# A STUDY OF THE BIOLOGICAL PROPERTIES OF MALIGNANT CATARRHAL FEVER VIRUS (ALCELAPHINE HERPESVIRUS-1) AND DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DETECTION OF ANTIBODIES TO ALCELAPHINE HERPESVIRUS-1 IN DOMESTIC AND EXOTIC RUMINANTS

By

SIAO-KUN WAN

Diploma
Taiwan Provincial Pingtung Institute of Agriculture
Pingtung, Taiwan
1975

Master of Science
Oklahoma State University
Stillwater, Oklahoma
1982

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
December, 1986

A STUDY OF THE BIOLOGICAL PROPERTIES OF MALIGNANT CATARRHAL FEVER VIRUS (ALCELAPHINE HERPESVIRUS-1) AND DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DETECTION OF ANTIBODIES TO ALCELAPHINE HERPESVIRUS-1 IN DOMESTIC AND EXOTIC RUMINANTS

Thesis Approved:

Anthony Castro

Thesis Adviser

Anthony Lopez

Charlotte L. Oaks

Robert A. Shelton

Margaret Essenberg

Norman N. Durham

Dean of the Graduate College

## ACKNOWLEDGMENTS

I thank the Zoologic Society of Oklahoma and the former Director, Mr. L. Curtis and the present Director, Mr. S. Wylie, of the Oklahoma City Zoo, for the financial support provided over a two year period for my investigations on malignant catarrhal fever (MCF). I also thank Dr. Dan Goodwin, Director of the Oklahoma Animal Disease Diagnostic Laboratory (OADDL), Stillwater, Oklahoma, for the laboratory materials, space and animals provided during the period of this study.

I express sincere appreciation to Dr. A. E. Castro, my major professor, for his diligent guidance during the period of my research. He not only served as an academic adviser, but also provided scholarly advice during personal crises. His patience and daily encouragement were instrumental in my success in completing this graduate program.

To the members of my committee, Dr. A. W. Confer, Dr. M. K. Essenberg, Dr. R. W. Fulton, and Dr. C. L. Ownby, a sincere gratitude is extended for their patience, advice, encouragement, and time spent to develop my research goals. I thank especially Dr. Essenberg for her sage counsel in the area of biochemistry.

I acknowledge gratefully Dr. W. P. Heuschele, Research Director of the San Diego Zoo (SDZ), for providing a major portion of the test sera from exotic ruminants and comparative serologic data. I also thank the research staff at the SDZ for their assistance during my visit to their laboratory.

My appreciation is also extended to Dr. Ed Carbary, Director of National Animal Diagnostic Center (NADC, Ames, Iowa), Dr. Gene Erickson, Head of the Bovine and Porcine Section, and Dr. C. Metz of NADC for their valuable comments and assistance, and providing sera, the WC-11 strain of MCF virus and bovine turbinate cells. I also thank the personnel at the diagnostic virology laboratory at NADC for their technical assistance during my visit. I thank Dr. James A. House of Plum Island Animal Disease Center, Greenport, NY, for the data provided on the transmission of MCF virus (greater kudu isolate) in cattle.

I thank Dr. J. Storz, Head of the Department of Veterinary Microbiology and Parasitology, Louisiana State University and Dr. J. C. DeMartini, Department of Pathology, Colorado State University, for the sera provided from cattle from outbreaks of MCF, and for their valuable comments and discussions.

I especially wish to express my gratitude to Dr. Ed Ramsay of the Oklahoma City Zoo for his encouragement and initiative in seeking the research funds and providing me with his vast knowledge on zoo species, and to Dr. M. Burton, Ms. B. M. Baumeister and other zoo staff for their invaluable help.

Of the many individuals who have directly and indirectly contributed their time and assistance, I wish to express a personal thanks to the staff of the OADDL and the professors and staff of the Department of Parasitology, Microbiology and Public Health. I thank Dr. D. L. Whitenack and Dr. B. Johnson of the OADDL for the necropsy and the histopathology data, and Mrs. D. Rex and Mrs. J. Pennington of the Electron microscopy (EM) laboratory in the Department of Physiology at Oklahoma State University for the preparation of viral specimens for EM.

I also thank Mrs. M. Castro for providing me with the comfortable accouterments of living during my completion of this thesis at the University of California at Davis.

A final thanks is given to my parents, Mr. and Mrs. F. P. Wan, my brother, Mr. Y. E. Wan, and sister, Ms. S. C. Wan, for their continued moral support and understanding.

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

LIST OF FIGURES

# CHAPTER I

# LITERATURE REVIEW

## Introduction

Malignant catarrhal fever (MCF) is a usually fatal disease of domestic cattle and also of a wide variety of captive and free-living ungulates (32, 36, 82). The clinical syndrome of MCF is characterized by a prolonged high fever, oral, nasal and ocular discharges, bilateral corneal opacity, photophobia, generalized lymph nodes enlargements and inflammation of the upper respiratory and digestive tracts (36, 48). According to its clinical manifestation, MCF has been differentiated by Gotze into four clinical forms: peracute, head and eye, alimentary and mild forms but there is considerable overlap of the clinical syndromes observed (18). The characteristic pathologic lesions of the four forms of MCF are a vasculitis with a lymphoid proliferation and an associated mononuclear infiltrate (36, 48).

The distribution of MCF is world-wide and most reports indicate a low morbidity and high mortality in affected species (84). Two forms of the disease have been described: namely, "wildebeest-associated" MCF which is termed "African" MCF and later called alcelaphine herpesvirus type 1 by Mushi (64) and "sheep-associated" MCF also known as "European or American" MCF (64). The classification of MCF has usually been based on the suspected source of the MCF infection.

Nevertheless, the clinical signs and histopathological lesions of both forms are indistinguishable.

For centuries, the Masai herdsmen of Africa had observed a connection between herds of wildebeest and a respiratory disease in cattle, later called "snotsiekte" by the Boers of South Africa (18). The disease was most prevalent in cattle during their association with female wildebeest in parturition. Masai herdsmen believed that cattle acquired the disease by the ingestion of placentas or hairs from neonate wildebeest. By the inoculation of whole blood from a clinically healthy black wildebeest (Connochaetes gnou) into cattle, Mettan was able to reproduce MCF (18). This experiment established the role of African wildebeest as the carriers of the infectious agent. In 1960 in Africa, following the discovery and introduction of methods to propagate living cells in culture (23), a highly cell-associated herpesvirus of MCF was isolated from the blood of a blue wildebeest (Gorgon taurinus taurinus) by Plowright et al. (84). Subsequently, MCF was reproduced by inoculation of this virus into cattle (84).

Although the majority of adult wildebeest possess virus neutralizing (VN) antibody throughout their lives indicating exposure to the MCF virus, MCF is rarely seen in wildebeest (79, 80). Virus neutralizing antibody has also been found in hartebeest (Alcelaphus buselaphus Cokei Pallas) and topi (Damaliscus korrigum Ogliby) (91) which are also members of the subfamily of Alcelaphinae, and oryx (Oryx beisa callotis) (60, 91) a member of the subfamily Hippotraginae. Herpesviruses which have been isolated from both hartebeest (90) and topi (69) have been found to be serologically related to the herpesvirus of MCF from

wildebeest. However, these herpesviruses from hartebeest and topi have not consistently produced MCF in cattle (69, 90).

In the United States prior to 1979, MCF had been described in a greater kudu (Tragelaphus strepsiceros) at the St. Louis Zoo (4), an Indian gaur (Bos gaurus), a barasingha deer (Cervus duvaucelis) and 4 Javan banteng (Bos javanicus) in the San Diego Wild Animal Park (SDWAP) (31), and a bison (Bison bison) from South Dakota (113). At the Oklahoma City (OKC) Zoo in 1979, serologically identical herpesviruses were isolated from clinical cases of MCF in an Indian gaur and a greater kudu (9). This outbreak of MCF coincided with the calving period of a captive herd of wildebeest (Connochaetes gnou) housed at the zoo (9). Subsequently, a serologically related herpesvirus was also isolated from a neonate and a healthy 12-day old calf in the wildebeest herd (12). Following the report of MCF from the OKC zoo, herpesviruses were also isolated from several exotic ruminant species located in the SDWAP which were shown to be serologically related to the alcelaphine herpesvirus of MCF (32, 33, 38).

