Characterization of a persistent infection with bovine herpesvirus type-4

by

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INTRODUCTION

Herpesviruses have been isolated from almost all species and give rise to a variety of disease syndromes. The bovine herpesviruses especially are associated with a diversity of clinical disease unparalleled by any other group of viruses affecting cattle.

Infectious bovine rhinotracheitis and bovine herpes mammillitis are two examples of relatively new and important diseases recognized since the 1950s. Under more traditional systems of livestock production, as in many Third World countries, the infections caused by bovine herpesviruses have rarely been of great economic significance. Only with the development of more intensive systems of animal production have problems emerged. In many developing countries of Africa, South America and Asia, the isolation of different bovine herpesviruses has been recorded (Gibbs and Rweyemamu, 1977a,b). This circumstance may hinder the attempts to develop intensive, more productive methods of cattle raising.

Though the economic importance of the diseases caused by bovine herpesviruses is a strong reason that warrants the continuing attention of veterinarians and virologists, it is not the only one. Actually, the diversity of disease associated with the bovine herpesviruses and human herpesviruses follows a similar pattern. In this respect, latent or chronic infections in cattle as well as the possible role of bovine herpesviruses in cattle oncogenic processes have become an appealing

subject for comparative virologists.

From all of this, it can be concluded that a more complete understanding of the bovine herpesvirus group is required.

During the last two decades the list of herpesviruses originating from cattle has been expanded considerably. Specifically, a large number of isolates recovered from diverse clinical syndromes as well as from clinically normal cattle have been placed in the bovine herpesvirus-4 (BHV-4) group (also referred as Bovid Herpesvirus-4) (WHO/FAO, 1976; Straub, 1978; Mohanty, 1980). The members of this group are geographically widespread (Africa, Near East, Europe and U.S.A.). The characteristic of poor inducer of neutralizing antibodies is common to all of these isolates. This last feature, in addition to a limited sensitivity of the standard seroneutralization assays in use, determined that not all of them have been compared serologically. In most cases, there is no evidence to implicate them as the primary etiological agents of the clinical syndromes from which they were isolated and experimental inoculation with these agents in all but a few instances has failed to elicit any clinical disease.

The purpose of this work was to investigate the response of cattle and rabbits experimentally infected with one strain of BHV-4 (75-3374) isolated by Reed et al. (1977) from a case of mammary pustular dermatitis. Although no evidence of the pathogenicity of this strain could be found, it has been possible to characterize an association of the virus with lymphoid organs and peripheral blood leukocytes in vivo and demonstrate persistence of the virus for extended periods of time.

Explanation of Thesis Format

This thesis consists of an introduction, a literature review, two separate manuscripts, a general conclusion, references and acknowledgements. The master's candidate, Fernando Abel Osorio, is the senior author and principal investigator for each of the manuscripts.

LITERATURE REVIEW

Family Herpesviridae

The International Committee on Taxonomy of Viruses (ICTV) (1979) has defined the DNA-containing viruses with envelopes or complex coats as belonging to one of 3 large families: Herpesviridae, Iridoviridae and Poxviridae.

The main characteristics of the family Herpesviridae include: an enveloped icosahedral capsid with 162 capsomers; an average diameter of 100 nm for the naked virus and up to 200 nm for enveloped virus; assembly of the capsid in the cell nucleus with envelopment of the nucleocapsid at the nuclear membrane; extreme sensitivity to ether; a genome composed of one molecule of linear double stranded deoxyribonucleic acid of molecular weight ranging from 80 to 150 x 10^6 daltons and a ratio G + C between 32 and 82% (ICTV, 1979).

Three subfamilies have been delineated for members of Herpesviridae, leaving some herpesviruses unclassified and no genera formally named (ICTV, 1979). The three subfamilies have been defined mainly on the basis of the replicative characteristics of the virus members and their persistence features and consist of 1) Alphaherpesvirinae, comprising the herpes simplex virus group, of relatively short ($<$24 h) replicative cycle and latent virus infection frequently demonstrated in ganglia and other nervous tissue, 2) Betaherpesvirinae, the cytomegalovirus group, of slow reproductive cycle ($>$24 h), with enlargement of the infected

cell *in vivo* and often *in vitro* (cytomegalia) and persistence of the virus in epithelia (salivary glands) and other (e.g. lymphoid) tissues, 3) Gammaherpesvirinae, comprising the lymphoproliferative group, characterized by restricted replication in lymphoblastoid cells and lytic infections in some types of epithelioid cells and fibroblastic cells. The latent virus is frequently demonstrated in lymphoid tissue. Prototype viruses have been named for each subfamily and other members have been assigned where information was sufficient to make this possible. These reference prototypes have been taken from among the more studied and well-characterized human herpesviruses. For Alphaherpesvirinae, the prototype is human (alpha) herpesvirus-1 (herpes simplex virus). For Betaherpesvirinae, the prototype is human (beta) herpesvirus-5 (human cytomegalovirus) and for Gammaherpesvirinae human (gamma) herpesvirus-4 (Epstein-Barr virus) is the prototype.

Herpesviruses of Cattle

Under the provisional classification described above, other members of each subfamily include not only other herpesviruses of man but also herpesviruses of other hosts, each of which is given a numerical designation among the herpesviruses named according to their natural host species. This is at variance with the previous proposals of the Herpesvirus Study Group of the ICTV (Roizman, 1973) which had suggested that all herpesviruses should be named according to the taxonomic unit-- the family-- of the hosts. This last system was not ratified by the

ICTV (Fenner, 1976) and was considered inconvenient (Gibbs and Rweyemamu, 1977a) in that most of the mammalian families are extremely diverse (i.e. family Bovidae includes 128 species).

The co-existence of both classification criteria during a certain period of time created some degree of confusion between the different authors who attempted to bring order to the great number of herpesviruses isolated from cattle. McKercher (1973), in the first systematic review published on herpesviruses of veterinary importance, used the species name to denominate the two well-known herpesviruses of cattle: bovine herpesvirus-1 (infectious bovine rhinotracheitis virus) and bovine herpesvirus-2 (bovine herpes mammillitis/Allerton virus).

The Herpesvirus Study Group of the ICTV (Roizman, 1973) included the malignant catarrhal fever virus and the virus of ovine pulmonary adenomatosis (Verwoed et al., 1979) within the Bovid herpesvirus group and called them Bovid herpesvirus 1, 2, 3 and 4 respectively.

Smith (1977), following this family-based pattern, proposed the designation Bovid herpesvirus-5 for a group of different isolates serologically related to the European isolate Movar 33/63 (Movar group) which had demonstrated no relationship to the previously known bovine herpesviruses. At the same time, and despite this proposition, some other authors referred to this Movar group as bovine herpesvirus III (Gibbs and Rweyemamu, 1977b; Eugster, 1978). The rationale for this conflicting classification was that these authors did not include in their listing the virus of malignant catarrhal fever as a herpesvirus

of cattle which was considered to be mainly a virus from wildebeest and other Alcelaphinae.

The Consultation on the WHO/FAO Programme on Comparative Virology, held in 1976 (WHO/FAO, 1976), although deciding nothing conclusive about herpesvirus naming and classification, provided a tentative working list of the herpesviruses of cattle. The FAO list recognized 4 antigenically different types of bovine herpesviruses, (Table 1) and indicated a prototype strain in each. The virus of pulmonary adenomatosis of sheep was not included. This tentative classification became generally accepted and was used by the more recent reviews in the field (Mohanty, 1978, 1980; Straub, 1978).

The antigenic differences among these 4 serotypes of bovine herpesviruses correlate with distinctive patterns of pathogenesis, immunogenicity and persistence that are well-characterized for BHV-1, BHV-2 and BHV-3. An attempt will be made to summarize the differential characteristics of each of these groups and a special emphasis will be given to the available information on the less well-defined and newly recognized BHV-4 group.

Bovine herpesvirus-1 (BHV-1)

BHV-1 was the first viral agent shown to cause respiratory infection in cattle. The virus is associated with rhinotracheitis, conjunctivitis, vulvovaginitis, balanoposthitis, meningoencephalitis, and neonatal infection (Kahrs, 1977). From these respiratory and reproductive

Table 1. Herpesviruses of cattle

Type*	Vernacular name	Reference strain
BOVINE HERPESVIRUS - 1	Infectious bovine rhinotracheitis virus (IBRV)	COLORADO I (York et al., 1957) USA
BOVINE HERPESVIRUS - 2	Bovine herpes mammillitis/ Allerton virus (BHMV)	BOVINE MAMMILLITIS (Martin et al., 1964) U.K.
BOVINE HERPESVIRUS - 3	Malignant catarrhal fever virus (MCFV)	W C II (Plowright, 1968) Africa
BOVINE HERPESVIRUS - 4	Movar/Orphan herpesviruses	MOVAR 33/63 (Bartha et al., 1966) Hungary
UNCLASSIFIED	"Epivag" associated slow CPE viruses	(Maré and Van Resburg, 1961; Theodoridis, 1978) S. Africa
	Syncytial herpesvirus from lymphosarcomatous cattle	(Van der Maaten and Boothe, 1972) USA

*The four types with numerical nomenclature correspond to the groups proposed by the WHO/FAO Consultation on Comparative Virology (WHO/FAO, 1976) and they are referred also as Bovid herpesvirus 1, 2, 3 and 4.

symptoms were derived the vernacular names of the virus: infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (IBR/IPV) virus. However, isolates from the genital tract were shown to produce respiratory illness and vice versa (McKercher, 1973) demonstrating the etiological uniqueness of the virus for both syndromes.

The virus has a fast cytopathic effect (CPE) in infected tissue culture cells, being complete in less than 24 hrs. The infected cells become rounded, frequently ballooned and some not infrequent small refractile syncytia are present. As the CPE progresses, strands of cytoplasm are seen linking affected cells (Gibbs and Rweyemamu, 1977a).

The humoral immune response to IBR virus is characterized by high titered serum-neutralizing antibodies, as well as antibodies with cytolytic and antibody-dependent cell-mediated cytotoxicity (ADCC) inducer capabilities (Rouse et al., 1976). The cell-mediated immunity has been demonstrated to play a principal role in the prevention of virus spread and recovery from disease (Davies and Carmichael, 1973; Rouse and Babiuk, 1974).

The virus has the ability to persist after acute infection in inoculated animals. As other members of the alphaherpesvirus group, BHV-1 persists in sensory and autonomic nerve ganglia (Narita et al., 1978; Homan and Easterday, 1980a). The persistence in trigeminal ganglia and recurrence also has been demonstrated in rabbits (Rock, D. L., 1981. Persistent infection with bovine herpesvirus-1: a rabbit model. Ph.D. dissertation, Iowa State University, Ames, IA, USA).

Bovine herpesvirus-2 (BHV-2)

BHV-2 causes severe skin lesions on the udder and on the teats of cows and heifers shortly after calving. The virus has been isolated in eastern and southern Africa, Europe, the United States, and Australia (Smith, 1977). The disease occurs in 2 distinct forms: 1) generalized nodular lesions on large areas of the skin over various parts of the body, 2) localized necrotic ulcerations on the skin of the teats, udder, escutcheon and perineum.

The first isolation of the virus causing generalized skin disease (Allerton virus) was by Alexander et al. (1957) from cases of lumpy skin disease in southern Africa. It emerged from this work that the disease, once considered as one etiological entity could be caused by a pox virus (prototype Neethling) or by a herpesvirus (BHV-2: Allerton virus). The disease caused by Allerton virus was demonstrated to be benign and in order to distinguish it from the more severe poxvirus infection it has generally been called pseudo-lumpy skin disease (PLSD). The nodules in the skin due to BHV-2 appear most frequently on the face, neck, back, and perineum. The nodules are firm, round, raised areas presenting a flat surface with a slightly depressed center (Gibbs and (Rweyemamu, 1977b).

The mammary form of BHV-2 was known for a long time, but the significance of BHV-2 as a specific cause of mammillitis was not realized until the work of Martin et al. (1964) in England, who reported the isolation of BHV-2 from teats. Bovine herpesvirus-2 has also been

isolated from oral and facial lesions of cattle and buffalo calves. In all the cases where the dam has been examined, a concurrent mammillitis has been confirmed (Gibbs and Rweyemamu, 1977b).

The cytopathic effect of BHV-2 in tissue cultures is characterized by the formation of large syncytial masses. The syncytia may be either globose or stellate with or without transyncytial vacuolation (Gibbs and Rweyemamu, 1977b). The CPE is fast, (<24 h) and can be evident as early as 12 hours after inoculation. Often the syncytia may involve practically the whole monolayer before the sheet disintegrates and becomes detached from the glass in balls (Alexander et al., 1957; Gibbs and Rweyemamu, 1977b).

