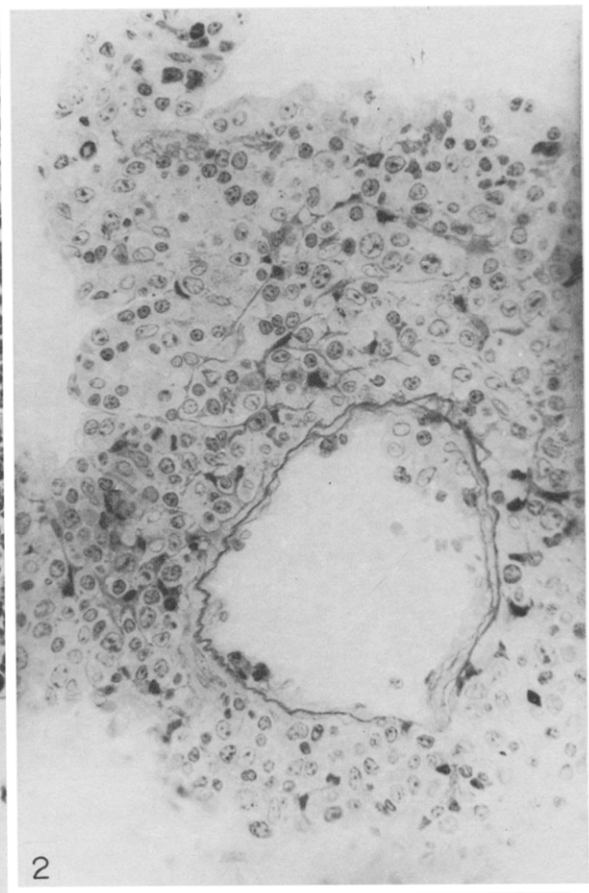
Marek's disease, [12] lymphoma's in the marmoset [13], Burkitt's lymphoma [14] and a lymphoreticular disorder in rabbits [15] are excessive proliferations of undifferentiated lymphoreticular cells associated with herpesvirus infections. In these disorders virions were not routinely observed within the replicating lymphoid cells, yet virus was recovered either by cultivation or co-cultivation of lymphoid tissues *in vitro*. All these cardinal features were observed in rabbits infected with MCFV, where the appearance of the lymphoblastoid cells coincided with the recovery of virus from lymphoid tissues *in vitro* and in detection of circulating IIF antibody.

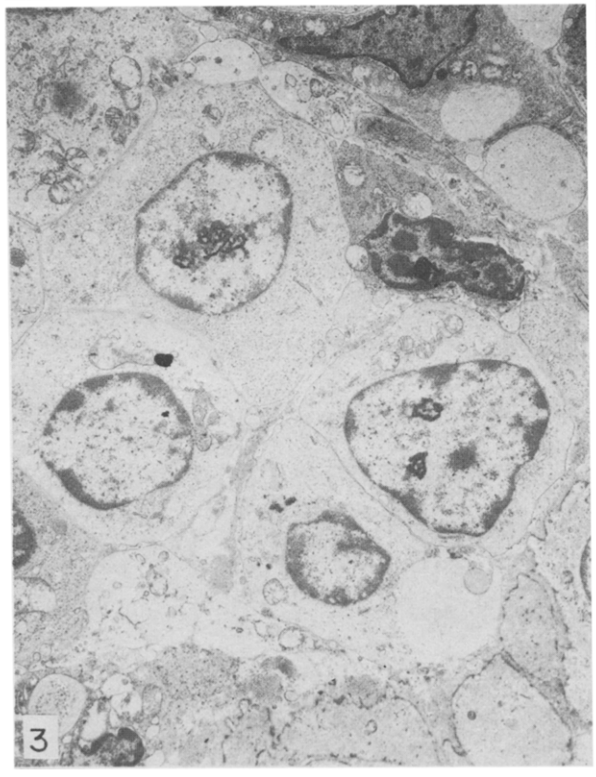
Morphologically the cells resembled lymphoblasts in size, in nuclear to cytoplasmic ratio, in their prominent reticulated nucleolus within a vesicular nucleus, and in the relative lack of cytoplasmic differentiation. In infected rabbits the lymphoblast-like cells appeared first in the paracortical sinuses of lymph nodes and around the Malphigian corpuscles of the spleen. Their proliferation was in excess of that seen in an inflammatory response and they obviously infiltrated and continued to divide in other tissues. This was particularly noticeable in the lung, where the perivascular proliferation extended into alveolar septa and must have induced severe anoxia. While these cells resembled transformed cells, in as much as they multiplied in an uncontrolled manner and invaded and continued to divide in other tissues, it is not known whether they contain tumour-specific membrane antigens as in MD [16]. It would therefore seem judicious at this stage to still describe the lymphoid lesions in MCFV as acute proliferative [5] rather than neoplastic changes.