In Europe, MCF was first described in 1798 in cattle by Colson (76). However, most European or North American clinical cases of MCF in cattle or deer were associated with contact with particular flocks of healthy sheep during their lambing season (3, 28, 57, 59, 77, 95). This sheep-associated form of MCF has been reported in cattle or deer in England (44, 93), the United States (2, 28, 56, 77, 95), Canada (3, 59), Finland (119), New Zealand (43, 46), and Indonesia (41). Outbreaks of the sheep-associated form of MCF in California (70), Colorado (77), Minnesota (29), and Arizona (70) have led to numerous losses in domestic herds of cattle. This form of MCF also has been associated with death

in banteng (Bos sondaicus) and zebu (Bos indicus) cattle (87), Pere David's deer (Elaphurus davidianus) (44, 117), red deer (Cervus elephus) (93), white-tailed deer (Odocoileus virginianus) (14, 133), axis deer (Axis axis) (14), and Indonesian swamp buffalo (Bubalus bubalis) (41). In all cases of sheep-associated MCF, the putative diagnosis was based on the clinical signs, histopathological lesions and a history of contact with lambing sheep. However, despite the increasing number of reports the etiologic agent of sheep-associated MCF has yet to be isolated or identified.

Since 1979, several virulent strains of MCF virus have been isolated from certain captive exotic ruminants at the OKC zoo (9, 12) and San Diego Zoo (32, 33, 35, 38). But because MCF has been considered a foreign animal disease in the United States, a paucity of research on MCF has been done in this country. The restrictive handling of MCF virus in the United States has negated a thorough investigation as to the potential threat of MCF to the domestic livestock industry.

To minimize the potential losses of endangered exotic ruminants in zoological parks and domestic cattle and deer, reliable virus isolation and serologic procedures for the diagnosis of MCF required investigation. Thus, the purpose of this research was: (a) to study certain biologic properties of specific isolates of alcelaphine herpesvirus; (b) to develop an enzyme-linked immunosorbent assay (ELISA) which could be used in the detection of antibody specific for alcelaphine herpesvirus; (c) to evaluate the sensitivity and specificity of the ELISA as compared to other serologic assays for the determination of MCF antibody in domestic and exotic ruminants; (d) to determine by serology the prevalence of antibody to MCF virus in domestic and exotic ruminants;

and (e) to study the clinical and immunological response of lambs inoculated with the virulent alcelaphine herpesvirus.

## Species Susceptible for MCF

Malignant catarrhal fever is a disease primarily of ruminants, and certain exotic, wild and domestic ruminant species are highly susceptible to MCF (32). In most susceptible species, MCF has a high mortality but a low morbidity (84). In the past 40 years, most of the reported cases of MCF have been based on histopathological observations. Species in which MCF has been documented included cattle (Bos taurus) (2, 3, 11, 29, 46, 54, 56, 57, 59, 76, 118, 123, 125), white-tailed deer (Odocoileus virginianus) (127, 133), farmed Rusa deer (Cervus timorensis) (20), Indonesian swamp buffalo (Bubalus bubalis) (41), bison (Bison bison) (53, 113), Pere David's deer (Elaphurus davidianus) (44, 117), Shira's moose (Alces alces shirasi) (130), sika deer (Cervus nippon) (131), red deer (Cervus elaphus) (93). Heuschele (32) has reported that the following wild ungulate species in captivity or free living are susceptible to MCF: Arabian oryx (Oryx leucoryx), axis deer (Cervus axis), banteng (Bos Sondaicus, Bos javanicus), barasingha deer (Cervus duavauceli), Blesbok (Damaliscus forcas), blackbuck (Antilopo cervicapra), bongo (Taurotragus eurycerus isaci), bushbuck (Tragelaphus scriptus), Cape buffalo (Syncerus caffer), Congo buffalo (Syncerus caffer nanus), duiker (species not indicated), Eld's deer (Cervus eldi thamin), European bison (Bison bonasus), greater kudu (Tragelaphus stresiceros), Indian gaur (Bos gaurus), lechwe (Kobus lechwe), lama (Liama quanicoe glama), mule deer (Odocoileus hemionus), nilgai (Boselaphus trageocamelus), Sambar deer (Cervus mariannus), sitatunga (Trangelaphus

spekii), slender-horned gazelle (Gazella leptoceros), water buffalo (Bubalus babalis), and zulu suni (Nesotragus moschatus zuleunsis).

Although reports of clinical MCF in wildebeest have appeared, this species in addition to the hartebeest and topi rarely demonstrate clinical disease (69, 79, 90). However, alcelaphine herpesvirus-1 or a serological similar herpesvirus has been isolated from each of these 3 species (69, 84, 90). Sheep and goats also appear to be carriers of a form of alcelaphine herpesvirus, as determined by serology (100); however, clinical signs of MCF in these species have not been observed (75, 119).

In laboratory animals, rabbits have been experimentally inoculated with blood or tissues collected from MCF-infected cattle or deer and have developed a fatal MCF infection (5, 126). Furthermore, the infectivity and virulence of the herpesvirus has been retained after several passages in rabbits (5, 126). Rabbits also have been an excellent animal model for the study of the pathogenesis of sheep-associated MCF (6, 21).

Attempts at the reproduction in other laboratory animals of MCF by the inoculation of guinea pigs, unweaned white mice and hamsters have been uniformly negative (40, 49, 126). The use of embryonated chicken and duck eggs for the propagation of alcelaphine herpesvirus-1 has also been unsuccessful (49).

## Wildebeest-Associated Malignant Catarrhal Fever

### Etilogy of MCF

The first viral isolate of MCF was characterized as a herpesvirus

by its morphology and size as determined by electron microscopy (EM) and the type of cytopathic effects (CPE) the virus produced in host cells (84, 85). The virus of MCF was found in its early passages in cell cultures to be highly cell-associated (85). The CPE observed in cell cultures infected with MCF virus were characterized by the formation of syncytia or multinucleated cells and eosinophilic intranuclear inclusion bodies (84, 85). The virus of MCF closely resembled the herpesviruses of varicella-zoster, cytomegalovirus and Marek's disease based on cytopathologic changes in infected cells (83). The serial passage of cell cultures infected by MCF virus usually resulted in the production of cell-free virus with a CPE characterized by rounded, refractile cells and the simultaneous loss of syncytia between cell culture passages 7 and 30 (85).

The herpesviruses isolated from each of two captive neonate wildebeest from the OKC Zoo (12, 13) produced clinical MCF which was fatal when each virus was inoculated into a white-tailed deer (12, 13). A herpesvirus was then reisolated from the buffy coat cells from each of the inoculated deer (12, 13). The viral isolates from each white-tailed deer were identified as the alcelaphine herpesvirus of MCF following their inoculation into cell cultures by a positive nuclear immunofluorescence using a reference serum to MCF virus, by EM (12) and by the typical vascular lesions of MCF observed in the tissues of the infected deer (12, 13).

Previously, the same investigators (9, 11) in 1980 had reported on the isolation of MCF virus from clinically ill exotic ruminants in captivity at the OKC Zoo. Herpesviruses were isolated from an adult Indian gaur and a young greater kudu which had clinical signs of MCF.

The diagnosis of MCF in each species was also confirmed by histopathologic lesions typical of MCF (134). Each herpesviral isolate produced clinical MCF in both domestic cattle[1] and white-tailed deer (11, 127). The fulfillment of "Koch's postulates" was done by the re-isolation of a herpesvirus from the tissues of each experimentally inoculated animal and the identification of each viral isolates as an alcelaphine herpesvirus by EM and serology (7, 8, 10).

Recently, herpesviruses have been isolated from clinical cases of MCF in a nilgai (Boselaphus tragocamelus), two Formosan sika deer (Cervus nippon), an axis deer (Cervus axis), a scimitar-horned oryx (Oryx gazella dammah), a Jimela Topi (Damaliscus lunatus jimela), a Cape hartebeest (Alcelaphus buselaphus caama) and an asymptomatic blue wildebeest (Connochaetes taurinus albojubatus) which were in captivity at the SDWAP (32, 34, 38). These viral isolates were identified as herpesviruses by their size and morphology on EM and their CPE in cell cultures (10). Each viral isolate was related serologically to the alcelaphine herpesvirus as determined by indirect immunofluorescence (IIF) and neutralization of virus (37) with antiserum to WC-11 strain of alcelaphine herpesvirus isolated by Plowright et al. (85). Although the wildebeest isolate appeared to have been involved in a subsequent outbreak of MCF in the zoo nursery at the SDWAP (10, 32), the virulence of the other herpesviral isolates from ruminants at the SDWAP for susceptible ruminants has yet to be determined.

---

[1]Dr. James A. House, Plum Island Animal Disease Center, Greenport, NY, 1985, personal communication.