In natural infections, the development of antibody is rapid and can be assayed by seroneutralization tests. Some authors found persistence of neutralizing antibodies for at least 8 months (Gibbs and Rweyemamu, 1977b); others reported antibody to decline to barely detectable levels at four months after inoculation (Kalunda and Plowright, 1972).

There is good evidence demonstrating persistent infection with BHV-2. Martin and Scott (1979) demonstrated recrudescence of the in calves following corticosteroid administration, 10 weeks or later after initial infection. Although it is not possible to indicate in which tissues or organs BHV-2 remains latent, there is evidence that the lesions recurs at the same site as the initial lesion. BHV-2 has been included in the alphaherpesvirus group together with BHV-1

(ICTV, 1979).

Bovine herpesvirus-3 (BHV-3)

BHV-3 causes a natural clinical disease called malignant catarrhal fever (MCF) only in cattle and domestic buffaloes. Nevertheless, the primary hosts for this virus are members of the family Alcelaphinae (wildebeest, hartebeest and topi). These animals are asymptomatic carriers for MCFV. For this reason some authors proposed that MCFV not be considered a bovine herpesvirus (Gibbs and Rweyemamu, 1977a), but as an Alcephalinae herpesvirus.

The disease is worldwide in distribution, but its incidence is generally sporadic and low. It often terminates in fatal encephalitis, usually 5 to 12 days after onset of pyrexia (Mohanty, 1978). It is believed that the causative agent is acquired by cattle from inapparently infected sheep or from wildebeest (in areas like Africa, where a cattle-wildebeest contact is likely) (Plowright, 1968).

The virus produces multinucleated, often vacuolated syncytia. The infectivity titer is extremely low, and the virus is highly cell-associated. In the beginning, MCFV induces very small non-progressive CPE and is not transmissible by cell-free fluids (Plowright, 1968).

The virus produces a fatal lymphoid proliferation and angitis after experimental infection in cattle and rabbits (Plowright, 1968). Virus may be recovered from leucocytes 3 to 15 days before the terminal

pyrexia, and then from all tissues invaded by proliferating lymphoid cells (Plowright, 1968). Edington and Patel (1981), working with rabbits as an experimental model, determined that the spleen is the target organ and primary site of virus replication. They postulated that the detection of an early cycle of replication indicates that MCFV has an early pathogenesis similar to that of other lymphotropic herpesviruses, i.e., Marek's disease virus (MDV) in poultry. However, in MD the replication in the bursa is followed by the production of infectious, cell-free virus from the feather follicle, whereas there has been no evidence of MCFV being produced in other than lymphoid cells, nor does horizontal transmission occur between either cattle or rabbits (Plowright, 1968). The target cell was determined by these same authors (Patel and Edington, 1980) as being associated with medium sized lymphocytes and not with the proliferative lymphoblastoid cells. A similar observation took place in MCFV infected cattle (Patel and Edington, 1981) and evidence exists for a latent state of MCFV in the bovine medium-size lymphocyte. It was not possible either in cattle or rabbits to determine the lymphocyte subpopulation carrying the genome of MCFV (Patel and Edington, 1981).

Bovine herpesvirus-4 (BHV-4)

A large number of isolates recovered from diverse clinical syndromes as well as from clinically normal cattle in Africa, the Near East, Europe and Asia have been placed in the bovine herpesvirus-4 group

(WHO/FAO, 1976; Straub, 1978; Mohanty, 1980). The European and American isolates have been demonstrated to be serologically related to the strain Movar 33/63 (Bartha et al., 1966) which has been designated the prototype strain of the BHV-4 group (Table 2).

Bartha et al., in Hungary in 1966, isolated a herpes-type virus, Movar 33/63, from ocular discharges of calves that had developed a severe keratoconjunctivitis 2-4 weeks after recovery from pneumoenteritis due to bovine adenovirus type-4. The virus was non-pathogenic to calves and, with respect to keratoconjunctivitis, no etiological role could be attributed to the Movar isolate. A serologically related virus was isolated from tongue and palate material of a case of malignant catarrhal fever found in East Germany (Liebermann et al., 1967). The virus, designated Reims isolate, was nonpathogenic for calves, rabbits and mice. Luther et al. (1971) reported the isolation of a strain of herpesvirus different from other known bovine herpesviruses from calf kidney cell culture derived from a normal animal in England. This took place during the course of routine work in their laboratory on preparation of cell cultures. The isolate was called CK-54. This virus was similar to Reims and Movar isolates when analyzed by cross neutralization tests performed by reduction of 50% plaque titer. For all three of the isolates the conventional seroneutralization test was not sensitive enough to detect significant antibody titer, confirming previous observations of Bartha for Movar (Bartha et al., 1966). The isolation of this virus from apparently normal healthy cattle suggested

Table 2. Isolates grouped within bovine herpesvirus-4 (BHV-4)

Clinical disease/source from which virus was isolated	Denomination of the isolate	Geographical location and reference
Keratoconjunctivitis	Movar 33/63 (Reference strain)	Hungary (Bartha et al., 1966)
Respiratory disease	DN 599	USA (Mohanty et al., 1971)
	FTC1 and 2	USA (Smith et al., 1972)
	ST-66	Russia (Bodon et al., 1971)
Tongue and palate (malignant catarrhal fever-like case)	Rheims	E. Germany (Liebermann et al., 1967)
	Non-syncytia forming (NSF) herpesvirus	Tanzania (Rweyemamu and Loretu, 1973)
Kidney	CK-54	England (Luther et al., 1971)
	BPX-11	Hungary (Belak and Palfi, 1974)
	Orphan herpesvirus	Kenya (Plowright and Ferris, cited by Alexander et al., 1957)
Lymphoid tissues (leukocytes, lymph nodes and/or spleen)	LK	Russia (Bodon et al., 1970)
	V 11	USA (Van der Maaten and Boothe, 1972)
	Non-syncytia forming (NSF) herpesvirus	Tanzania (Rweyemamu and Loretu, 1973)
	Reticulosarcoma isolate	Russia (Surin et al., 1969)
	BZD (Orphan herpesvirus)	South Africa (Alexander et al., 1957; Prydie and Coackley, 1959)

Female Genital Tract	DDV-71 H. 1/80	USA (Parks and Kendrick, 1973) USA (M. J. Van der Maaten, National Animal Disease Center, Ames, IA, USA. Personal Communication, 1980)
Semen	Unnamed	Tanzania (Loretu et al., 1974)
Skin	Orphan herpesvirus NSF Herpesvirus 2274	S. Africa (Alexander et al., 1957) Tanzania (Rweyemamu and Loretu, 1973) USA (Reed et al., 1977)
Trigeminal ganglium	Unnamed	USA (Homan and Easterday, 1980a)

the possibility of latency of the virus for long periods. A similar isolation from the kidney of a 2 month-old calf showing no clinical signs was reported by Bélak and Pálfi (1974) in Hungary. This strain—BPX/11—showed very low antigenicity in rabbits and failed to infect two calves. They reported serological relationship with Movar 33/63 as demonstrated by serum neutralization.

Three isolates of bovine herpesviruses antigenically related to Movar were reported in Russia. One of these strains—-LK —(Bodon et al., 1970) was isolated from leukocytes of calves with respiratory disease but no data were presented about experimental pathogenicity. The other strain — ST/66 — (Bodon et al., 1971) was recovered from the pharynx, pleura, nasal cavity and lymph nodes of a calf with bronchopneumonia. In this case, when young cattle were inoculated intratracheally, animals developed signs of pyrexia, rhinitis and conjunctivitis. The third Russian isolate, reported by Surin et al. (1969), was recovered from the axillary lymph node of a cow affected with lymphosarcoma of reticular cells. Rabbits, guinea pigs and mice inoculated intravenously and intramuscularly with the virus failed to reproduce any disease.

Agents similar to Movar 33/63 have been isolated in Africa. These African isolates constitute a group generically designated "bovine orphan herpesviruses" due to their apparent lack of pathogenicity for cattle (Alexander et al., 1957; Gibbs and Rweyemamu, 1977b; Mohanty, 1980). They have been known and described mainly in Tanzania, Kenya

and South Africa since well before the isolation of Movar 33/63 (Weiss, 1963), but it was not until recently that their relationship to the Movar group was reported and their inclusion in the BHV-4 group proposed (Mohanty, 1980; Gibbs and Rweyemamu, 1977b).

This group of "orphan bovine herpesviruses", whose African prototype strain was called BZD, was first described by Alexander and Haig in 1957, in South Africa. They isolated this virus on five different occasions from suspensions of skin nodules or lymph nodes of animals suffering from lumpy skin disease (Alexander et al., 1957).

The widespread frequency of these orphan viruses among cattle in Africa is a striking feature of the group. From 27 specimens of field cases of lumpy skin disease, Prydie and Coackley (1959) isolated, in Kenya, 4 "orphan" viruses from skin lesions as well as from lymph nodes. They obtained two other isolations of similar "orphan" virus during serial passage of lumpy skin disease in cattle. This mixing of "orphan" herpesviruses with the true etiological agent of lumpy skin disease, the poxvirus denominated Neethling, constituted a common event in the characterization of that disease. In fact, the first "orphan" virus isolated by Alexander and Haig (Alexander et al., 1957) produced typical cases of lumpy skin disease with the 1st, 4th, and 8th tissue culture passage levels of the virus and doubtful reaction with the 15th passage level. The 26th and subsequent passage levels failed to produce any clinical reaction or antibodies to Neethling virus. It was later reported (De Lange, 1959; Weiss, 1963) that a mixed culture of Neethling and an

"orphan" virus were transferred simultaneously through at least 15 passages and that the poxvirus was eventually eliminated or outgrown by the "orphan" virus.

During 1956 to 1962 orphan viruses were isolated in 10 of 77 specimens from outbreaks of lumpy skin disease examined in South Africa (Weiss, 1963). In 7 of the 10 specimens, only orphan viruses were isolated, including one from a fresh salted hide. From the 3 other specimens, mixed cultures of orphan and LSD viruses were obtained. Furthermore, Alexander and Weiss, in 1959, isolated orphan viruses in 3 out of 136 hides as reported by Weiss (1963). These hides originated from 3 different farms in the Northern Transvaal province of South Africa.

Although orphan viruses have been repeatedly reported to be non-pathogenic for cattle, sheep, goats, rabbits and mice and to have failed to produce neutralizing antibodies (Alexander et al., 1957; Haig, 1957; Prydie and Coackley, 1959; Capstick, 1959), it appears that the simultaneous inoculation of lumpy skin disease agent (Neethling virus) and an "orphan" bovine herpesvirus into cattle reduced the incubation period of lumpy skin disease but had no effect on its severity (Capstick, 1959). Alexander and Weiss, in 1959, cited by Weiss (Weiss, 1963), infected cattle with both viruses (orphan and Neethling). The cattle developed generalized lumpy skin disease. The orphan virus could be recovered from skin nodules between days 12 and 26 post-infection and could be recovered sporadically from blood.

One of the animals was slaughtered 24 days after inoculation, and the orphan virus was isolated from lymph nodes, salivary glands, saliva, skin and muscle, but not from the gastrointestinal tract.

Isolation of bovine herpesviruses related to orphan herpesviruses took place in Africa from several other sources. Haig (1957) isolated this virus from a case of vaginitis in cattle and Plowright and Ferris, in Kenya, (cited by Alexander et al., 1957), isolated a virus serologically indistinguishable from orphan viruses from uninoculated calf kidney tissue culture.

Schiemann et al. (1971), working with Plowright and Jesset during the investigation of an outbreak of a severe disease in buffaloes at Serengeti National Park in Tanzania, isolated orphan viruses from the supraescapular, mesenteric and hepatic lymph nodes of 3 animals. The isolate was non-pathogenic for rabbits and no neutralizing antibody activity could be detected. Furthermore, Kaminjolo, working in Kenya, in 1972, isolated an orphan-like herpesvirus from tumors of urinary bladders from several steers. Subsequently, Rweyemamu and Loretu (1973) reported the isolation of what they called non-syncytia forming (NSF) herpesvirus from cattle in Tanzania. These isolations were from a skin nodule suspected of Lumpy skin disease, pharyngeal mucosa of a heifer with diarrhea, a tongue scraping for foot-and-mouth disease investigation and from the buffy coat layer of blood samples of animals with jaundice with possible cause of death due to chlorinated hydrocarbons. After further characterization of these isolates Rweyemamu and Loretu

concluded that these non-syncytial forming herpesvirus isolates were identical and indistinguishable from the African "orphan" herpesviruese. At the same time they called attention to the similar cytopathic effect of these isolates, Movar 33/63 and human cytomegalovirus. Nevertheless, they could not carry out any critical comparison with the European Movar 33/63 because of the impossibility of preparing antisera of good titer in rabbits for either Movar or NSF isolates. They suggested the use of a plaque reduction neutralization test.