While the predominant changes in rabbits fatally infected with MCFV were lymphoproliferative there was a concurrent focal necrosis within the paracortical areas of lymph nodes and this was followed by a progressive destruction of the cortex of the thymus. These latter lesions suggest that there was a predominant involvement of T lymphocytes which are indicated as the source of transformed cells in Marek's disease (MD) [16].

It has been established in MD that a primary cycle of virus replication occurs in the bursa in day-old chickens [17]. The detection of IIF antibody in parallel with or prior to the development of the lymphoproliferative lesions in MCFV-infected rabbits [11] was strong evidence that in this syndrome a preliminary cycle of virus replication had also occurred. Transmission of infection to receptor rabbits from rabbits killed at 2 and 4 days after inoculation of MCFV is further evidence that this has occurred. While it is possible that there may have been a persistence of the inoculum in the host at 2 days p.i. it is most unlikely that this was so at 4 days p.i., particularly in suspension B which consisted of peripheral lymph nodes and nasal mucosa.







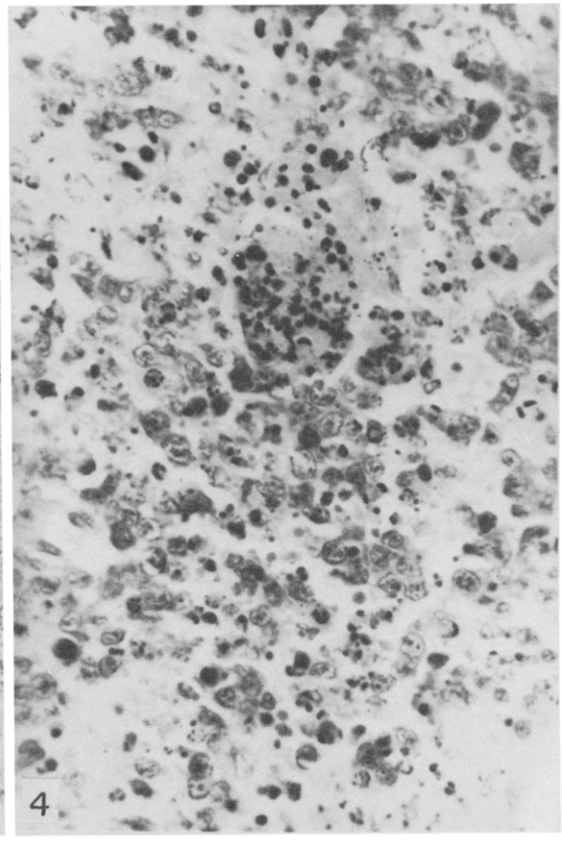


Fig. 1. *Rabbit thymus* 4 days *after i.p. inoculation of MCFV. Macrophages and neutrophyls have accumulated around the congested capsular vessels of the thymus. H and E,* ×420.

Fig. 2. *The pulmonary vein is surrounded by primitive reticular cells in this rabbit at the terminal lymphoproliferative phase of infection with MCFV. Several mitotic figures can be seen while the normal alveolar architecture is distorted. Methylene blue,* ×650.

Fig. 3. *The invading proliferating cells in this febrile rabbit* (12 days) *p.i. have a prominent reticulated nucleolus, a sparse distribution of mitochondria and little evidence of endoplasmic reticular activity. Uranyl acetate: lead hydroxide,* ×5000.