## Classification of MCF Virus

The virus of MCF was initially grouped within the herpesvirus of bovidae (58). Subsequently, MCF virus was classified as bovid herpesvirus type 3 by a nomenclature committee of the World Health Organization (64). Ludwig (55) has also classified MCF virus as bovid herpesvirus 3 based on the clinical disease produced. Reid et al. (91) proposed that MCF virus should be designated as "alcelaphine herpesvirus" because antibody to MCF virus was frequently detected in 3 species in the subfamily Alcelaphinae (i.e., wildebeest, hartebeest and topi) and only in the oryx species of the subfamily Hippotraginae. Plowright (82) suggested that since both subfamilies belong to the Hippotraginae family, the MCF virus should be called "Hippotragine herpesvirus". Mushi and Rurangirwa (64) reported that the alcelaphine ungulates are the natural hosts of MCF virus, therefore, MCF virus should be classified as "alcelaphine herpesvirus type 1". In 1985, at a conference on MCF held in the United States, researchers on MCF from the United States and England agreed that the wildebeest-associated MCF virus should be referred to as "alcelaphine herpesvirus-1" in their publications (8). Therefore, in an attempt at standardization of the nomenclature of MCF virus each isolate of MCF virus in each chapter of this thesis will be referred to as alcelaphine herpesvirus-1.

By restriction-enzyme analysis of the extracted DNA from isolates of alcelaphine herpesvirus-1, the virus of MCF (Oklahoma and WC-11 strains) were separated from other isolates of bovine herpesviruses which are classified within the bovid herpesvirus group IV (71). In a

comparison of restriction endonuclease digests, DNA fragments of WC-11 strain which is considered the prototype virus of MCF migrated similarly to those from the herpesviral isolate from an Indian gaur (Oklahoma strain) (71). However, the DNA digests of both alcelaphine herpesviruses were sufficiently different in comparison to the migration patterns of the DNAs of other bovine herpesviruses (71). Although a high degree of DNA homology exists between the Oklahoma and WC-11 strains of alcelaphine herpesvirus-1, minor diversities were found. These diversities may be a reflection of a host-dependent modification of the virus.[2]

Morphology and Size of MCF Virus

The morphologic appearance of the virion of the alcelaphine herpesvirus-1 in ultrathin stained sections of bovine fetal kidney (BFK) cells (7, 10, 85) is indistinguishable from other herpesviruses (55, 98). Four major structural components have been observed by EM on the WC-11 strain in BFK cells (85) and several other isolates of alcelaphine herpesvirus-1 in BFK cells (7, 9, 13) or fetal aoudad (Ammotragus lervia) kidney (FAK) cells (10). In each EM study, the viral particles had a dense central core which contained the viral DNA woven around a cylindrical "toroid-shape" structure of possible protein composition (7, 10). Hexagonal or icosahedral nucleocapsids appeared empty or complete with DNA and such virions appeared to be released from "ring-like" structures located in the nucleus of the infected cell (10). The viral envelope was shown to be acquired by the budding of virions through either the nuclear, inner cellular or plasma membranes

---

[2]Dr. Werner P. Heuschele, San Diego Zoo, CA, 1985, personal communication.

(7). In one ultrastructural study (7), an electron translucent area typical of the herpesvirus tegument previously described by Roizman (96) was seen between the nucleocapsid and envelope of the alcelaphine herpesvirus-1. Minute projections (peplomers) on the surface of the envelope have been reported for other herpesviruses (98); however, peplomers were not observed ultrastructurally on alcelaphine herpesvirus (10).

Castro et al. (7) reported that immature intranuclear virions seen in the nuclei of BFK cells infected with alcelaphine herpesvirus-1 ranged in size from 109 to 120 nm, immature intracytoplasmic virions measured 128 nm, and the enveloped viral particles ranged in size between 141 and 194 nm. Earlier, Plowright et al. (85) had described two morphologic forms of the virus as seen in ultrathin sections of BFK cells infected with WC-11 strain of alcelaphine herpesvirus-1. One viral form consisted of naked (single-ringed) nucleocapsids which were 90 nm in diameter while the enveloped (double-ringed) particles seen measured 130 nm in diameter (85). The discrepancies in the size of the virions described in each report (7, 85) may be a reflection of the different procedures employed for the fixation and staining for EM. Nevertheless, the morphology and size range of those strains of alcelaphine herpesvirus-1 studied were in agreement with morphologic features described for other herpesviruses (97).

In preparations of virus examined by EM (85) at the 49th passage in BFK cells which were stained with phosphotungstic acid or negative staining, enveloped cell-free WC-11 virus had a size range of 140 to 220 nm. Plowright et al. (85) also described the nucleocapsid of the

alcelaphine herpesvirus-1 as composed of 162 capsomeric subunits which were 19.5 nm in diameter and 12.5 nm in length and appeared hollow and elongated.

Cytopathology of MCF Virus in Cell Cultures

The characteristic CPE of alcelaphine herpesvirus-1 in bovine thyroid or BFK cells is the formation of macro or microsyncytia, a multinucleated cell (9, 12, 13, 30, 33, 84, 85, 128). The syncytia are found usually within 6 to 8 days following the infection of cells by virus or by co-cultivation of intact virus-infected cells with susceptible cells (85). The syncytia formed by alcelaphine herpesvirus-1 were described as finite with some recruitment of surrounding cells which eventually contracted into a rounded, highly refractile "balloon-like" appearance (33). These refractile masses after formation usually detached from the cellular monolayers within 12 to 14 hours leaving a cell-free areas which occasionally contained cell debris (9). In instances where the viral CPE were not progressive the healthy surrounding cells multiplied and replaced the empty "plaque-like" areas. However, with most isolates of alcelaphine herpesvirus-1, syncytia usually reappeared in adjacent areas or when the cell monolayer was subcultured (9). The size and number of viral-induced syncytia usually varied from one subculture to another because the input of cell-associated virus was dependent on the number of virus-infected cells at each cell transfer (13, 33, 85). Because of the slow development of syncytia in virus-infected cells and the absence of cell-free virus in the supernatant fluids, virus-infected cells were shown to undergo several passages by co-cultivating with fresh uninfected cells at each passage (11). However, in one cell culture study with

alcelaphine herpesvirus-1, viral CPE was not present throughout 16 cell culture passages (9).

The early passages of 4 Oklahoma isolates of alcelaphine herpesvirus-1 were characterized by a lack of cell-free virus and the formation of syncytia (9, 13). In other studies (33, 128) after extensive cell culture passages, the virus produced a CPE which contained rounded, refractile cells and no syncytia. Syncytia in virus-infected cells appeared to be temperature-dependent because viral-induced syncytia were seen at 37 C (12, 30), but not at 32 to 34 C since only foci of "balloon-like" rounded cells were described in bovine thyroid cells (30) and FAK cells infected with alcelaphine herpesvirus-1 (33). In 2 separate studies (30, 33), investigators have reported that at incubation temperatures of 32 to 34 C enhancement of the production of cell-free virus occurred. Hence, the type of viral CPE observed may be an indicator of the presence of cell-free virus (30, 33).

If the monolayers of alcelaphine herpesvirus-1 infected cells which contained syncytia were stained with hematoxylin and eosin, trichrome or May-Grünwald-Giemsa stains, intranuclear inclusion bodies characterized as "Cowdry type A" were observed (11, 13, 30, 85).

## Replication of MCF Virus in Cell Cultures

The replicative cycle of most isolates of alcelaphine herpesvirus-1 has not been thoroughly studied because of the highly cell-associated nature of these viral isolates and the slow development of CPE. The adaptation of the WC-11 isolate of MCF to a continuous cell line and the production of cell-free virus has enabled investigators to study certain molecular events in the replication of the virus (85). In

studies on the replication of alcelaphine herpesvirus-1, the morphogenesis of the virus has been deduced from ultrastructural observations using EM (10, 85).

By EM studies, the method of viral attachment, penetration, and uncoating of MCF virus have not been revealed by EM (10, 85). Investigations with herpes simplex virus have shown that the entry of herpesvirions into cells is a consequence of "viropexis" or cell engulfment of particles which eventually become entrapped in cytoplasmic vesicles (24, 42). The fate of each herpesviral particle in the cytoplasm of the cell is unknown; however, a digestive process by cellular enzymes is involved in the uncoating process of viral particles in vesicles (85). Since viral DNA and the nucleocapsids of herpesviruses are synthesized in the nucleus (96), the de novo synthesis of alcelaphine herpesvirions was described by the appearance in the nucleus (10) of 43 nm viral cores, which were located within concentric dark-staining rings. Evidence presented in this EM study (10) indicated that the assembly of viral nucleocapsids occurred within these same rings and immature virions appeared as single particles or occurred in aggregates. Occasionally, the nucleocapsids appeared to bud into vesicles located within the nucleus (10).