Loretu et al. (1974) isolated from bovine semen a non-syncytia forming herpesvirus that was homologous to the previously described isolates. The first reported isolation in America of a virus related to Movar 33/63 took place in 1971, when Mohanty and co-workers (Mohanty et al., 1971) isolated a herpesvirus (DN-599) from nasal swabs taken from an 18-month-old steer with clinical symptoms of respiratory disease. They reported that experimental infection via the respiratory route caused overt respiratory disease in young calves (Mohanty et al., 1971, 1972). The inoculated calves developed severe respiratory disease accompanied by pyrexia, hyperpnea, dyspnea, cough, and profuse nasal discharge. Conjunctivitis was noticed in the majority of calves and some of them died. The virus was recovered from the nasal discharge of the affected animals for up to 17 days postinfection and from the lungs of those that died. The lungs of those that died had areas of consolidation, atelectasia and emphysema. Pasteurella multocida was also isolated

from these lungs (Mohanty, 1973). This virus was not neutralized by antisera to herpes simplex virus, IBRV, Movar 33/63, MCFV, Allerton, pseudorabies, BHV-2 and canine, equine, and avian herpesviruses (Mohanty et al., 1971,1972). Recovered calves did not develop neutralizing antibodies to DN-599 virus (Mohanty et al., 1972) nor were antibodies encountered in 84 sera from Maryland cattle (Mohanty et al., 1971). An antibody response was not detected in calves, goats, rabbits, chickens and rats after multiple inoculations even when adjuvant was used in some cases (Mohanty, 1973). It was demonstrated later that antibody titers in fact do rise, and can be demonstrated by ferritin conjugated IgG or by immune electron microscopy (Mohanty, 1975). The indirect immunofluorescence test was also demonstrated to be very sensitive for detection and assay of antibodies to DN-599 (Sass et al., 1974).

Smith and co-workers (1972) isolated a bovine herpesvirus from the tissues (ulcers of the larynx and pharynx and retropharyngeal lymph nodes) of 4 of 19 steers from an outbreak of respiratory disease characterized by hemorrhagic necrotizing tracheitis. The agent, called FTC, was similar in many respects to the Movar 33/63 and DN-599 isolates. Subsequent experimental infection of colostrum-deprived calves with both FTC and DN-599, caused little or no clinical illness. Repeated injections of virus suspensions into calves, goats, and rabbits elicited very little or no measurable neutralizing or complement fixing antibody.

Parks and Kendrick (1973) isolated a herpesvirus from the uterine exudate of a dairy cow with metritis. The infected animal was one of a

herd of 1800 dairy cows that had experienced an abnormal incidence of postpartum metritis. The virus had no serologic relationship to several known herpesviruses. When injected into young animals, (Kendrick et al., 1976) intranasally, intramuscularly and in the conjunctival sac, no reisolation of the virus was possible and serum neutralizing antibody could not be identified. The only response was a very uniform peak of pyrexia in all the animals between days 7 and 10 postinfection. The possibility of some role of this isolate (DDV-71) in bovine fetal disease was investigated by inoculation into bovine fetuses of various gestational ages. This resulted in the death of some fetuses in the 3rd and 4th months of gestation. Some fetuses in the 3rd and 4th month and all older than 4 months survived the inoculation. These fetuses experienced a lymphoreticular activation. Virus was isolated in 4 fetuses, from lung or amnionic fluid. The fact that only fetuses of 3 to 4 months were affected made unlikely any important role of this virus in bovine abortions, because most undiagnosed bovine abortions occur in mid-gestation (Kendrick et al., 1976).

Potgieter and Maré (1974) demonstrated an antigenic relationship among the isolates DN-599, FTC and V11. This last isolate was a herpesvirus isolated by Van der Maaten (cited by Potgieter and Maré, 1974) from leukocytes and lymph nodes of a lymphosarcomatous cow in Iowa. They could demonstrate, by reciprocal virus neutralization and indirect fluorescent antibody test, that the 3 isolates were antigenically related. They were successful in demonstrating neutralizing activity of the specific antisera by using plaque reduction (50%) neutralization test

with an incubation of 4 C overnight followed by 2 hours at room temperature. They verified that the rate at which DH-599, FTC and V11 viruses were neutralized by specific antiserum was exceptionally slow but relatively high antiserum titers were observed.

Smith published in 1976 the results of his studies on the serologic relatedness of several isolates related to Movar. He claimed to have modified standard serologic techniques which enabled him to group these agents. Details of these modifications were not published. As a result of this analysis, he concluded that the European isolates Movar 33/63, CK-54 and BPX/11, and the American isolates DN-599, FTC, DDV-71 and V11 were serologically related and proposed the establishment of a Bovid herpesvirus-5 group in which would be included all of these isolates. He reported that by his modified serologic techniques he was able to detect subgroup differences within this group. Further details about these differences were not published.

Other isolates of BHV-4 have been reported in America. One of them was made by Van der Maaten from a uterus of a normal cow (M. J. Van der Maaten, National Animal Disease Center, Ames, Iowa, USA, Personal communication, 1980). This isolate was serologically indistinguishable from V11 and DN-599 (Osorio and Reed, Vet. Med. Res. Inst., Iowa State University, Ames, Iowa, unpublished data 1980). Eugster (1978) isolated a virus from the feces of a cow with diarrhea. This virus was serologically identical to DN-599. In experimental inoculation of cattle with this fecal strain the only clinical sign noted was a fever after 3 to 4 days postinoculation. Virus was recovered from the nose 2 to

10 days postinfection.

Reed et al. (1977) isolated a strain (3374) serologically similar to DN-599 from 2 separate enzootics of mammary pustular dermatitis. This same author reported the isolation of a similar agent from a bovine fetus aborted in the 6th month of gestation (Reed et al., 1979). The virus was mixed with a cytopathic strain of bovine viral diarrhea virus.

In 1980, Krogman (Krogman, L., 1980. Host responses of calves to a BHV-5 Herpesvirus. Master of Science Thesis, South Dakota State University, Brookings, South Dakota, USA) studied the possible reactivation of DN-599 after corticosteroid treatment. Experimental infection of cattle was performed by intratracheal, intranasal and intradermal inoculation. During the period of acute infection no virus was detected from any source and in 2 of 4 inoculated calves mild respiratory signs were evident. After repeated dexamethasone treatments, she could isolate (post-mortem) virus from the thoracic lymph node of one of the calves, 7 days after the last dexamethasone treatment. From another calf the virus was isolated from organ cultures of spinal cord and trigeminal ganglion after 5 days of incubation. This detection in neural ganglia took place almost 50 days post-dexamethasone treatment. The source of DN-599 used for this experiment was an isolate from South Dakota (77-4019) recovered from a pool of lung spleen and liver of a five-month-old calf.

During a survey to detect persistent BHV-1 in neural tissues, Homan and Easterday (1980a) reported the isolation of DN-599 virus from explant

and cocultivation of trigeminal ganglia of 2 out of 43 abattoir slaughtered cattle.

On the basis of the similarities among all these European, African and American isolates it has been proposed that all of them be placed in the bovine herpesvirus-4 group with Movar 33/63 as prototype reference strain (WHO/FAO, 1976; Gibbs and Rweymamu, 1977b; Mohanty, 1978; Straub, 1978). The relationship between the American and European strains has been confirmed by serology and widely published (Smith, 1977). Although the same relationship was reported between African and European isolates, no details about the serological procedures used have been published (Gibbs and Rweyemamu, 1977b; Mohanty, 1980).

All of the isolates have common characteristics that constitute the features of the BHV-4 group. These are:

1) Morphology and physicochemical characteristics of herpesviruses which are indistinguishable from other members of the family Herpesviridae.

2) Ability to grow to high titer in cell monolayers derived from bovine tissues but also in unestablished kidney cell cultures of several other species (Bartha et al., 1966; Luther et al., 1971; Sass et al., 1974; Gibbs and Rweyemamu, 1977b).

3) The cytopathic effect is slow when compared with BHV-1 and BHV-2 and is apparent after 3-4 days of incubation. Although the time of appearance of CPE can be shortened, it will be always greater than 24 hours.

4) Most authors agree that BHV-4 group do not produce syncytia, with the exception reported by Luther et al. (1971) for the isolate CK-54.

5) In most cases there is no evidence to support the role of the isolates as primary etiological agents and, with some exceptions, the experimental inoculation of cattle with these isolates do not produce any symptoms. Two American isolates: DN-599 and FTC (Mohanty et al., 1971; Smith et al., 1972) and a Russian isolate: ST-66 (Bodon et al., 1971) demonstrated experimental pathogenicity when inoculated into cattle.

6) None of the isolates produced disease in laboratory animals. The species assayed by different workers were rabbits, guinea pigs, hamsters, rats, neonatal and adult mice and chickens (Gibbs and Rweyemamu, 1977b).

7) All of the isolates demonstrated limited ability to induce detectable titers of neutralizing antibodies, in cattle and rabbits, by the standard serum neutralization assays. However, high titers of antibodies are present in this sera, when assayed by other means such as indirect immunofluorescence (Sass et al., 1974) and immunoelectron microscopy (Mohanty, 1975). Potgieter and Maré (1974) demonstrated that, for three American isolates, the specific neutralization of the viruses was extremely slow. To explain the lack of SN titers, they postulated the induction of BHV-4 of low avidity antibodies, which do not irreversibly inactivate viral infectivity.

8) The members of the BHV-4 group are characterized by a widespread distribution, having been reported in Africa, Europe, America and

recently in the Near East (Erhan and Onar, 1977). Potgieter and Aldridge (1977) demonstrated antigenic homogeneity among 3 American isolates originating from 3 very distinct points of the United States.

Ungrouped bovine herpesviruses

There are still some isolates of bovine herpesviruses that demonstrate some different characteristics from the members of the four groups previously described.

In the U.S.A., Van der Maaten and Boothe (1972) isolated a strongly cell-associated syncytial herpesvirus from leukocytes of 2 cattle with lymphosarcoma. When examined by electron microscopy, this virus demonstrated herpes viral structure. In tissue culture, syncytia developed but intranuclear inclusions were not encountered. Experimental animals inoculated by a variety of routes with this virus remained normal. Calves were infected with no apparent disease, but the virus was reisolated from the circulating leukocytes of these calves 4-12 months post-inoculation. Sera from the original herd demonstrated high titers of antibodies by the IFA test for this virus. No evidence linked this herpesvirus with the lymphoid neoplasm of cattle. Although the authors postulated some possible relationship with BHV-2, because of similar cultural characteristics, there is no supplementary information in the literature about a comparison between both viruses.

Finally, there is a group of South African viruses whose isolation by Maré and Van Resnburg (1961) and by Theodoridis (1978) took place

during an investigation of the viral etiology of a regional reproductive disease known as "Epivag syndrome" (epididimitis-vaginitis). These viruses share several features with BHV-4, principally their slow cytopathic effect and induction of low avidity neutralizing antibodies. These viruses have been described (Theodoridis, 1978) as producing many enlarged cells (giant cells) in infected tissue cultures, when stained with hematoxylin-eosin. This characteristic was the reason by which some authors (Mohanty, 1980) described them as the cytomegalovirus of the bovine species. The existence of the bovine cytomegalovirus was inferred by Schiefer (1974) when he found cytomegalic inclusion bodies by histopathology in an aborted fetus in Canada. He found dense particles, believed to be virus, when observing the cytoplasm of these cytomegalic cells by electron microscopy.

The Epivag-related slow herpesviruses were mainly isolated from reproductive tissues: vagina, semen, testicles and placenta. Sometimes they were mixed with IPV virus and their experimental pathogenicity consisted of a mild anterior vaginitis. The pathological significance of these viruses becomes apparent when inoculated together with IPV virus (Theodoridis, 1978). There are no references in the literature concerning their comparison with BHV-4 group members or their characterization as bovine cytomegalovirus.