Fig. 4. *The cortical sinuses of a lymph node is engorged with the lymphoblastoid cells while concurrent focal necrosis occurs*

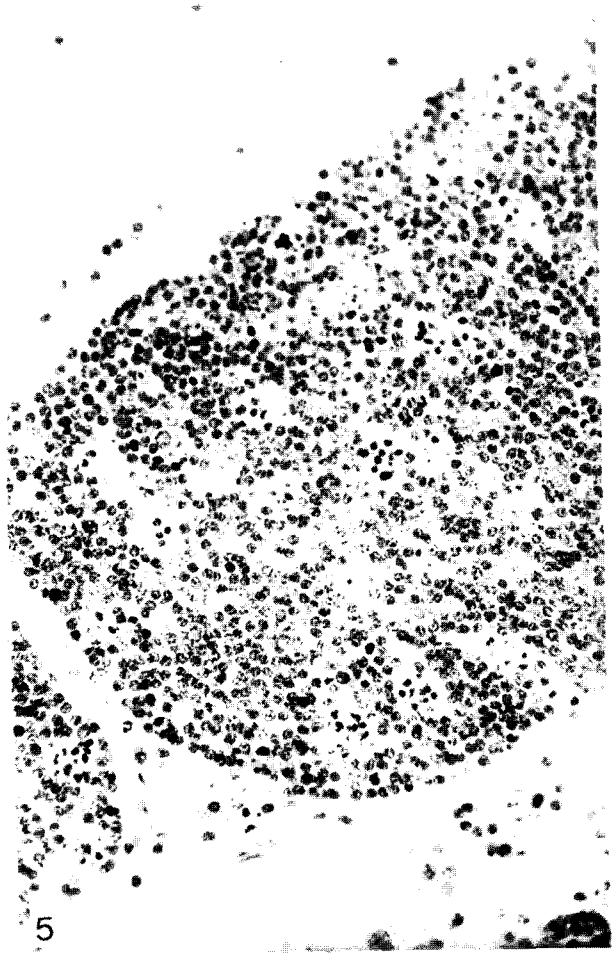


Fig. 5. Clusters of degenerating lymphocytes appear in the cortex of the thymus in a rabbit. 10 days *p.i. H and E,* × 420.

Pathologically, petechiae and focal necrosis at 2, 4 and 6 days p.i., were evidence that early inflammatory reaction had occurred in the retropharyngeal lymph nodes and thymus, yet no intranuclear inclusions, positive fluorescence, or recovery of virus from tissue culture was recorded at this stage. These latter observations possibly reflected the insensitivity of the BT cells and the fluorescent system in relation to animal inoculation.

While virus has been recovered from terminal tissues it has not been possible to locate the sites of expression either with an immunofluorescent technique or by electron microscopy. In Burkitt's lymphoma virions were detected in less than 1.0% of cultivated tumour cells [14] and similarly in Marek's disease, [16] but in 5–10% of cells from *H. saimiri* induced lymphomas [18]. The demonstration of viral antigen in each disorder has been associated with the establishment of lymphoblastoid lines. Despite repeated attempts it has not yet been possible to establish persistent cultures of lymphocytes from MCFV infected rabbits. Thus while MCFV infection resembles the herpes induced lymphoproliferative disorders in that a primary cycle of viral replication is followed by a widespread proliferation of lymphoblastoid cells, it differs in that these primitive cells do not appear to contain viral antigen, nor give rise to discrete tumours, nor to establish cell lines. It may be that the acute lethal course of the disease precludes these features developing, and it is hoped that further studies will both define the nature of the target cell in the primary and secondary phases of MCFV replication and their relationship to the lymphoid proliferation. The lesions of MCFV infection in rabbits do however parallel the "malignant lymphomas" induced by *H. saimiri* in New Zealand white rabbits [19, 20] in which the oculo-nasal involvement and acute nature of the syndrome is commented on by the authors. The two conditions would seem to differ from the "malignant lymphoma" described in the cottontailed rabbit [15, 21] where the lymphoblastoid proliferation commencing 2–3 weeks after inoculation reaches a peak at 8–10 weeks following which most animals recover. In this condition rabbits are viraemic throughout and thus the syndrome has several features in common with infectious mononucleosis in man. Thus, while three herpesviruses induced a proliferation of lymphoblastoid cells in rabbits the role of these cells and the site of viral replication should be more clearly defined before a neoplastic nomenclature is used.

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