The principal site for the envelopment of the nucleocapsids of herpesviruses has been found to be the inner lamella of the nuclear membrane (17, 96). In an EM study on alcelaphine herpesvirions, viral nucleocapsids were seen pinching off the nuclear membrane or budding into the perinuclear cisternae thus acquiring an envelope (10). The extensive de novo synthesis of portions of the nuclear membrane as determined by incorporation of a radio-label in cells in tested with

pseudorabies virus and seen by EM as thicken areas have demonstrated the nuclear membrane as the principal site for viral budding (1). The further acquisition of envelopes by alcelaphine herpesvirions appeared to occur by the budding through cytoplasmic and plasma membranes (10). Unenveloped alcelaphine herpesvirions have been seen by EM in the cytoplasm of infected cells and their presence in the cytoplasm of the cell has been suggested to occur by the rupture of the nuclear membrane (10). The unenveloped nucleocapsids of alcelaphine herpesvirus-1 were described as acquiring envelopes by budding through such cytoplasmic structures as the Golgi apparatus, vesicles, and membranes of the endoplasmic reticulum (10). The passage of virions through the cytoplasm appeared to occur by the movement of virions through channels formed by the Golgi apparatus or the endoplasmic reticulum (10). The release of alcelaphine herpesvirions from the cell occurred by budding through the plasma membrane or by the rupture of the cell membrane. A method for the morphogenesis of the alcelaphine herpesvirus-1 in infected bovine cells has been proposed (10) which has morphogenic features similar to those proposed for cytomegalovirus (122) and herpes simplex virus (98).

Three fluorescent stained viral antigens have been described for alcelaphine herpesvirus-1 in infected BFK cells (105, 107). Cytosine arabinoside is an inhibitor for the production of late antigens which are essential for the maturation of herpesvirions (107). By adding the cytosine arabinoside into alcelaphine herpesvirus-infected BFK cells, Rossiter et al. (107) observed 2 types of immunofluorescent early antigens (IIF(E)); one was diffuse and distributed throughout the cells, the other was particulate and intranuclear. Acute and hyperimmune sera obtained from rabbits and cattle experimentally

infected with alcelaphine herpesvirus-1 reacted with particulate IIF(E), whereas only hyperimmune sera reacted with diffuse IIF(E) (107). Immunofluorescent late antigens (IIF(L)) appeared as particulate or diffuse were found in cytosine arabinoside untreated but alcelaphine herpesvirus-1 infected BFK cells (105).

Stability of Alcelaphine Herpesvirus-1

The infectivity of the alcelaphine herpesvirus-1 is destroyed rapidly at a frozen storage temperature of -20 C and also by freeze-drying (81). The infectivity of cell-associated alcelaphine herpesvirus-1 appears associated with the viability of the host cells because viral infectivity was destroyed by ultrasonic treatment of infected cells (81). Cell-associated alcelaphine herpesvirus, however, retained its infectivity when stored at -70 C as intact cells in medium supplemented with 10 to 20% fetal bovine serum (FBS) and 5 to 15% of a cryopreservative (e.g., glycerol or DMSO) (11, 49, 81). Cell-free virus at passage level > 10 can be stored for a month at 4 C for 30 days with a minimal loss of viral infectivity (49). Monolayers of BFK cells infected with alcelaphine herpesvirus-1 at passage level of 2 were also stored at 4 C for 30 days with a minimal loss in viral infectivity (11).

The addition of chloroform or ether to cell-free alcelaphine herpesvirus-1 will destroy totally the infectivity of the virus (85). When the virus was treated with 5-iodo-2' deoxyuridine (IUDR) or 5-fluoro-deoxyuridine (FUDR), virus infectivity was also lost (85).

To maintain virus viability during the isolation of alcelaphine herpesvirus-1 from tissues, tissue specimens were kept at 4 C for less

than 24 hours prior to virus isolation (81). The infectivity of alcelaphine herpesvirus-1 in blood has been shown to be associated with the cells of the buffy coat or specifically the lymphocytes. Furthermore, Plowright (79) found that the half-life of the infectivity of virus was 0.83 days when an anticoagulent like EDTA was added to the blood and 0.67 days when heparin was used. Mushi and Wafula (65) reported that cell-free virus in nasal and ocular secretions which were stored at -70 C retained their infectivity over 24-month period. Because of the fragility of the cell-free enveloped virus to extremes in temperatures, retention of viral infectivity outside the host at 32 C or freezing temperatures remains highly unlikely. Thus, this natural temperature barrier has been postulated as a factor for the absence of documented horizontal transmission of virus in the field (81).

## Shedding of Alcelaphine Herpesvirus-1

The shedding of cell-free virus in nasal and ocular secretions in neonates and wildebeest calves was first described by Mushi et al. (66, 67, 68). Explant cultures of cornea and nasal turbinates from wildebeest calves less than 3 months of age also contained and released infectious alcelaphine herpesvirus-1 (67). When the immunoglobulin G class of VN antibody began to appear in the nasal secretions of young wildebeest over 3 months old, shedding of infectious virus from nasal secretions was diminished (67, 111). It appears that during this brief period of shedding of cell-free virus by wildebeest neonates that infection of susceptible species is at its greatest risk.

Although infectious virus was demonstrated in nasal secretions from wildebeest calves, alcelaphine herpesvirus-1 was not isolated

from tissue specimens from such calves and only 2 of 11 calves were found to be viremic (68). In another study, Plowright (79) reported that the infection rate in wildebeest calves was 31%. Castro et al. (12) were unable to isolate the alcelaphine herpesvirus-1 from the nasal secretions of a newborn wildebeest, but infectious virus was isolated from its tissues. Nevertheless, viremia is rare in wildebeest over 3 months of age or when demonstrable VN antibody appears in the sera (67, 111). Thus, the cessation of secretion of cell-free infectious virus by wildebeest calves coincided with the presence of VN antibody in their sera.

In adult blue wildebeest, cell-associated MCF virus had been isolated from nasal secretions following the exposure to stress factors (e.g., by changing environment, feed, or by injection of betamethasone) (115). Cell-associated virus has been isolated also from the nasal secretions of a pregnant wildebeest (115). A viremia was not seen among those wildebeest injected with betamethasone, although in wildebeest Plowright (79) has demonstrated viremia during late pregnancy. This finding suggests that the stress induced by a pregnancy may reactivate latent virus present in wildebeest.

The virus of MCF has not been isolated from the urine (66, 79) or saliva (66) of wildebeest calves which were viremic, although Heuschele (39) reported the isolation of MCF virus from the feces of a sick young Formosan sika deer. In contrast, the bovine cytomegaloviruses (herpesvirus) are usually found in both saliva and urine (132).

The most successful method for the isolation of virus from MCF-affected ruminants is from buffy coat cells obtained by the peak temperature during the febrile period. In contrast to the MCF

carrier species, ruminant species including domestic cattle when infected by alcelaphine herpesvirus-1 rarely shed cell-free virus in their secretions, although the whole blood is highly infectious when inoculated into a susceptible ruminant (49, 79). Nevertheless, Kalunda (49) reported on the isolation of the alcelaphine herpesvirus-1 from 32 to 53 (60%) nasal secretions and 8 of 10 saliva samples obtained from viremic cattle which were experimentally infected by intranasal inoculation of the virulent virus. A herpesvirus has also been recovered from nasal and ocular secretions from a Formosan sika deer which had recovered previously from clinical MCF then given dexamethasone (0.1 mg/kg body weight) treatment daily for 6 consecutive days (39).

The alcelaphine herpesvirus-1 is similar to other herpesviruses which can establish a persistent infection in their hosts (58, 64). In a study of MCF in cattle (83), a cow was experimentally infected with alcelaphine herpesvirus-1 and subsequently the cow developed a viremia at 9 to 16 days post infection but the cow had no overt clinical signs of MCF. Furthermore, the cow resisted a challenge by virulent alcelaphine herpesvirus-1 which was given at 13 weeks post-infection and did not subsequently develop a viremia following inoculation of virulent virus. This cow became persistently-infected but gave birth to 6 calves, each of which developed clinical MCF. Infectious alcelaphine herpesvirus-1 was subsequently identified in cultured thyroid cells from 1 calf and isolated from the whole blood of another calf. The cow never demonstrated a viremia during each calving and alcelaphine herpesvirus-1 could not be isolated from its tissues (83). In another investigation (90), two steers were inoculated with a cell-free attenuated isolate of alcelaphine

herpesvirus from a hartebeest and each steer survived after a challenge with virulent alcelaphine herpesvirus-1. A viremia was subsequently detected in both steers. Three months following the challenge, the "immune" steer was introduced into a herd of wildebeest and the animal subsequently developed a persistent viremia which lasted for 5 weeks prior to death (114). This preceding study with steers suggested that the antibody or cell-mediated immunity raised against the alcelaphine herpesvirus-1 was either of a transient nature or that cross-protection was not complete for different isolates of alcelaphine herpesvirus-1.