Persistent Infections By Herpesviruses

A persistent infection is defined as one in which virus (or at least the viral genome) is conserved in the host for long periods of

time (Fenner, 1974). A chronic infection is a persistent infection in which infectious virus is present and can be recovered by conventional virological methods from appropriate tissues at any time (Fenner, 1974). A latent infection is a persistent infection in which infectious virus is not present, or is present only intermittently in tissues harboring the viral genome in a "reactivable" state (Stevens, 1978).

The herpesviruses, particularly as exemplified by herpes simplex, have long been considered to be the classic examples of latent viruses. The herpesviruses have been divided (Stevens, 1978) into three subgroups according to the tissue harboring latent viruses (Table 3): neurotropic herpesviruses, those that are associated principally or exclusively with nervous tissues; lymphotropic herpesviruses, are those that persist in association with lymphoid tissue and epitheliotropic herpesviruses are those persisting in epithelial tissues.

Persistence of herpesviruses in nervous tissue

Herpes simplex virus (HSV) represents a well-studied member of the neurotropic group. The latent infection established by HSV appears to be specific for nervous tissues, since after inoculation of large doses of virus intravenously, only nervous tissues were found to harbor the virus (Stevens, 1978). Viral genetic information can be found only in neurons when in situ hybridization techniques are applied to latently infected ganglia (Stevens, 1975). The state in which the viral genome persists in these neurons is not definitely known but there is evidence

Table 3. Herpesviruses grouped by organ or tissue in which they persist*

NERVOUS TISSUE (NEUROTROPIC HERPESVIRUSES)	Herpes simplex virus types 1 & 2	
	Herpesvirus simiae and others simian herpesviruses	
	Bovine herpesvirus - 1†	
	Pseudorabies virus†	
LYMPHOID TISSUE (LYMPHOTROPIC HERPESVIRUSES)	Epstein - Barr virus	Herpesvirus ateles
	Murine cytomegalovirus	Herpesvirus saimiri
	Marek's disease virus	Malignant catarrhal fever‡
	Herpesvirus sylvilagus	Human cytomegalovirus (possible)
	Equid herpesvirus 2§	
EPITHELIAL TISSUE	Lucke's carcinoma virus of the frog	
	Epstein - Barr virus	

*Taken from Stevens (1978) with additions.

†Taken from McKercher, 1973.

‡Taken from Patel and Edington, 1981.

§Taken from Coggins and Gleeson, 1980.

that it does not persist as replicating virus (Stevens, 1978). The postulated non-replicating virus would persist as an entire virion or as viral DNA only.

The possible relevance of some special features of the viral genome of herpesviruses for their need of integration for latency has been postulated by Ritchie and Timbury (1980) who remark that similarities exist between the structure of HSV-DNA and that of a transposable element (transposon). These unique sequences bracketed by repeated sequences exist for all the herpesviruses studied and could stand for a molecular explanation of establishment of latency as well as reactivation. In the case of HSV, integration into cellular DNA is not a required mechanism for perpetuation of infection, since neurons do not divide.

The mechanism by which the latent infection is maintained has not been defined. Two major hypotheses have been proposed: the "dynamic state" and the "static state" hypotheses. The dynamic state hypothesis (Hill and Blyth, 1976) suggests that virus replication and shedding from infected ganglia is occurring continuously but at a slow rate. The virus migrates to the skin and produces microfoci of infection but the host's immune response, if uncompromised, can control the degree of virus-induced cytopathology and no clinical disease occurs. Support for this hypothesis comes from observations that virus shedding does occur intermittently or continuously in the absence of visible lesions (Hill and Blyth, 1976).

The static state theory suggests that the virus is present in the

ganglia in some unknown state, either integrated or free, but not replicating, possibly because the specific neurons harboring the virus lack a functional transcriptase required for replication (Roizman, 1974) or because of some immunological control of replication (Lehner et al., 1975). Following some reactivation stimulus, altering the biochemical or immunological balance, the virus migrates centrifugally along the axon to initiate an active site of replication in epithelial cells.

Persistence of herpesviruses in lymphoid tissues

Gammaherpesvirinae (Epstein-Barr virus group) and Betaherpesvirinae (cytomegalovirus group) present evidence for association with lymphoid cells during persistent infection (Stevens, 1978). In the first case, the evidence is strong. The well-characterized Epstein-Barr virus (EBV) was initially found in a persistently infected lymphoblastoid cell line. In fact, lymphoblastoid cell lines can be established from essentially any person with positive EBV serology or by infecting *in vitro* cultures of lymphocytes derived from seronegative individuals (Ho, 1981). These cell lines constitute "immortalized" cultures that carry the viral genome; all are characterized as being of B-cell origin (T cells do not possess viral receptors).

Unlike tissues carrying herpes simplex virus, Epstein-Barr virus can not be recovered directly from lymphocytes at the time they are taken from the infected individual. Viral replication takes place only following the stress or derepression of *in vitro* cultivation

(Stevens, 1978). However, two antigens, Epstein-Barr nuclear antigen (EBNA) and membrane antigen (MA), are present in latently infected cells examined directly after removal from the patient. The different cell lines carrying the EBV genome differ in the degree to which the virus is expressed, varying from a very limited expression to replication of infectious virus (Ho, 1981).

The state of the viral DNA in latently infected cells is well-understood in the case of EBV. The genetic information for EBV exists in lymphocytes in two forms: a circular, extrachromosal plasmid of length equivalent to that of an intact EBV genome and an integrated form of unknown length (Adams and Lindahl, 1975). EBV is unique among human viruses in that it produces disease by immortalizing or transforming cells. Thus, the lymphocytes transformed by EBV multiply like tumor cells and a small number of lymphocytes can produce cell-free virus capable of infecting other lymphocytes. This infection and transformation by EBV of antibody producing B cells results in polyclonal expansion with many types of antibodies produced. The cells producing heterophil antibodies to sheep or horse red blood cells are always affected and cause the increase of these antibodies in EBV-infected patients (Ho, 1981).

The relationship of Betaherpesvirinae with lymphoid cells is less clear than that of the EBV group. There is as yet no concrete evidence that human cytomegalovirus (HCMV) can even infect lymphocytes. Although HCMV can be detected in the buffy coat cells it presently is unclear in

which leukocyte fraction HCMV resides. Rinaldo et al. (1977) isolated HCMV from both mononuclear and polymorphonuclear leukocyte fractions. Virus was not detected in washed erythrocytes, plasma or leukocyte lysates.

Lang and Noren (1968) first detected HCMV in association with leukocyte cells in infants infected congenitally with HCMV. This was the basis for a further hypothesis regarding the relevance of latently HCMV-infected lymphoid cells in organ transplantation and post-transfusion infections (Lang, 1972). In fact, Lang proposed that since transfused individuals receive unmatched leukocytes, this could constitute an in vivo mixed lymphocyte reaction with consequent enhanced replication of lymphoid elements. When the donor or recipient is carrying HCMV in his lymphocytes, it would result in a reactivation of the virus, with the expression of clinical disease. Joncas et al. (1975) reported the detection by DNA-DNA reassociation kinetics of unexpressed HCMV genome in an EBV positive lymphoblastoid cell line which was established from a 9-month male child with congenital defects from whom was isolated cytomegalovirus. Furthermore, Pagano (1975) found several genome equivalents of HCMV DNA in the buffy coats of one of three blood donors but it is not known what cells they were in.

Plotkin (1979) reported a line of B lymphoblasts that were permissive for HCMV. Ho (1981) reported that several attempts to infect normal as well as B or T lymphocyte cell lines with HCMV resulted in no infection, incomplete infection, or a small amount of virus replication, but no

evidence of viral persistence or transformation after freshly donated lymphocytes were infected.

Other members of the Betaherpesvirinae group show more evidence than HCMV for lymphoid-associated persistence. Murine cytomegalovirus (MCMV) establishes a special relationship with B lymphocytes in the mouse. Virus attenuated by passage through cell cultures can establish a latent infection in B lymphocytes of neonatal mice. The latent infection can be demonstrated by cocultivation on allogeneic fibroblasts or by molecular hybridization (Hudson, 1979; Wise et al., 1979). Explants of abdominal lymph nodes and spleens of chronically infected mice resulted in reactivation of MCMV. Unattenuated virus will produce latent infection in B lymphocytes, T lymphocytes and macrophages, and other cells may be involved as well (Ho, 1981). For now, latent MCMV infection can only be defined operationally; it is not possible to demonstrate lytic virus by routine laboratory methods and additional measures of activation, such as treatment with immunosuppressants or cocultivation, are needed.

Nothing is known yet about the molecular basis of latency of MCMV, particularly about the state of viral genome and limitation of its expression. A big difference between EBV (Gammaherpesvirus) and MCMV (Betaherpesvirus) is that the lesions of MCMV were not demonstrated to be the result of lymphocyte infection (Hudson, 1979).

PART I. EXPERIMENTAL INFECTION OF CATTLE WITH BOVINE HERPESVIRUS-4: EVIDENCE FOR A LYMPHOID-ASSOCIATED PERSISTENT INFECTION

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EXPERIMENTAL INFECTION OF CATTLE WITH BOVINE HERPESVIRUS-4: EVIDENCE FOR A LYMPHOID-ASSOCIATED PERSISTENT INFECTION

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SUMMARY

A strain of bovine herpesvirus-4 isolated from cases of mammary pustular dermatitis has been used for experimental infection of cattle. This strain is serologically indistinguishable from the group prototype Movar 33/63 and from strain DN-599. Serologically negative cattle were infected intravenously or by simultaneous intranasal, intravenous, intramammary (via teat channel) and intradermal inoculations. All isolated cattle seroconverted. No clinical signs or lesions were evident, except for a dermal lesion corresponding with one intradermal inoculation point. Virus was recovered from the dermal lesion and was excreted in milk for 17 days. Virus was recovered from esophagus-pharyngeal fluid at 9 and 13 days post-inoculation. At different times of slaughtering (2 to 14 months post-inoculation) virus was recovered from cocultures with bovine lung cells and/or explant cultures of lymph nodes, spleen, tonsils, and in one case, kidney. In 2 of the cattle, the virus was recovered repeatedly throughout one year from peripheral blood leukocytes by cocultivation with bovine lung cells. The number of infectious leukocytes, as determined by Infectious Centre Assay, ranged from less than 1 to 6 infectious cells per 10^7 leukocytes.

INTRODUCTION

The type 4 of bovine herpesvirus also is referred to as Bovid herpesvirus-4 (BHV-4) and includes a large number of isolates recovered from diverse clinical diseases as well as from healthy cattle (WHO/FAO, 1976; Straub, 1978). Members of this group are geographically widespread and have been reported in Africa (Alexander et al., 1957; Rweyemamu and Loretu, 1973), in Europe (Bartha et al., 1966; Liebermann et al., 1967; Luther et al., 1971; Bélak and Pálfi, 1974), and in America (Mohanty et al., 1971; Smith et al., 1972; Kendrick et al., 1976; Reed et al., 1977). In most of the cases, serological relationship to the European isolate Movar 33/63 (Bartha et al., 1966) has been reported (Smith, 1977; Gibbs and Rweyemamu, 1977). This strain constitutes the prototype reference for the group (WHO/FAO, 1976).

Distinctive features of the BHV-4 group are a slow cytopathic effect (CPE) with no syncytium development (Gibbs and Rweyemamu, 1977) and a humoral immune response characterized by the production of low avidity neutralizing antibodies which are barely detectable by standard seroneutralization assays (Potgieter and Maré, 1974).

Although a possible role in respiratory disease has been reported (Mohanty et al., 1971; Smith et al., 1972) most of the isolates of BHV-4 group exhibited a general failure to elicit clinical disease by experimental inoculation of cattle and laboratory animals (Alexander et al., 1957; Liebermann et al., 1967; Luther et al., 1971; Schiemann

et al., 1971; Bélak and Pálfi, 1974) and the pathogenic role of the group is still ill-defined.

The frequency with which members of the BHV-4 group have been recovered from tissues, mainly kidney, of apparently normal healthy cattle (Alexander et al., 1957; Luther et al., 1971; Bélak and Pálfi, 1974) suggested the possibility that these viruses could establish latency for long periods of time (Bélak and Pálfi, 1974). The isolation of BHV-4 in 2 of 44 trigeminal ganglion explants was reported in a survey to detect naturally occurring latent bovine herpesvirus infections in neural tissue of abattoir cattle (Homan and Easterday, 1981). These authors inferred that BHV-4 could establish neural latency.