Transmission of Virus

The mode of transmission of MCF in nature is yet to be defined. Rossiter et al. (104) reported on the futile attempts to isolate alcelaphine herpesvirus-1 from wildebeest fetal membranes and fluids. These workers (104) collected specimens from wildebeest located in various areas in East Africa and were unable to isolate alcelaphine herpesvirus-1 from any fresh or aged specimen. The negative viral isolation results by Rossiter et al. (104) did not exclude the presence of virus in the specimens, because earlier investigations (79, 86) had indicated that a certain proportion of wildebeest are infected in utero with alcelaphine herpesvirus-1. Rossiter et al. (104) postulated that the difficulties in the isolation of infectious virus may have been due to: (1) the low concentration of infectious virus in the clinical material, or (b) since alcelaphine herpesvirus-1 has a tropism for lymphoid cells (72, 81), the virus may not replicate productively in the cells of the fetal membrane of the wildebeest.

Similar to the dissemination of infectious virus from the upper respiratory as by infections of herpes simplex virus, infectious bovine rhinotracheitis virus and feline herpesvirus, the probable mode of transmission for alcelaphine herpesvirus-1 may be through oral or nasal secretions (66). This was substantiated by the isolation of cell-free and cell-associated alcelaphine herpesvirus-1 from nasal secretions of young and adult wildebeest (67, 115). In a report from Kenya (115), a steer that was grazed in a paddock adjacent to breeding wildebeest did develop fatal MCF. The epidemiologic data indicated that alcelaphine herpesvirus was naturally transmitted by either virus contaminated aerosals or droplets shed by young or neonate wildebeest (49, 66, 67, 81, 115).

However, in other attempts at transmission into susceptible cattle by prolonged exposure to wildebeest which were actively shedding alcelaphine herpesvirions, none of the exposed cattle became ill (83). The absence of transmission of virus to cattle suggested that either a low concentration of cell-free virus was present in the secretions of the wildebeest or the virus shed was mostly cell-associated which is routinely non-infectious by an aerosol route. Although alcelaphine herpesvirus-1 had been reportedly isolated from crude nasal and ocular secretions from MCF-affected cattle (49), the presence of cell-free virus in the secretions was not determined. Experimentally, white-tailed deer exposed over 30-day period to MCF-infected deer in an isolation facility did not develop clinical disease and were subsequently fully susceptible to infection by alcelaphine herpesvirus (11). Contact transmission between MCF-infected cattle and healthy cattle is rare (49, 64), because cattle are a dead-end host for the alcelaphine herpesvirus-1.

## Immunity of Animals with MCF

Humoral Immunity. Cattle which had survived a natural infection of MCF later developed a solid immunity against the virulent alcelaphine herpesvirus-1 (81). This immunity was retained in most survivors for a year or longer without clinical evidence of a secondary infection. Piercy (74) postulated that the persistence of MCF infection in the host probably contributed in a general way to this prolonged protection. In a study by Plowright (81) on the development of antibody to alcelaphine herpesvirus-1, VN antibody was undetectable in cattle which survived an infection by virus until the 8th week after the onset of viremia. The VN antibody titer in the sera of cattle survivors of a MCF infection, however, was low (1:6), but a 10-fold increase in titer (1:60) occurred after a challenge dose of alcelaphine herpesvirus (81).

Experimentally, cattle artificially infected with virulent alcelaphine herpesvirus-1 developed antibodies which were detected by an IIF assay (106). The IIF titers increased rapidly over a 6 to 7 day period prior to pyrexia in the animal; whereas, measurable VN antibody was not detected in these cattle prior to their death (106). In a series of experiments, Rossiter (105, 108) measured the humoral response in African cattle infected with alcelaphine herpesvirus-1, and he found a similar delay in the rise of VN antibody. In this study (105) 16 cattle naturally infected with alcelaphine herpesvirus-1, antibodies against alcelaphine herpesvirus-1 were detected by complement fixation and IIF assays; however, minimal levels of VN antibody were detected in only 6 of 13 clinical cases of MCF. Most of the sera from cattle infected with sheep-associated MCF also contained antibody to

the IIF (L) antigens of alcelaphine herpesvirus-1; but similar to cattle infected with alcelaphine herpesvirus-1 these sera also lacked detectable VN antibody (101, 105).

By IIF assay rabbits experimentally inoculated with virulent alcelaphine herpesvirus-1 developed measurable IIF antibody to the virus at 5 to 6 days prior to the onset of pyrexia and antibody titers continuously increased until the death of the rabbits (106). The mean time to onset of pyrexia after inoculation was 15.6 days, the mean time to the death was 18.9 days. In these rabbits, VN antibody was not detected until the pyrexia period.

Mushi and Rurangirwa (64) reported that neonate wildebeest acquired a high level of colostral antibody to alcelaphine herpesvirus-1 from their dams but the colostral antibody did not prevent an infection by alcelaphine herpesvirus-1 since most wildebeest calves were viremic and did shed cell-free infectious virus until 3 months of age. Because of the widespread nature of alcelaphine herpesvirus-1 in herds of wildebeest, most wildebeest tested possessed VN antibody in their sera by 7 months of age (80). This VN antibody persisted at high levels throughout the adult life of the wildebeest (80, 108). In other African ruminant species, 60% of the sera tested from hartebeest were positive for VN antibody to alcelaphine herpesvirus-1 (91). Virus neutralizing antibody was also detected in the serum of young oryx (Oryx eisa callotis) in captivity in Africa (60).

Cell-Mediated Immunity. Antibody to alcelaphine herpesvirus-1 in susceptible ruminant species is not the sole defense against a fatal MCF infection, since low levels of VN antibody have been found

in just a few survivors (81). Similar to certain herpesviruses (47), the humoral immune response to an infection by alcelaphine herpesvirus-1 may not be a major factor involved in the protection from infection. Although a paucity of information is available on the role of cell-mediated immunity in MCF, investigations on cell-mediated immunity in rabbits inoculated with alcelaphine herpesvirus-1 have shown that the blastogenic transformation of lymphocytes in response to stimulation by a mitogen (PHA:phytohemagglutinin) which is used as a measure of lymphocytic activity was inhibited (129). This lymphocytic inhibition of blastogenic transformation by virus was nonetheless removed by several washes with a phosphate buffered solution. The mitogen-induced blastogenic transformation of unaffected lymphocytes was also inhibited, when acute serum from an MCF-affected rabbit was present. Based on their findings, these investigators (129) suggested that the inhibition of blastogenic transformation of the non-infected lymphocytes was a response to immunosuppressive factors in the acute MCF serum.

In contrast, Russell (112) was unable to demonstrate an immunosuppressive effect in acute sera of calves and rabbits experimentally infected with alcelaphine herpesvirus-1. Furthermore, in the same study the whole blood from calves infected with MCF virus had a high metabolic response following their stimulation by a mitogen (112). Hence, the findings obtained by these latter studies indicated that the inhibition of blastogenic transformation of lymphocytes was not a consequence of an immunosuppressive effect in acute MCF serum as previously described (129).

In another cell-mediated immunological study on MCF (110), a reduced antibody response to sheep red blood cells was demonstrated

when rabbits were inoculated simultaneously with MCF virus and sheep red blood cells. Based on the pathogenesis and immunological mechanism of MCF proposed by Edington et al. (22), Rurangirwa et al. (110) postulated that the dysfunction of the T-lymphocytes caused by alcelaphine herpesvirus-1 resulted in a reduced immune response to sheep red blood cells (110). At present, the role of alcelaphine herpesvirus-1 in the development of the cell-mediated immune response in an infected host remains unclear.

## Clinical Signs of MCF

Gross Lesions. In most ruminants, the clinical signs of MCF have been described as occurring in any of the following four forms: (a) the common "head and eye", (b) peracute, (c) intestinal, and (d) the mild forms as cited by Daubney et al. (18). Because of known differences in the virulence of isolates of alcelaphine herpesvirus-1, the duration of clinical MCF and the variation in susceptibility of individual animals, a combination of any of the 4 clinical forms may be observed in either natural or experimental infections. However, the rate of mortality of the "head and eye" form is usually greater than any of the other clinical forms (18).

The clinical syndrome of MCF has been described for several different ruminant species (2, 18, 20, 27, 41, 44, 46, 48, 51, 52, 53, 54, 59, 78, 113, 119, 123, 127, 130, 131, 133, 134). The most common clinical manifestations of MCF seen in cattle have been extensively summarized (36, 48), however, a review of the pathogenesis is herewith indicated. In an MCF-affected ruminant, a persistent high fever (above 40.5 C) is a prelude to the first signs of illness.