The present study was designed to characterize the experimental infection of cattle with a strain of BHV-4 isolated from cases of mammary pustular dermatitis in Iowa and South Dakota (Reed et al., 1977). This isolate is serologically indistinguishable from the prototype strain Movar 33/63 and the DN-599 isolate (Reed et al., 1977).

MATERIALS AND METHODS

Clinical and Virological Procedures

Cell culture

Bovine lung (BLG) cells were grown in Eagle's minimum essential medium with Earle's salts (MEM) supplemented with 10% betapioprolactone treated fetal bovine serum (FBS), 0.16% sodium bicarbonate, 8 mM N-2-hydroethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and antibiotics (100 I.U. pencillin, 100 ug streptomycin culfate and 100 ug kanamycin sulfate per ml). Cells were grown and maintained at 37 C in a humidified air atmosphere containing 5% carbon dioxide (CO_2).

Virus

The strain 3374 (Reed et al., 1977) of bovine herpesvirus was recovered from scrappings of udder lesions in bovine fetal spleen (BFS) cells and was passed three times in BLG cells. The stock preparation used for inoculation contained $10^{7.8}$ median tissue culture infective doses ($TCID_{50}$)/ml.

Experimental animals

Nine Hereford cattle from one herd were used in this experiment. All of them were serologically negative for 3374 strain of BHV-4, as determined by the indirect fluorescent antibody test. Five of the cattle were used to infect and four were held as uninoculated controls.

The infected cattle were: two adult (6 years old) cows in lactation;

one 1-week-old calf born from a serologically negative mother, kept in isolation after delivery, and fed with colostrum for the first 3 days; and one pregnant cow (8 years old) that delivered a heifer calf while in isolation, 25 days after infection. In the latter case, the heifer calf remained in isolation in contact with the dam. The control animals were 4 calves born from the same seronegative herd, placed in isolation immediately after delivery and fed with colostrum for the first 3 days.

Inoculation procedure

The 2 adult cows in lactation received a total inoculation dose of $5 \times 10^{7.8}$ $TCID_{50}$ each. The inocula were administered in both animals by various simultaneous routes, as follows: 2 ml of the stock virus suspension was inoculated into the mammary gland, via teat channel (0.5 ml each quarter); 1 ml was inoculated intradermally in the skin of the udder (0.25 ml into each of 4 sites) and 1 ml was inoculated intravenously. The 1-week-old calf and the pregnant cow were injected with $10^{7.5}$ $TCID_{50}$ each, intravenously.

Clinical observation and collection of specimens

Clinical observation of inoculated and control cattle was carried out daily, beginning one week before infection, with collection of rectal temperatures and heparinized blood samples for hematology. Nasal, vaginal, ocular and rectal swabs as well as milk samples were collected for virus isolation. Esophagus-pharyngeal fluid samples

for virus isolation were collected by means of a cup probang. After day 30 post-infection (PI), clinical samples were taken with a 4 day frequency. Samples of serum were collected at inoculation time, at 21 days PI, and every week thereafter. Total and differential leukocyte counts were made daily for 30 days PI.

Necropsy

The date of sacrifice for each of the inoculated animals is presented in Table 1. One uninoculated control calf was sacrificed simultaneously with cattle Nos. 1 through 4. The animals were killed by intravenous inoculation of sodium phenobarbital.

At necropsy, samples for virus isolation were taken from the following organs: lung, kidney, adrenal, lymph nodes (peripheral and visceral), spleen, liver, pancreas, tonsils, thymus, cervix, endometrium, and trigeminal and paralumbar ganglia.

Virus isolation procedures

The nasal, vaginal, ocular and rectal swabs were immersed in 2 ml of MEM containing penicillin, streptomycin and kanamycin at a concentration 3 times higher than that described for cell cultures, plus 10 µg of amphotericin B per ml and vigorously shaken for 15 seconds prior to inoculation of 0.5 ml onto BLG monolayer cell cultures.

The samples of whole milk and esophagus-pharyngeal fluid were diluted in a ratio 1:15 with MEM containg 3X regular antibiotics and

Table 1. Experimental infection of cattle with bovine herpesvirus-4 (Strain 3374): Experimental design, antibody conversion and viral recovery

Number and type of animal	Inoculation route	Serology*		
		Preinoculation antibody titer	21 days PI antibody titer	Antibody titer at sacrifice
1. Adult cow	Simultaneous† Inoculations: I.V.	Neg	80	320
2. Adult cow	I. MAMM: I.D.	5	160	640
3. Calf	I.V.	Neg	80	80
4. Adult cow	I.V.	Neg	160	160
5. Heifer	Contact	Neg‖	NT	80
Controls 6 through 9	None	Neg	Neg	Neg

*Data express the reciprocal of the indirect fluorescent antibody test titer.

†I.V. = Intravenous; I. MAMM. = Intramammary; I.D. = Intradermal.

‡NT = Not tested.

§MNC = mononuclear cells.

‖Preinoculation antibody titer stated for heifer No. 5 corresponds to birth time and following 32 days.

¶Precise date of infection of heifer No. 5 is unknown.

#NA = not applicable.

Virus recovery during early post-infection period (31 days PI)	Virus assayed in leukocytes	Necropsy (days PI)	Tissues of post-mortem virus recovery
Milk	NT[‡]	101	Lymph nodes & spleen
Milk and dermal vesicle	NT	132	Lymph nodes, spleen & tonsils
Neg	NT	54	Lymph nodes, spleen, tonsils & kidney
Esophagus-pharyngeal fluid	(+) MNC[§]	401	Lymph nodes & spleen
Neg	(+) MNC	>376[¶]	Lymph nodes & spleen
Neg	Neg	NA[#]	None

amphotericin B. Two ml of this dilution was inoculated onto BLG monolayer cell cultures. These were observed daily for the presence of CPE and were discarded if negative after 14 days.

All solid tissues taken at necropsy were washed in MEM and cut into 1-5 mm^3 pieces. Explant cultures of all the tissues were established by culturing these pieces in MEM supplemented with 10% FBS and 3X regular antibiotics and amphotericin B. The explant cultures were maintained at 37 C in a humidified air atmosphere containing 5% CO_2, with medium changes twice a week. Samples of culture fluid were tested for infectivity on BLG cells every third day.

For the preparation of cocultures, the constituent cells of tissue fragments (1-3 mm^3) of lung, kidney, adrenal, tonsil, pancreas, genital tract and ganglion were dispersed by trypsinization in 0.25% trypsin at 37 C. Trypsinization was in 3 consecutive steps of approximately 5, 15 and 45 minutes, or until turbidity was noticed. All the visible particulate material was discarded, saving the supernatants rich in single cells. These cells were rinsed three times in MEM, centrifuged at 150 xg for 5 min. and suspended in MEM with antibiotics and 10% FBS. Each of these batches of cells was mixed with trypsinized BLG cells in proportions 1:2 and incubated as described for other cell cultures. The suspension of single cells of lymphoid organs other than tonsils (spleen, thymus and lymph nodes) was prepared by mincing the tissue samples and repeatedly aspirating the suspension through a 5 ml syringe until the tissue pieces were thoroughly dissociated. Large,

non-dissociated clumps of cells were allowed to settle out and the suspended single cells were saved and rinsed 3 times in MEM, and processed as described above for other cell types.

Cell-free homogenates from the different tissues were prepared by mechanical disruption (Stomacher Blender, UAC House, London, UK) and further homogenization by Ten Broeck tissue grinders of a 10% suspension in MEM of 1-3 mm^3 pieces of tissue. In some cases, three cycles of freezing and thawing were added as an additional step. A sample of 0.5 ml of these homogenates was inoculated onto BLG monolayers, which were watched for CPE as described.

Investigation of the virus in blood

The association of the virus with blood elements was studied in two of the inoculated animals, Nos. 4 and 5 (Table 2), throughout more than one year of isolation post-inoculation.

Samples of blood ranging from 10 cc to 100 cc were collected by jugular venipuncture in tubes containing preservative-free heparin (5 IU per ml of blood collected). Plasma and platelet fraction and packed red blood cell (RBC) fraction were separated by centrifugation at 100 xg for 20 min. Both fractions were assayed by inoculating 1 ml of each on BLG cells monolayers.

The isolation of polymorphonuclear fraction (PMN) was as described by Roth and Kaeberle (1981). After centrifugation of the blood sample at 1,000 xg for 20 minutes, the plasma, buffy coat and top few mm of

Table 2. Isolation and quantitation of BHV-4 associated with peripheral blood leukocytes in experimentally infected cattle

Animal No.	Days[†] PI	Blood Fraction				Virus Titer in MNC[*]		
		Plasma & Platelets	RBC	PMN	MNC	Total MNC	Adherent	Non-adherent
Cow No. 4	13	Neg	Neg	Neg	+	NT[‡]	NT	NT
	20	Neg	Neg	Neg	+	NT	NT	NT
	52	Neg	Neg	Neg	+	0.37	NT	NT
	186	Neg	Neg	Neg	+	0.08	NT	NT
	386	Neg	Neg	Neg	+	0.59	NT	NT
Heifer No. 5	130	Neg	Neg	Neg	+	0.41	0.16	0.29
	247	Neg	Neg	Neg	+	0.36	Neg	0.45
	330	Neg	Neg	Neg	+	0.19	0.24	0.33
Controls: Calves Nos. 6 thru 9	NA[§]	Neg	Neg	Neg	Neg	NT	NT	NT

[*]The titer of BHV-4 in mononuclear cell (MNC) fraction is expressed as the median of the number of focus forming units per 10^6 MNC as determined by Infectious Centre Assay.

[†]Days post-infection.

[‡]NT = not tested.

[§]NA = not applicable.

packed RBC were aspirated and discarded. The packed RBC fraction was then measured and the erythrocytes in the suspension were lysed by adding two volumes of cold phosphate buffered (0.0132M, pH 7.2) deionized water with gentle mixing. Isotonicity was restored after 45 seconds by the addition of one volume of phosphate buffered (0.0132M pH 7.2) 2.7% NaCl solution. This preparation was poured into centrifuge tubes and centrifuged at 250 xg for 10 minutes. The supernatant fluid was poured off and the pellet of cells was resuspended in phosphate buffered saline (PBS), rinsed two times in that buffer and finally resuspended in Hanks balanced salt solution (HBSS) without Ca^{++} and Mg^{++}. The cells were counted with a Neubauer's hemocytometer. A smear of the cells was made and stained with a modified Wright's stain (Marleco Diff-Quik, Dade Diagnostics Lab., Aguada, Puerto Rico).

The PMNs were coated on BLG monolayers, in a ratio no higher than 8×10^6 PMNs per 75 cm^2 flask of BLG monolayer, incubated at 37 C and watched for CPE.

For isolation of virus from the buffy coat layer, this fraction was aspirated after the centrifugation of blood at 1000 xg for 20 minutes and diluted in HBSS without Ca^{++} and Mg^{++}. The suspension was counted and a smear of the cells was made and stained with modified Wright's stain. The buffy coat cells were inoculated onto BLG monolayers not exceeding the inoculum of 15×10^6 cells per 75 cm^2 of BLG monolayer.

When enrichment in mononuclear cells was required, a Ficoll-Hypaque flotation was used as described by Rouse and Babiuk (1974). The buffy

coat cells obtained by centrifugation of heparinized blood, suspended in 10 ml of HBSS without Ca^{++} and Mg^{++} were layered on 3 ml of Ficoll-Hypaque (density at 25 C, 1.077 g/cm^3) (Ficoll-Pharmacia, Piscataway, NJ., Hypaque-Winthrop Lab., New York, NY). and centrifuged at 400 xg for 20 min at room temperature. The mononuclear cell (MNC) enriched (mainly lymphocytes) band was collected from the interface of plasma and Ficoll-Hypaque. These cells were washed once in HBSS without Ca^{++} and Mg^{++} and a total (hemocytometer) and a differential (stained smear) count was performed. The cells harvested by this technique contained always greater than 96% MNC.

When isolation of adherent and non-adherent subpopulations from the MNC-enriched fraction was required, an additional rinse and resuspension of the MNC fraction in medium 199 (Grand Island Biological Co. (GIBCO), Grand Island, NY) was performed before the counts. For this separation the suspension of cells was placed in 60 mm plastic tissue culture Petri dishes and incubated for 2 hr with intermittent agitation at 37 C in a humidified atmosphere containing 5% CO_2. Non-adherent cells were removed by three vigorous washes with medium 199 and were saved. The adherent cells were removed by gentle scrapping, pooled, and resuspended in medium 199. Both adherent and non-adherent cell fractions were counted as described.