Serous bilateral nasal and ocular discharges occur first and subsequently become mucoid to mucopurulent. Scleral congestion may be observed in both eyes. Corneal opacity is a common lesion in MCF-affected species. This corneal opacity usually begins at the peripheral limbus and progresses centripetally with a resultant blindness. Erosions of the buccal papilla and nasal mucosa may lead to anorexia and dyspnea with an associated excessive lacrimation. Incoordination with convulsions and paresis occur in the latter stages of MCF and the superficial lymph nodes are usually enlarged and easily palpable. Constipation is common in the early stage of MCF; however, diarrhea can be seen prior to death.

In most clinical cases of MCF, the animal carcass is dehydrated and the hair coat is roughened. The lesions on the muzzle vary from a thickened and scaled appearance to a complete erosion of the epithelium. If these erosive areas are wiped, an irregular raw surface is revealed. The skin of the udder, teats, coronary bands, and interdigital spaces are usually reddened and cracked and have a crusty appearance. In cases of prolonged disease, the animals are routinely emaciated (95). Cutaneous edema is prominent around the light areas of the skin and the edematous lesion commences either at the base of the horns or around the loin, perineum or cervical areas. Numerous petechial hemorrhages with extensive erythema can be found on the tongue, buccal mucosa and nasal cavities. The erosions of the mucosa are usually covered with mucopurulent exudate. Multiple erosions or ulcerations with an associated diphtheric membrane are also present on the mucosa of the pharnyx and larnyx.

Histopathological Lesions. The pantropic nature of MCF is reflected by the consistency of lesions in each tissue examined

(48, 51). Significant differences in the histopathological descriptions of naturally occurring cases of MCF in cattle, deer and rabbits have not been found (2, 6, 22, 48). Similarly, a description of the histopathological lesions of MCF observed in experimentally inoculated cattle (2, 18, 27, 51, 52, 53, 54, 78, 134), white-tailed deer (14, 127) and rabbits (5, 6, 22, 126) have varied little from those described for natural-occurring cases of MCF.

The histopathologic changes described in most tissues involved a proliferation of connective tissue, endothelial and epithelial cells, with an associated infiltration of monocytes and lymphocytes into the lamina propria. The accumulation of an infiltrate of endothelial cells in the adventitial layer of blood vessels in most affected organs is routinely followed by a necrotizing fibrinoid vasculitis which appears to be typical for MCF.

Goss et al. (27) have reported the presence of cytoplasmic inclusions in the epithelial cells of mucous membranes from calves infected with MCF. Berkman et al. (2) reported that tissues from 73% of MCF-affected cattle when examined histologically had inclusion bodies in the cytoplasm of the neurons located in the vagoglosso-pharyngeal nucleus. In a recent report (39), "Cowdry type A" intra-nuclear inclusion bodies and multinucleated giant cells (syncytia) have been described in tissues from a clinically ill Formosan sika deer.

Pathogenesis. Although the histologic changes of MCF in tissues are nearly pathognomonic, the pathogenesis of the lesions of MCF still remain obscure.

A transformation of lymphoid cells as an initial virus-caused lesion based on histopathologic changes in tissues from MCF-affected cattle and rabbits has been postulated (22, 45). The lymphoid proliferation observed in various organs in MCF-affected tissues by Edington et al. (22) and Hunt and Billups (45) was similar to that described for the herpesviruses of Marek's disease (domestic fowl), Herpesvirus saimiri (marmoset), Herpesvirus sylvilagus (cotton-tailed rabbits), and the Epstein-Barr herpesvirus (man) by de The et al. (19).

The fatal lymphoproliferation observed in MCF-affected rabbits initially occurred in the paracortical zone of the lymph nodes and around the Malphigian corpuscles of the spleen, but rapidly spread to the lung, kidney, liver, peripheral nerves and retina (22). The cells involved in this lymphoproliferation resembled lymphoblasts which multiplied in an uncontrolled manner and subsequently invaded the tissues. Edington et al. (22) proposed based on the histopathology of their examined that the lymphoid lesions of MCF were due to an acute proliferative change rather than to a neoplastic process.

Buxton et al. (6) believed that the lymphoproliferation seen in MCF-affected tissues was due to hyperplastic process, because most tissues examined had retained their normal architecture. In subsequent investigations, the role of the lymphoproliferative effects in the production of a fatal infection by MCF were studied (6). Rabbits were inoculated with washed mesenteric lymph node cells from rabbits previously infected with sheep-associated MCF and then treated with 20 mg/kg of cyclosporin-A, a potent suppressive agent of T-lymphocytes. The inoculated rabbits did not develop a lympho-proliferative response yet succumbed to which MCF suggested that the

lymphoproliferative response of MCF in rabbits was involved in only a peripheral way in the destruction of host tissues and the death of the animal (6).

In another study of MCF (39), a young Formosan sika deer in a zoologic park (SDWAP) which had developed a mild infection characteristic of MCF subsequently recovered. Several months later this sika deer was inoculated with dexamethasone (0.1 mg/kg body weight) and recrudescence of MCF occurred. Histopathologic examination of tissues from the sick deer which was euthanatized at moribund revealed an extensive vasculitis with a focal neoplastic lymphoproliferation in the small intestine and also an associated granulomatous inflammation in the vascular system (39). There was no evidence of bovine leukemia virus in the deer and only alcelaphine herpesvirus-1 was recovered from its tissues. The histologic findings in the Formosan sika deer supports the hypothesis of Hunt and Billups (42) regarding the oncogenic potential of the alcelaphine herpesvirus-1.

In cattle affected with MCF, the histopathological changes reported resembled the description of the lesions seen in an immune complex disease (114). Because of such observations, Rweyemamu et al. (114) postulated that MCF is an immunopathologic disease. Subsequent investigations on the pathogenesis of MCF have focused on finding the depositions of immune complexes (IC) in tissues; however, IC have not been demonstrated in either the circulation or tissues in cattle infected with "sheep-associated" MCF (51) or in cattle infected with alcelaphine herpesvirus-1 (63). Using IIF test to identify the C3 component of complement and conglutinin, Patel and Edington (73) detected conglutinin deposits along the basement membrane in the

glomeruli and in endothelial cells of cattle infected with alcelaphine herpesvirus-1. The fluorescence found in this preceding study (73) was occasionally associated with the vascular lesions of MCF but also was found in normal glomeruli of cattle with terminal MCF. Additionally, neither MCF viral antigens nor virus-specific antibodies were demonstrated within these deposits of immunoglobulin and conglutinin (73).

Because of the inability to detect IC in MCF, an acute form of IC disease has been proposed (63). However, since MCF virus is highly host cell-associated, Mushi and Rurangirwa (63) regard it highly unlikely that the quantity of extracellular antigen required for acute immune complex formation can be produced. Furthermore, neither viral antigens, host immunoglobulins nor the C3 component of complement have ever been detected in the severe vasculitis associated with MCF (51, 63).

Generally, isolates of alcelaphine herpesvirus-1 have been obtained from peripheral lymphocytes or from suspensions of lymphoid organs (81). To ascertain which cells in the host provided the support for viral replication, rabbits were inoculated intravenously (i.v.) with alcelaphine herpesvirus-1 (72). These workers (72) found that initial appearance of immunofluorescent viral antigens occurred in the spleen of the rabbits. The immunofluorescing cells in the spleen had the appearance of differentiated, medium-sized lymphocytes but were not associated with proliferating lymphoblastoid cells (72). The presence of small numbers of immunofluorescing cells in the spleen of the MCF-infected rabbits implicated the spleen as a primary site of viral replications. Small numbers of cells containing viral immunofluorescing antigens were also detected in the thymus and lymph

nodes. These observations indicated that these tissues were areas of viral synthesis or reservoirs for rapidly disseminated virus (72).

The viral infectivity also was demonstrated in macrophages/monocytes from alcelaphine herpesvirus-1 infected rabbits (62). These virus-containing cells had unusually large cytoplasmic vacuoles but otherwise lacked any other cellular lesions. Although the infectivity of alcelaphine herpesvirus-1 was associated primarily with the macrophages, the role of macrophages in the pathogenesis of MCF remains unclear.

In a report by Rurangirwa and Mushi (109), the viral infectivity of alcelaphine herpesvirus-1 associated with the lymph nodes and the spleen was found to reside in lymphocytes within these tissues. Rossiter (102) in his studies reported that most proliferating lymphocytes from cases of MCF did not adhere to plastic, phagocytized bacteria or carry any surface immunoglobulin. Because of the morphologic appearance of MCF-infected lymphocytes, the high incorporation of $^{3}$H-thymidine into these infected lymphocytes and the ability of MCF-infected lymphocytes to form non-immune rosettes with rabbit erythrocytes, Rossiter (102) suggested that the cells involved in the lymphoproliferation were T-lymphoblasts.