For Infectious Centre Assay (see below) the adherent cells were not removed from the 60-mm plastic Petri dishes, the assay instead was performed <u>in situ</u> after removal of the non-adherent fraction.

The number of adherent cells per plate was calculated by removing and counting the cells of a representative sample of plates.

Infectious Centre Assay (ICA)

Virus present in the MNC populations, as well as in adherent and non-adherent subpopulations was quantified by ICA. Dilutions of the MNC-enriched population and the non-adherent subpopulation were prepared either with HBSS without Ca^{++} and Mg^{++} or medium 199. The dilutions of the total MNC cells contained respectively 10^8, 10^7 and 10^6 viable cells per ml whereas the dilutions corresponding to non-adherent cells contained 10^7, 10^6 and 10^5 viable cells per ml. Immediately before plating on the monolayers, 0.1 ml of each dilution was thoroughly mixed with 0.5 ml of fluid agar overlay medium: MEM with 0.7% agar (Seaplaque, Marine Colloids, FMC Co., Rockland, MA), 5% FBS, 100 µg per ml of Protamine sulfate, 100 IU penicillin, 100 µg streptomycin sulfate and 100 µg kanamycin sulfate per ml) at 38-39 C and plated onto freshly seeded (2 hour prior) BLG cell monolayers in 60 mm tissue culture plastic Petri dishes. After this layer had solidified, a second 4 ml of agar overlay medium was added. Four monolayer cultures of BLG were used per dilution of total or non-adherent MNC suspensions. After incubation for 9 days, the monolayers were fixed with 10% formalin and stained with 1 percent crystal violet in 10% ethanol.

The plates containing the adherent subpopulation were seeded with BLG cells, incubated 2 hrs at 37 C and coated with agar overlay

medium (4 ml). Plates were incubated 9 days, fixed and stained as described previously. After staining, foci of CPE were counted and expressed as focus forming units (FFU) per 10^6 MNC inoculated.

Serology

Identification of isolates All the isolates recovered from clinical samples, explants and cocultivations were identified as BHV-4 by the indirect fluorescent antibody (IFA) test, as previously described (Reed et al., 1977). Slide cultures of BLG cells were inoculated with the isolate, incubated at 37 C until 50% CPE was observed, and then fixed in acetone for 1 minutes. Fixed slide cultures were flooded with a 1:20 dilution of a rabbit antiserum against the 3374 isolate of BHV-4 and incubated at 37 C for 1 hour. Slides were rinsed 3 times in 0.01 M (pH 7.2) phosphate-buffered physiologic saline solution (PBS), flooded with fluorescein-conjugated goat anti-rabbit globulin (Cappel Lab., Downington, PA) and incubated at 37 C for 20 minutes. Slides were rinsed 3 times in PBS and mounted in borate buffered glycerin (pH 8.6) for examination by fluorescence microscopy.

Antibody titers The IFA antibody titers of inoculated and control cattle were determined as described above with the following modifications: BLG slide cell cultures inoculated with the 3374 strain of BHV-4 were treated with dilutions of serums from inoculated and control cattle and then stained with fluorescein-conjugated rabbit anti-bovine

globulin (Cappel Lab., Downington, PA). All the serum samples from different times (pre and post-inoculation) corresponding to the same animal were titrated simultaneously.

RESULTS

Clinical, Serological and Virological Observations

Clinical findings and serology

Inoculated animals showed no clinical symptoms other than increased body temperature (41.5-42 C) by day 4 and 5 PI. One of the cows that received intradermal inoculation of the virus in the udder developed a vesicular lesion at one of the 4 injection points. The lesion consisted of a circumscribed area of cutaneous congestion (3 mm in diameter) that was apparent by day 6 PI and developed into a vesicle 48 hours later. No significant changes were detected in the total and differential white blood cell counts carried out during the first 30 days PI.

Serology

The serologic results are given in table 1. All inoculated animals developed specific antibodies by day 21 PI. These titers persisted and sometimes increased throughout the experiment. Heifer No. 5, which remained in contact with her infected mother, seroconverted 42 days post-birth.

Virus isolations

Early post-infection period Table 1 summarizes the viral isolation results. A homogenate prepared from a scraping taken from the single vesicular lesion in the udder of cow No. 2 produced BHV-4

CPE when inoculated onto BLG cell monolayers.

Virus was isolated from the milk of cows Nos. 1 and 2 from day 2 PI to day 17 PI. The virus was isolated from esophagus-pharyngeal fluid only on days 9 and 13 PI from cow No. 4 which had been injected intravenously. Virus was not isolated from any fluid or mucous membranes of inoculated calves Nos. 3 and 5 or from the control calves Nos. 6,7, 8 and 9.

Isolation of BHV-4 from peripheral blood leukocytes Table 2 summarizes the results of virus assay of each of the blood fractions of two of the animals (Nos. 4 and 5) at different times throughout a period of more than one year after infection.

Early attempts to assay BHV-4 in leukocyte fraction were not successful because the initial high input of MNC or PMN produced toxic effects when cocultivated with BLG cells. Therefore, satisfactory results were not obtained until 52 days PI.

The BHV-4 specific CPE developed in monolayers after incubation which was never less than 7 days and rarely longer than 10 days. When ICA technique was used, foci of BHV-4 CPE also were evidenced within this time range. It was not possible to keep the ICA monolayers in incubation longer than 10 days without appearance of degenerative changes in the BLG cells. We, therefore, fixed and stained the plaques at day 9 PI.

The virus titer in the MNC population, as well as in the adherent and non-adherent subpopulations was low ranging from 0.08 to 0.69

FFU/10^6 MNC. Virus was isolated from none of the other cellular or plasma fractions of the blood of cattle Nos. 4 and 5, or from the blood elements of control calves Nos. 6,7, 8 and 9, when assayed at necropsy time.

Post-mortem virus recovery Table 1 summarizes the tissues from which BHV-4 could be recovered in each of the infected animals at necropsy time. Virus was isolated from lymph nodes and spleen of all infected cattle (cattle Nos. 1 through 5), both from cocultures of dispersed cells with BLG cells and from explant cultures.

In the cocultures, CPE characteristic of BHV-4 was detected after 6 to 8 days of incubation. Similar CPE was evident in BLG cells inoculated with supernatant fluid of spleen and lymph node explant cultures after incubation of the explant ranging from 6 to 16 days. In addition, outgrowths of cells (fibroblastic, in most cases) from these same explants became cytopathic between 12 and 20 days after explantation.

The lymph glands from which BHV-4 was isolated included: submaxillary, retropharyngeal, cervical, suprascapular, bronchial, mediastinal, mesenteric and prefemoral lymph nodes. Explants of kidney tissue from calf No. 3 shed BHV-4 as detected by assay of the supernatant fluid and CPE in the outgrown cells was observed after 19 days of incubation. Virus was not isolated from explant cultures or from single cell cocultivation of any other tissue.

All the organs sampled presented normal macroscopic characteristics

and no evidence for any pathologic alteration was found at necropsy of infected and control animals. Virus was not isolated from tissues of uninoculated control calves. The isolates recovered from milk, pharynx, udder, leukocytes, explant culture and cocultures were confirmed as BHV-4 by IFA tests.

DISCUSSION

The results of these experiments, although they do not demonstrate a definite role in disease for the isolate 3374 of BHV-4 in cattle, demonstrate that this virus is capable of establishing a persistent infection.

The single vesicular lesion which developed in the udder of one of the experimental animals was considerably less severe than the pustular dermatitis seen in the animals from which the strain 3374 was isolated (Reed et al., 1977).

Based on the circumscribed character of this dermal lesion and its location at one of the intradermal inoculation points, we believe that its development is more the consequence of intradermal inoculation rather than the expression of a true natural dermotropism of the virus.

BHV-4 isolations from bovine skin and hides have been reported previously, especially during investigations of lumpy skin disease and pseudolumpy skin disease in Africa (Alexander et al., 1957; Prydie and Coackley, 1959; Weiss, 1963; Rweyemamu and Loretu, 1973). Also, in these cases the dermal syndrome could not be reproduced by experimental inoculation of cattle and it was concluded that those BHV-4 isolates were "orphan" viruses (Alexander et al., 1957; Haig, 1957; Weiss, 1963).

The most significant finding of this study was the demonstration that BHV-4 can establish a persistent infection in lymphoid tissues

and a prolonged viremia associated with the MNC fraction. The virus was detected in fluids of explant cultures and in the cocultures of lymphoid tissue, as well as in the blood, only after an incubation period which was never less than 7 days. At the same time, lysates of the original tissues failed to yield infectious virus. These two circumstances suggest a requirement for integrity of the cell to obtain expression of infectious virus. This persistent infection also is characterized by constant presence of circulating antibodies. Isolation of the virus from the MNC fraction of the blood in the presence of high titers of circulating antibody could also suggest that BHV-4 in the blood is probably intracellular, and therefore protected from the action of antibody.

The requisite of integrity of the cell and incubation to recover infectious BHV-4 is a characteristic we detected also in spleen tissues of persistently infected rabbits after experimental inoculation with BHV-4 (F. A. Osorio and D. E. Reed, Veterinary Medical Research Institute, Iowa State University, Ames, IA, submitted for publication, 1982). This would suggest that the virus may persist in the lymphoid cell in a possible quiescent or latent state. Evidence for this feature has been found in, and extensively described for, other herpesviruses, specifically Betaherpesviruses such as murine cytomegalovirus (Henson et al., 1972; Wise et al., 1979; Ho, 1981) and possibly human cytomegalovirus as well as Gammaherpesviruses such as Epstein-Barr virus (Ho, 1981) and malignant catarrhal fever virus (Patel and

Edington, 1981). Alternatively, it also is possible that the virus is present in an actively replicating state, but in such a low titer that it was undetectable in the cell-free homogenates.

The virus associated with the MNC fraction of the blood, when titrated by ICA, was found in a very low titer not higher than 6 FFU (or infectious cells) per 10^7 MNC. These findings are similar to the titers observed in the MNC-associated persistent viremias described for equine herpesvirus-2 (Coggins and Gleeson, 1979) and human cytomegalovirus (Fiala et al., 1975).

The fact that the virus is present in low titer in the buffy coat requires that a large sample ($\geqslant$ 50 cc) of blood be processed or some enrichment procedure be utilized. In this respect, the enrichment through Ficoll-Hypaque gradient proved to be highly efficient, confirming previous results (Howell et al., 1979) in viremias due to human cytomegalovirus and other viruses.

We are still unable to define the type of MNC carrying the virus. Although the results presented in Table 2 show that BHV-4 was shed in higher titer by the non-adherent subpopulation (lymphocytes) of animal No. 5, the conclusion that lymphocytes constitute the target cell for BHV-4 would require more rigorous statistical analysis, employing more experimental animals. In addition, the possibility of contaminant subpopulations in each fraction can not be discarded and the fact that some subpopulations of lymphocytes can also be adherent (Benacerraf and Unanue, 1980) makes it desirable to characterize further the

cell type harboring BHV-4.

The herpesvirus of African malignant catarrhal fever (Bovid herpesvirus 3 - BHV-3) is also characterized by cell-associated viral infectivity, with virus recovery from lymphocytes and lymphoid tissues (Edington and Patel, 1981; Patel and Edington, 1981). Nevertheless, we could not detect in our experiments with BHV-4 any evidence of the extensive lymphoid proliferation that constitutes the distinctive feature of malignant catarrhal fever, Marek's disease, Epstein-Barr virus infections, and all the other diseases produced by members of the Gammaherpesvirus group (Ho, 1981; Patel and Edington, 1981).

The association of BHV-4 with lymphoid organs and the MNC fraction of blood, with its consequent permanent transport through lymphatic and hematogenous routes for long periods of time, could explain the great diversity of tissues (and syndromes) from which BHV-4 has been recovered (Straub, 1978). Specifically, several isolates of BHV-4, although coming from very dissimilar diseases, were obtained from some type of lymphoid organ or peripheral blood leukocytes. (Alexander et al., 1957; Prydie and Coackley, 1959; Bodon et al., 1970; Schiemann et al., 1971; Smith et al., 1972; Rweyemamu and Loretu, 1973; M. J. Van der Maaten, National Animal Disease Center, Ames, IA, Personal communication, 1980).

Until more information becomes available about the definite pathogenic role of BHV-4, the presence of this virus in cattle used for immunological experiments can not be disregarded. In fact, the

close association demonstrated between BHV-4 and the bovine lymphoid tissues may have significant implications in immunological disorders of cattle.