## Isolation of Herpesviruses from Other Species Which Resemble Alcelaphine Herpesvirus-1

A recent serologic survey indicated that most animals belonging to species in the subfamily of Alcelaphine (i.e., wildebeest, hartebeest, and topi) and the subfamily of Hippotragiane (i.e., oryx) possess VN antibodies to alcelaphine herpesvirus-1 (60, 91). A herpesvirus

serologically related to alcelaphine herpesvirus-1 was isolated from a hartebeest by Reid and Rowe (90). The virus was propagated in bovine thyroid cells and viral-induced syncytia were observed (90). The hartebeest isolate remained highly cell-associated but when this hartebeest herpesvirus was inoculated parenterally into cattle, severe MCF developed (90). Subsequently cell-free form of the hartebeest herpesvirus was obtained after continued passage in bovine thyroid cell cultures (90). Paradoxically, clinical MCF did not occur in cattle inoculated with the cell-free hartebeest virus (90). These viral transmission experiments in cattle implied that attenuation of the hartebeest-derived herpesvirus occurred during serial passages in vitro and that the virulence of the hartebeest herpesvirus was associated with the cell-associated state of the virus (90). This hartebeest viral isolate was later designated as alcelaphine herpesvirus-2 (97).

Mushi et al. (69) reported on the isolation of an "MCF-like" herpesvirus from a young (6 months) topi (Damaliscus horrigum, Ogilby) calf. The topi-derived virus could only be propagated in cell cultures derived from kidney, thyroid and lung of topi origin and the viral CPE in topi-derived cells was characterized by the formation of small syncytia with intranuclear inclusion bodies typical of "Cowdry type A". When cattle were inoculated with either cell-associated topi virus or with virus-infected tissue specimens from the topi, clinical disease was not observed. Hence, the role of the topi virus in the production of an MCF infection in cattle is unclear. Of interest is the reported finding (69) of the absence of clinical cases of MCF in areas inhabited only by topi. The isolation of a herpesvirus from a captive oryx has been reported (34); however, the role of this oryx-derived

herpesvirus in MCF is also unknown. Whether these herpesviral isolates from the hartebeest, topi, and oryx, which are serologically similar to the alcelaphine herpesvirus-1, are etiologic agents in the transmission of MCF infection into cattle is yet to be determined. However, results on transmission experiments with these herpesviruses indicated that the herpesvirus isolate from the hartebeest was less virulent in cattle than the alcelaphine herpesvirus-1 (90) and the topi isolate was avirulent (69).

## Etiology of Sheep-Associated MCF

### Etiology

In contrast to wildebeest-associated MCF, the etiologic agent of sheep-associated MCF has yet to be isolated. Worldwide epidemiological evidence (3, 27, 46, 56, 59, 100, 101) has implicated sheep as the source of infection, therefore, the commonly used terminology applied to this syndrome as sheep-associated MCF. The diagnosis of sheep-associated MCF is based on clinical signs and histopathological lesions in affected tissues which are similar to the wildebeest-associated MCF (48, 51, 52, 78).

Specific IIF antibody to the IIF(L) antigens of alcelaphine herpesvirus-1 was detected in 162 of 167 sheep sera (100). Serum samples for this MCF antibody study had been collected from flocks of sheep present during outbreaks of MCF in England, United States, Australia, and Africa (100). In addition, antibody to alcelaphine herpesvirus-1 has also been detected by IIF in sera from cattle in which sheep-associated MCF had been diagnosed (101). Because of these

serological findings, Rossiter (100) concluded that the etiology of sheep-associated MCF was a virus which was serologically related but not identical to the alcelaphine herpesvirus-1. Nevertheless, all attempts at isolation of the etiological agent of sheep-associated MCF have been uniformly unsuccessful.

Several different viruses, however, have been isolated from clinical cases of MCF in domestic ruminants in the United States (14, 15, 16, 50, 120, 121, 122). In Colorado, from several outbreaks of MCF in cattle, an enterovirus, a bovine syncytial virus, a parvovirus and a herpesvirus of the bovine herpesvirus group IV (or Movar) have been isolated (121). Other viruses have also been isolated from isolated cases of MCF in ruminants which have included a herpesvirus from cattle (50), a cytomegalovirus from cattle (120), a bovine syncytial virus from cattle (15), a togavirus from a white-tailed deer and an Axis deer (Axis axis) (14), a morbilli virus from an experimentally infected calf (16), and a cytomegalovirus from an American bison (122).

In 1979, Hamdy and coworkers (29) reported on the isolation of a herpesvirus from a sick cow from a Minnesota dairy herd. The cow had clinical signs of MCF and had been in contact with lambing ewes during a 4-month period. The Minnesota-herpesvirus was found to be serologically related to the alcelaphine herpesvirus-1 by complement fixation, IIF and serum-virus neutralization (SVN) assays. However, cattle inoculated intramuscularly with the herpesviral isolate from Minnesota which had been passaged in bovine thyroid cell cultures did not produce clinical MCF, and cattle inoculated with the herpesviral isolate were found to be resistant to challenge by virulent cell-

associated alcelaphine herpesvirus-1 (29). In summary, in none of these reported cases of sheep-associated MCF, where a virus has been isolated, has clinical MCF been reproduced by inoculation of the viral isolate into a susceptible ruminant.

It has been suggested that bovine viral diarrhea (BVD) virus and/or border disease (BD) virus may play a synergistic role in the etiology of sheep-associated MCF (25). However, in the Colorado or Minnesota outbreaks of MCF, the togavirus of BVD virus was not detected (29, 121). Neither has the togavirus of BVD been found in known clinical cases of wildebeest-associated MCF (9, 11, 82).

Recently, a thymus derived lymphocyte cell line (T-cells) from a rabbit was described by Reid et al. (94) which produced MCF when inoculated i.v. into rabbits. The T-cell line was derived at the 57th serial passage in rabbits of sheep-associated MCF materials derived from a MCF-affected farmed red deer by the i.v. inoculation of rabbit with a suspension of mesenteric lymph node (MLN) cells. To propagate these T-cells, a feeder layer of fetal ovine kidney cells was required and the T-cells possessed T-lymphocyte markers, but produced no immunoglobulins (94). The T-cells also contained large, dense intracytoplasmic granules and a non-specific esterase (94). The T-cells which was cultured in artificial medium did not exhibit CPE, but did contain cytotoxic activity against primary fetal ovine kidney, bovine embryo kidney, and baby rabbit kidney cells and also a rabbit kidney (RK-13) and a baby hamster kidney (BHK-21) cell line (94). Herpesvirus particles were not detected within the T-cells by EM (94), but when rabbits were inoculated i.v. with at least 100 cytotoxic T-cells clinical MCF developed (92). Finally, these

investigators found that the viral infectivity of the T-cells was destroyed by incubation with anti-T-lymphocyte serum and complement (90). In summarizing the experimental data on MCF obtained in rabbits, Reid et al. (92) concluded that the causative agent of sheep-associated MCF is a virus which may exist or is carried as an episomal DNA in the infected host cell.

## Transmission

Because the etiological agent of sheep-associated MCF has not been isolated, the mode of transmission of disease is unknown. Nevertheless, transmission by the direct inoculation of fresh whole blood from cattle affected with sheep-associated MCF into clinically healthy cattle has been successful in reproducing clinical MCF (3, 18, 53).

The prevalence of MCF frequently increases during the sheep lambing seasons (3, 75, 77). To study the possible mode of transmission of MCF into cattle, pregnant ewes were housed with a healthy steer and a calf (3). In another experiment, a steer and a calf were fed with ground placentas from lambing ewes and filtrates of the placental mixture were also inoculated i.v. into each animal. Of the cattle inoculated with the sheep materials, none developed any clinical signs of MCF (3).

Pierson et al. (76) also failed to reproduce clinical MCF in domestic cattle after exposing them to lambing ewes or by the i.v. inoculation of cattle with either blood or suspensions of fetal membranes from pregnant ewes. In New Zealand, Horner et al. (43) reported the reproduction of MCF in a 1-day-old calf following the inoculation of 70 ml of a mixture of whole blood in anticoagulant (EDTA)

which was obtained from 2 MCF-affected cows. However, a rabbit which was inoculated with unwashed buffy coat cells from the same cows remained healthy throughout a 60-day observation period (43). Blood et al. (3) also did not reproduce MCF in rabbits with blood or tissue specimens obtained from an MCF-affected steer.