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PART II. EXPERIMENTAL INFECTION OF RABBITS WITH BOVINE HERPESVIRUS-4: ACUTE AND PERSISTENT INFECTION

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EXPERIMENTAL INFECTION OF RABBITS WITH BOVINE HERPESVIRUS-4: ACUTE AND PERSISTENT INFECTION

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ABSTRACT

A strain of bovine herpesvirus-4 (BHV-4) isolated from bovine cases of mammary pustular dermatitis was used for experimental infection of rabbits. The strain is serologically indistinguishable from the group prototype Movar 33/63 and from the American isolate DN599.

Groups of rabbits were inoculated by various routes. Intravaginal and conjunctival inoculations resulted in vulvovaginitis and conjunctivitis, respectively, and in shedding of virus. The rabbits seroconverted for the virus, with high titers of antibodies (indirect fluorescent antibody test) that persisted throughout the experiment. Treatment with dexamethasone, beyond the acute infection, did not produce recrudescence of disease or shedding of the virus. Rabbits were killed at various times, from three to six months post-infection, and the virus was recovered from explant cultures of spleen and by co-cultivation of spleen cells with bovine lung cells.

These results demonstrate the usefulness of the rabbit as a model for studying the pathogenesis of BHV-4 infections in cattle.

INTRODUCTION

The bovine herpesvirus 4 group (BHV-4) - also referred as bovid herpesvirus 4 - (WHO/FAO, 1976; Straub, 1978; Mohanty, 1980) includes a large number of isolates recovered from diverse clinical diseases as well as from healthy cattle. Members of this group are widespread and have been reported in Africa (Alexander et al., 1957; Rweyemamu and Loretu, 1973), in Europe (Bartha et al., 1966; Liebermann et al., 1967; Luther et al., 1971) and in the United States (Mohanty et al., 1971; Smith et al., 1972; Kendrick et al., 1976; Reed et al., 1977). In most of the cases, serological relationship to the European isolate Movar 33/63 (Bartha et al., 1966) has been reported (Smith, 1977; Gibbs and Rweyemamu, 1977) and this strain constitutes the prototype reference for the group (WHO/FAO, 1976).

The members of the BHV-4 group produce a slow cytopathic effect (CPE) in tissue cultures, with no syncytium development, (Gibbs and Rweyemamu, 1977) and they evoke a humoral immune response characterized by production of low avidity neutralizing antibodies which are barely detectable by standard seroneutralizing (SN) assays (Potgieter and Maré, 1974).

Although a possible role in respiratory disease has been reported (Mohanty et al., 1971; Smith, et al., 1972) most of the isolates of BHV-4 group exhibited a general failure to elicit clinical disease by experimental inoculation of cattle and laboratory animals. All the

isolates of BHV-4 assayed have been reported apathogenic for rabbits, guinea pigs, hamsters, rats, neonatal and adult mice, and chickens inoculated by various routes (Alexander et al., 1957; Liebermann et al., 1967; Luther et al., 1971; Schiemann et al., 1971; Mohanty, 1973; Belak and Palfi, 1974).

In the experiments herein presented, we used an American strain of BHV-4 which was isolated from cases of mammary pustular dermatitis by Reed et al. (1977) and that is serologically indistinguishable from Movar 33/63 and DN599 isolates. Previous work in our laboratory demonstrated the ability of this strain to establish a lymphoid-associated persistent infection after experimental inoculation of cattle (Osorio and Reed, 1982, Vet. Med. Res. Inst., Ames, Iowa, submitted for publication 1982).

The purpose of the experiments that we report in this paper was to establish the feasibility of infecting rabbits with BHV-4. We were especially interested in determining if the rabbit could be used as a laboratory model to study the BHV-4 lymphoid association observed in cattle.

MATERIALS AND METHODS

Inoculation Procedures

Virus

The strain 3374 (Reed et al., 1977) of bovine herpesvirus was recovered from scraping of udder lesions in bovine fetal spleen (BFS) cells and was passed three times in bovine lung (BLG) cells. The stock preparation used for inoculation contained $10^{7.8}$ median tissue culture infective doses ($TCID_{50}$)/ml.

Experimental animals

Young adult white rabbits (2.5-3.0 kg) of either sex were purchased from a local commercial source and were housed in individual cages within an animal isolation room. All rabbits were free of antibodies to strain 3374, as determined by indirect fluorescent antibody tests prior to inoculation.

Experimental design

Rabbits (n=20) were divided into six groups and inoculated as detailed in Table 1.

The stock preparation of 3374 virus strain was inoculated according to the following procedures: The intravaginal inocula were spread in the anterior vagina and cervix; the marginal ear vein was used for intravenous inoculation; ocular inoculation was by instillation of the inoculum into the right conjunctival sac; intrapulmonar inocula

Table 1. Experimental infection of rabbits with bovine herpesvirus-4 (strain 3374)

Group	Number of Rabbits	Route of Inoculation Used	Virus dose ($TCID_{50}$)
1	6	intravaginal	$10^{7.5}$
2	3	intravenous	$10^{7.8}$
3	3	conjunctival sac	$10^{7.5}$
4	2	intrapulmonary	$10^{7.8}$
5	2	intradermal	$10^{7.8}$
6 (control)	4	All the previous routes	None*

*Each of the controls was mock-infected with a dose of Eagle's minimum essential medium (MEM) supplemented with 10%-Beta-propiolactone-treated fetal calf serum (FCS) equivalent to the infectious inocula, following the procedure of the inoculation route in each case.

were injected at the level of the 6th intercostal space of the right hemithorax and the intradermal injections were applied in the shaved dorsal skin in four points of 0.25 ml each.

Rabbits were observed for clinical signs of illness following virus inoculation. Conjunctival, vaginal and nasal swabs were collected for virus isolation daily during the first 32 days post-infection (PI) and weekly thereafter. These samples were harvested by saturation of cotton-tipped swabs with conjunctival, vaginal or nasal fluids.

Two rabbits of group 1 (Table 1) were killed at 89 days PI. At 137 days PI all the remaining rabbits from the inoculated and control groups were treated daily with 4 mg of dexamethasone (Azium, Schering Corp., Kenilworth, N.J., U.S.A.) for 5 days. During the period of dexamethasone treatment and the following twenty days, daily samples for virus isolation were collected. At 180 days PI, all the animals were killed by electrocution. Samples of spleen were taken from all the rabbits, trigeminal ganglia were taken from rabbits of group 3 (conjunctival inoculation) and group 6 (control), and paralumbar ganglia were taken from group 1 (intravaginal inoculation) and group 6 rabbits. Viral isolation was attempted from these samples by homogenization (splenic tissues), explantation (splenic and nervous tissues) or cocultivation (splenic cells). Samples of serum were taken at 20, 137 and 180 days PI.

Cell culture

Bovine lung (BLG) cells were grown in Eagle's minimum essential medium with Earle's salts (MEM) supplemented with 10% betapropiolactone treated fetal bovine serum (FBS), 0.16% sodium bicarbonate, 8mM N-2-hydroethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and antibiotics (100 I.U. penicillin, 100 µg streptomycin sulfate and 100 µg kanamycin sulfate per ml). Cells were grown and maintained at 37°C in a humidified air atmosphere containing 5% carbon dioxide (CO_2).

Virus isolation procedures

The ocular, nasal and vaginal swabs were immersed in 2 ml of MEM containing antibiotics at the concentrations described above plus 10 µg amphotericin B per ml and vigorously shaken for 15 seconds prior to inoculation of 0.5 ml onto BLG monolayer cell cultures. The remaining sample was stored at -70 C until titration. The titrations were performed by serial 10-fold dilutions in MEM on BLG monolayer cultures in 24-well plastic tissue culture plates. Inocula were 0.1 ml/well and 6 wells for each dilution. Virus titers were expressed as median tissue culture infectious doses ($TCID_{50}$) per ml.

Spleen and trigeminal ganglion samples for explant culture were washed in MEM, cut into 1-2 mm^3 pieces, and cultured in MEM supplemented with 10% FBS, and antibiotics (including amphotericin B). Cultures were maintained at 37 C in a humidified air atmosphere containing 5% CO_2. Samples of supernatant fluid were tested for infectivity on BLG

monolayers at 3-day intervals.

Cell-free homogenates from these spleens were prepared as 10% suspensions in MEM by mechanical disruption (Stomacher Blender, UAC House, London, U.K.) and further homogenization by Ten Broeck tissue grinders. In some cases, three cycles of freezing and thawing were added as an additional step. A sample of 0.5 ml of these homogenates was inoculated onto BLG monolayers.

Cocultivation of spleen cells was performed by mincing the spleen samples and repeatedly aspirating the suspension through a 5 ml syringe until the spleen was dissociated. Large, non-dissociated clumps were allowed to settle out and the suspended single cells were poured into a centrifuge tube. The cells were centrifuged at 1,500 rpm for 15 minutes, resuspended in 10 ml of MEM with FBS with antibiotics (including amphotericin B) and counted using a hemocytometer chamber. These cells were mixed with trypsinized BLG cells in a ratio spleen cells/BLG cells of 1:2. The cell mixtures were inoculated into small plastic flasks (25 cm^2) and incubated in a humidified air atmosphere containing 5% CO_2. All flasks were observed daily for cytopathic changes.

Serology

A) Identification of isolates: All the isolates recovered from swabs, explants and cocultivations were identified as BHV-4 by the indirect fluorescent antibody (IFA) test, as previously described (Reed et al., 1977). Briefly, bovine antiserums against the 3374 strain

of BHV-4 were obtained from cattle persistently infected with this virus in our laboratory (F. A. Osorio and D. E. Reed, Veterinary Medical Research Institute, Iowa State University, Ames, IA, submitted for publication, 1982). Those serums had IFA titers of 1:80-1:160. Slide cultures (Tissue Culture Chamber Slides, Lab-Tek Products, Division Miles Lab. Inc., Naperville, IL, USA) of 10-15th passage of BLG cells were inoculated with the isolates, incubated at 37 C until 50% CPE was observed, and then fixed in acetone for one minute. Fixed slide cultures were flooded with a 1:20 dilution of a bovine antiserum and incubated at 37 C for one hour. Slide cultures were rinsed three times in 0.01 M (PH 7.2) phosphate-buffered saline solution (PBS), flooded with fluorescein conjugated rabbit anti-bovine globulin (Cappel Lab., Downington, PA, USA) and incubated at 37 C for 30 minutes. Slides were rinsed three times in PBS and mounted in borate buffered glycerin (PH 8.6) for examination by fluorescence microscopy.

B) Antibody titers: The IFA antibody titers of serums of the inoculated and control rabbits were determined as described above with the following modifications: BLG slide cell cultures inoculated with the 3374 strain of BHV-4 were treated with dilutions of serums from inoculated and control rabbits and then stained with fluorescein-conjugated goat anti-rabbit globulin (Cappel Lab., Downington, PA, USA). All of the serum samples from different times (pre and post-inoculation) corresponding to the same animal were titrated simultaneously,

RESULTS

Clinical and Virological Observations

Clinical signs

All rabbits of group 1, inoculated intravaginally, developed vaginitis, characterized by hyperemia and edema that extended to the vulva. By day 4 PI, a mucopurulent and often hemorrhagic secretion was evident along with abundant pseudomembranes and desquamated material. The vaginitis decreased in severity by day 6 PI, and by day 12 PI the vaginal mucosa appeared normal. A 24 hour febrile response was noticed at day 4 PI (rectal temperature: 40.9-41.2C) in five of the 6 rabbits. The rabbits of group 3, infected in the right conjunctival sac, developed, at 48 hours PI, edema and hyperemia of the conjunctiva with injected scleral vessels in the eye infected. The left eye remained normal. The signs remitted by day 6 PI. No fever was detected in these animals. The vagina and eyes of the mock-infected control rabbits of group 6 remained normal.

One of the two rabbits of group 5 presented, at 48 hours PI, intense zones of hyperemia at the intradermal inoculation points. These areas became edematous nodules by day 3 PI and regressed by day 6 PI. Viral isolation was not attempted from these lesions. All the rabbits of group 2, 4 and 6 remained normal.

Virus isolation post-inoculation

The viral replication after intravaginal and conjunctival inoculation is given in Fig. 1.

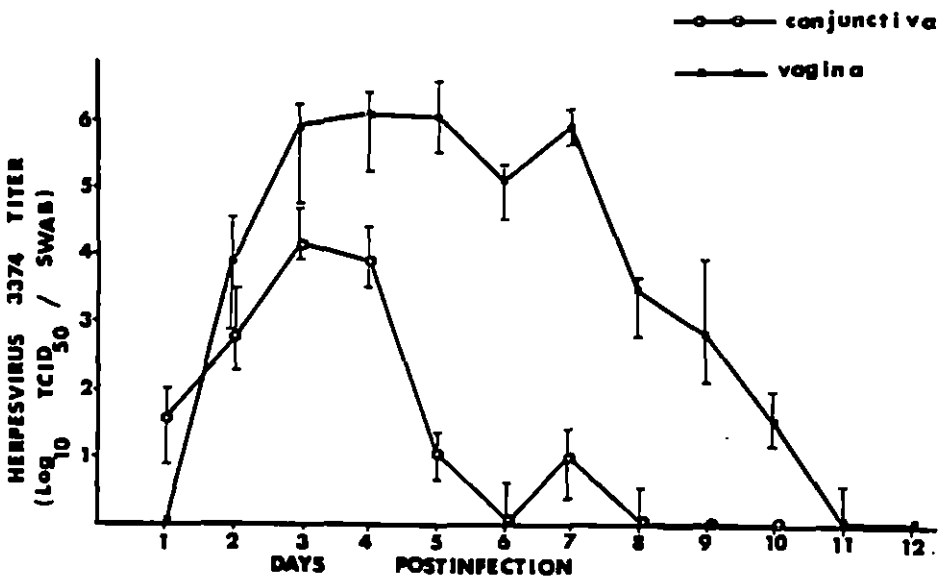

Figure 1. The mean and range of BHV-4 (strain 3374) titers in the right conjunctival sac and vagina of rabbits experimentally infected (N = 4 for conjunctival inoculation; N = 3 for vaginal inoculation). In both cases inoculum was $10^{7.5}$ $TCID_{50}$/animal.

Virus could not be recovered from the uninoculated left conjunctiva. Virus was isolated only at day 1 PI from a nasal swab of one of two rabbits inoculated by intrapulmonary route. Virus was not recovered from the rabbits in the groups inoculated intravenously, intrapulmonary and intradermally. No virus was recovered from the mock-infected control rabbits.

Dexamethasone treatment at 137 days PI caused neither recrudescence of disease nor reactivation of detectable virus in nasal, vaginal or ocular swabs, in any of the inoculated or control groups.

Post mortem viral isolation

A summary of virus isolation in the different groups of rabbits is given in Table 2. The virus was recovered from explants of spleen and cocultivations of splenic cells from all rabbits inoculated intravaginally (group 1) and intraconjunctivally (group 3) and from two of three rabbits inoculated intravenously (group 2). Virus was not isolated from explant cultures of spleen or splenic cell cocultivation of the remaining groups of rabbits and no virus was recovered from explant cultures of nervous tissues from rabbits of groups 1, 2 and 6.

The CPEs of BHV-4 were noticeable in BLG cells inoculated with supernatant of spleen explants after incubation of the explant ranging between 10 and 16 days. In addition, outgrowth of fibroblastic cells from the spleen explants became cytopathic between 12 and 20 days after explantation. Similar CPEs developed in the cocultures BLG/spleen

Table 2. Viral isolations and serologic conversions after experimental infection of rabbits with BHV-4 (strain 3374)

Group No. and Route of Inoculation	Post-inoculation viral isolation	IFA titers of the rabbit serums*			Post-mortem viral isolation	
		Preinoc.	20 Days PI	137 Days PI	Spleen†	Nervous tissue‡
1 (intravaginal)	6/6	5	80	160	2/2(89 DPI) 4/4(180 DPI)	0/2(89 DPI) 0/4(180 DPI)
2 (intravenous)	0/3	5	80	320	2/3(180 DPI)	NT§
3 (conjunctival)	3/3	Neg	80	160	3/3(180 DPI)	0/3(180 DPI)
4 (intrapulmonary)	1/2	Neg	80	113	0/2(180 DPI)	NT
5 (intradermal)	0/2	Neg	80	640	0/2(180 DPI)	NT
6 (control)	0/4	5	Neg	Neg	0/2(180 DPI)	0/4(180 DPI)

*Data are expressed as the geometric means of the reciprocals of the IFA titer.

†Explant cultures and cocultivation.

‡Explant cultures of paralumbar ganglia (goups 1 and 6) and trigeminal ganglia (groups 3 and 6).

§NT = not tested.

cells after at least eight days of culture. All the isolates were confirmed as BHV-4 by IFA tests. No CPE could be detected in the BLG monolayers inoculated with cell-free homogenates of the splenic tissue.

Serology

The results of the serologic tests of all the rabbits are presented in Table 2. The preinoculation IFA titers were not higher than 1:5. At 20 days PI,all the rabbits inoculated with virus seroconverted with titers ranging from 1:40 to 1:160. At 137 days PI,all these rabbits had increased antibody levels, with individual titers up to 1:1280.

DISCUSSION

The results of these experiments indicate that the 3374 strain of BHV-4 is capable of establishing infection in rabbits when inoculated by intravaginal, conjunctival or intravenous routes. Clinical signs of acute disease were more restricted to the mucosal surface in which the virus was inoculated (groups 1 and 3), with practically no symptoms of generalized disease beyond a 24 hour fever in the animals inoculated intravaginally. During this acute period of disease, virus was recovered from the vagina and conjunctiva in titers that permitted comparison of the replication in both tissues. Virus also was isolated at day 1 PI from the nose of one rabbit of group 4 (intrapulmonary). This may represent clearance of the inoculated virus through the upper respiratory tract and not actual replication of virus in the respiratory tract. Serologic conversions were detected by IFA tests in all groups of animals inoculated with the virus.

Previous works reported failure to establish an infection with BHV-4 in rabbits by several routes (Alexander et al., 1957; Luther et al., 1971; Mohanty, 1973; Belak and Palfi, 1974) including intracerebral (Liebermann et al., 1967) and intradermal routes (Schiemann et al., 1971). Those authors could not detect antibodies against this virus in the rabbit sera when using the standard SN assay. Our results with IFA test confirm the previous report of Sass et al. (1974) about the higher sensitivity of this technique when compared with the SN assay in ability to detect

antibodies against BHV-4. The antibody titers we detected in rabbits were, in some cases, even higher than the ones we observed in experimentally infected cattle (F. A. Osorio and D. E. Reed, Vet. Med. Res. Inst., Iowa State University, Ames, Iowa, submitted for publication, 1982) and they persisted at least for the duration of the experiment (6 months). Potgieter and Mare (1974) reported a rather transient antibody response, as measured by a 50% plaque reduction neutralization test with prolonged incubation of serum-virus mixtures, in rabbits hyperimmunized with the DN599 strain of BHV-4.

The most significant information derived from this study is the demonstration that BHV-4 can persist in the rabbit in association with lymphoid organs, specifically the spleen. These results are similar to those found in experimentally infected cattle in which the virus could be detected in peripheral and visceral lymph nodes, spleen, tonsils and circulating leukocytes for extended periods of time (Osorio and Reed, 1982, submitted for publication). The virus was detected in the supernatant fluids of explant culture and in the coculture of splenic/BLG cells only after a prolonged incubation period which was never less than eight days. At the same time, lysates of the original splenic samples failed to yield infectious virus. This requirement for integrity of the cell and incubation to obtain expression of infectious virus is a characteristic we detected also in lymphoid tissues and leukocytes of persistently BHV-4 infected cattle (F. A. Osorio and D. E. Reed, Veterinary Medical Research Institute, Iowa State University, Ames, Iowa, submitted for publication, 1982). This suggests that the virus may persist

in the splenic cell in a possible quiescent or latent state. Evidence for this feature has been found for other herpesviruses, especially in lymphoid tissues perisistently infected with Betaherpesviruses like murine cytomegalovirus (Henson et al., 1972; Ho, 1981) and (possibly) human cytomegalovirus (Lang and Noren, 1968; Rinaldo et al., 1977) as well as Gammaherpesviruses like Epstein-Barr virus (Ho, 1981) and malignant catarrhal fever virus (Patel and Edington, 1980). Alternatively, it also is possible that the virus was present in an actively replicating state, but in such a low titer that it was indetectable in the cell-free homogenates.

All the isolates were confirmed as BHV-4 by IFA tests. The possibility that the isolates were laboratory contaminants is minimal considering that control rabbit spleens yielded no virus and the BLG cells carried along for cocultures and supernatant assays never developed CPEs. Furthermore, the isolations were made at a time when BHV-4 was not being passed in the laboratory.

No persistent virus could be detected in groups 4 and 5 (intrapulmonar and intradermal inoculations), but a serologic response with high level of antibodies was detected in both cases. As the spleen was the only organ assayed for persistent virus in the rabbits of these two groups, the possibility that the virus did persist in other lymphoid organs (i.e., regional lymph nodes of the area of inoculation) cannot be discarded.

Although for groups 1, 2 and 3 the virus was actually demonstrated to be persisting in the animals, the treatment with dexamethasone

failed to reactivate the virus. The chance of the splenic viral isolation being due to a reactivation caused by the dexamethasone treatment is low since the virus was isolated in a similar manner from rabbits killed at day 90 PI, with no dexamethasone treatment, as well as from rabbits killed at day 180 PI which was 43 days after dexamethasone treatment. The use of corticosteroids to reactivate latent bovine herpesvirus-1 (BHV-1) was demonstrated to be very effective both in cattle (Davies and Carmichael, 1973) and in rabbits (Rock and Reed, 1982) where reactivation was obtained in essentially 100% of persistently infected animals. However, corticosteroids have poor efficacy of reactivation for latent Herpes simplex virus (HSV) in mice (Openshaw et al., 1979) and for latent bovine herpesvirus-2 (BHV-2) in cattle (Probert and Povey, 1975; Martin and Scott, 1979). Although the ultimate mechanism for costicosteroid viral reactivation has not been elucidated, this spectrum of responses could be due to a different susceptibility to the hormone of the diverse target cells harboring herpesviruses.

It is already known that BHV-1 (Lupton et al., 1980; Rock and Reed, 1980), BHV-2 (Castrucci et al., 1972) and bovine herpesvirus-3 (BHV-3) (Patel and Edington, 1980) have the ability to establish experimental infection in laboratory rabbits. This report provides evidence that BHV-4 also can be studied, in its acute and persistent infection, in a rabbit model. This will constitute a feasible tool to characterize the lymphoid association of BHV-4.

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SUMMARY AND CONCLUSIONS

This work involved investigation of the response of cattle and rabbits experimentally infected with a strain of BHV-4 isolated from a case of mammary pustular dermatitis.

After infection of cattle by several routes, no clinical signs or lesions were evident, except for a dermal lesion corresponding with one intradermal inoculation point. Seroconversions were detected by the Indirect Fluorescent Antibody Test. Virus was recovered from the dermal lesion and was excreted in milk for 17 days. Virus also was recovered sporadically from esophagus-pharyngeal fluid. At different times of slaughtering (2 to 14 months postinoculation), virus was recovered from cocultures with bovine lung cells and/or explant cultures of lymph organs. A persistent viremia was detected and studied in 2 of the cattle. The virus was recovered repeatedly throughout one year from peripheral blood leukocytes by cocultivation with bovine lung cells. The number of infectious leukocytes, as determined by Infectious Centre Assay, ranged from less than 1 to 6 infectious cells per 10^7 leukocytes.

Inoculation of rabbits by various routes resulted in seroconversions (Indirect Fluorescent Antibody Tests). After intravaginal and conjunctival inoculations, vulvovaginitis and conjunctivitis were evident and virus was shed.

The antibody titers persisted throughout the experiment. Treatment with dexamethasone, beyond the acute infection, did not produce recrudescence of disease or virus shedding. Rabbits were killed at various times from three to six months post-infection and the virus was recovered from explant cultures of spleen and by co-cultivation of spleen cells with bovine lung cells.

The following conclusions may be made:

1. No evidence for a definite role of BHV-4 in clinical disease of cattle can be raised from this work.

2. Bovine herpesvirus-4 is capable of establishing a persistent infection after experimental inoculation of cattle and rabbits.

3. This persistent infection is associated with lymphoid tissue in both species. In cattle there is, concomitantly, a persistent viremia associated with the mononuclear cell fraction of the blood.

4. Integrity of the lymphoid cell and long in vitro incubation appear to be requisites for recovery of infectious BHV-4. A quiescent or latent state of the virus within the lymphoid cell is possible.

5. The association of BHV-4 with lymphoid organs and the MNC fraction of blood could explain the great diversity of tissues (and syndromes) from which BHV-4 has been recovered.

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