A farmed red deer which had been associated with a flock of lambing ewes developed clinical MCF and tissue homogenates then were inoculated intraperitoneally into rabbits (5). Several of these rabbits inoculated with materials from the red deer later developed clinical MCF (5). In Indonesia, Ramachandran et al. (84) reported that successful reproduction of clinical MCF in a healthy banteng following the inoculation of blood from a banteng which had been diagnosed clinically as having sheep-associated MCF. Westbury et al. (126) reproduced MCF by the inoculation into rabbits of blood from an MCF-affected Rusa deer (Cervus timorensis); however, MCF was not transmissible into cattle by blood inoculation from the Rusa deer. Yet these workers (126) were able, by the inoculation of blood, to reproduce MCF throughout 11 serial passages in rabbits. In a 1984 report, Hoffmann et al. (40) successfully reproduced MCF in 2 species of cattle (Bos indicus and Bos javanicus) by the i.v. inoculation of blood obtained from an Indonesian swamp buffalo (Bubalus bubalis) which had clinical MCF; but he was unable to reproduce MCF with the same blood inoculum in either rabbits, guinea pigs or mice. Selman and coworkers (116) reported the reproduction of sheep-associated MCF in each of 10 serial passages in cattle, however, virus isolation was not attempted. Reid and Buxton (89) have been able to maintain the infectivity of an agent derived from sheep-associated MCF for 100 serial passages in rabbits.

The complexity associated with the transmission of sheep-associated MCF from one species to another may be a reflection of the concentration of infectious virus in the inoculum because excessively large volumes (i.e., 500 ml) of blood are routinely reported required to reproduce MCF (8, 116). However, even this "transfusion-like" procedure is not entirely successful in the reproduction of MCF. Therefore, transmission of clinical sheep-associated MCF is probably a function of the virulence, concentration and defectiveness of the putative virus within the inoculum.

## Diagnosis of MCF

### Clinical Signs

A presumptive field diagnosis of MCF is usually based on the anamnesis and clinical signs (35). In outbreaks of MCF in susceptible species, there is usually a history of an association with either wildebeest or sheep undergoing parturition (81). The clinical signs (see Clinical Signs of MCF) of MCF that are primarily described for cattle (2, 18, 27, 129, 134) are briefly included: a nasal and ocular discharge which progresses from serous to mucopurulent or purulent with an encrustation of the muzzle and nares, a high fever (above 40.5 C), a bilateral corneal opacity, a pseudomembrane associated with erosion of the oral epithelium and the enlargement of most lymph nodes. The affected animal has labored breathing and usually extends its neck. The animal usually becomes depressed and anorectic. At between 17 to 28 days, a fever (above 40.5 C) is measurable and thereafter the animal dies within 24 to 48 hours.

Laboratory Tests

The gross changes seen in the animal tissues have been previously described (48). The prominent microscopic lesions found in most affected tissues provide a tentative and usually confirmatory diagnosis of MCF. In general, the histopathologic changes in most organs are a disseminated vasculitis with an associated lymphoid proliferation and mononuclear infiltration (35, 48, 82).

As confirmation the histopathologic observations, attempts at virus isolation should be done in conjunction with serology to confirm an exposure to alcelaphine herpesvirus-1. Methods for virus isolation have been extensively described (9, 11, 12, 84). Briefly, buffy coat cells which contain the alcelaphine herpesvirus-1 are co-cultivated with either bovine fetal thyroid or kidney cells (9, 12, 84). Virus also may be isolated by the intraperitoneal or i.v. inoculation of whole blood from the MCF-affected animal into cattle (11, 84), rabbits (5), or deer (11). The specimens routinely collected from virus isolation included 300 to 500 ml blood in defibrinated in glass bead or EDTA or heparin and sections of spleen, lymph nodes, thyroid, brain, lung (36) and nasal turbinates (67). Tissues collected for virus isolation should be kept at 4 C and freezing should be avoided. Specimens must be processed within 24 hours to increase the success for virus isolation. Alcelaphine herpesvirus-1 in inoculated cell culture is identified by its unique CPE (micro or macro syncytia) which occurs during early passage levels in cells. Morphologic confirmation of a herpesvirus is accomplished by examination of infected cell cultures by EM. Serologic identification of alcelaphine

herpesvirus-1 is done by using IIF assay (11, 26, 99), immunoperoxidase (99) or a SVN test (37, 61). To demonstrate antibodies specific to alcelaphine herpesvirus-1 or exposure to sheep-associated MCF, IIF assay (11, 99), complement fixation (103), SVN test (37, 61), and an ELISA (124) have been reported.

## Differential Diagnosis

Because of the similar appearance of the erosive lesions of MCF to other mucosal diseases, the following conditions or diseases should be considered as differential diagnoses: BVD-mucosal disease, foot and mouth disease, vesicular stomatitis, rinderpest, bluetongue, ingested caustics, poisonous plants and mycotoxin (36). The laboratory diagnosis of MCF is an extensive and tedious process which usually requires submission of a thorough anamnesis with accompanying fresh tissues for histopathologic examination and unclotted blood for attempts at isolation of the putative virus.

## Prevention and Control

The mode of transmission of both forms of MCF in nature is unknown. However, because of the known outbreaks of MCF associated with sheep or wildebeest during their lambing or calving seasons respectively, susceptible ruminant species should be separated from areas in which these carrier animals are located. The finding by Mushi et al. (67) of cell-free virulent alcelaphine herpesvirus in nasal and ocular secretions of neonates and wildebeest calves under 3 months of age argues for the transmission of alcelaphine herpesvirus-1 by contaminated aerosols, droplets or drinking water. However, other possible modes

of transmission of alcelaphine herpesvirus-1 such as arthropods or mechanical vectors such as the handling adult carrier species have not yet been eliminated. Therefore, breeding or housing potential carriers (i.e., wildebeest, hartebeest or topi) in grazing areas in common with susceptible ruminants increases the potential danger of MCF in food animals and captive or endangered zoological species (33, 38, 88). Since cattle and certain highly susceptible antelope and deer species are routinely dead-end hosts, these animals pose a minimal thread of species to species transmission. In most of these dead-end hosts, the alcelaphine herpesvirus-1 has been found to be extensively cell-associated and rarely cell-free (83).

Because of the difficulties associated with the isolation of alcelaphine herpesvirus-1 or the sheep-associated herpesvirus, the measurement of antibodies specific for alcelaphine herpesvirus-1 is a logical vehicle to identify an MCF infection in a susceptible or carrier species, therefore, captive carrier ruminants (wildebeest, hartebeest and topi) should be serologically tested prior to their movement. Heuschele et al. (38) and Ramsay et al. (88) have suggested that these carrier species should be screened for antibodies to alcelaphine herpesvirus-1 by both SVN and IIF tests (37, 38, 88) and also by a recently developed ELISA (124). A handling period of 30 to 60 days between serologic tests has been recommended for the determination of the immune status of a carrier animal (88).

These recommendations for the movement of zoological species in the United States have developed for the OKC Zoo (88) and the SDWAP (32, 38) for prevention and control of MCF which originates from exotic carrier ruminant species.

## LITERATURE CITED


1. Ben-Porat, T., and A. S. Kaplan. 1972. Studies on the biogenesis of herpesvirus envelope. Nature 235:165-166.

2. Berkman, R. N., R. D. Barner, C. C. Morrill, and R. F. Langham. 1960. Bovine malignant caterrhal fever in Michigan. II. Pathology. Am. J. Vet. Res. 22:1015-1026.

3. Blood, D. C., H. C. Rowsell, and M. Savan. 1961. An outbreak of bovine malignant catarrhal in a dairy herd. II. Transmission experiments. Can. Vet. J. 2:319-325.

4. Boever, W. J., and B. Kurka. 1974. Malignant catarrhal fever in greater kudu. JAVMA 165:817-819.

5. Buxton, D., and H. W. Reid. 1980. Transmission of malignant catarrhal fever to rabbits. Vet. Rec. 106:243-245.

6. Buxton, D., H. W. Reid, J. Finlayson, and I. Pow. 1984. Pathogenesis of 'sheep-associated' malignant catarrhal fever in rabbits. Res. Vet. Sci. 36:205-211.

7. Castro, A. E., and G. G. Daley. 1982. Electron microscopic study of the African strain of malignant catarrhal fever virus in bovine cell cultures. Am. J. Vet. Res. 43:576-582.

8. Castro, A. E., and W. P. Heuschele. 1985. Conference on malignant catarrhal fever. Bovine Practitioner 20:162-168.

9. Castro, A. E., D. L. Whitenack, and D. E. Goodwin. 1981. Isolation and identification of the herpesvirus of malignant catarrhal fever from exotic ruminant species in a zoologic park in North America. Amer. Assn. Vet. Lab. Diag., 24th Ann. Proc., pp. 67-78.

10. Castro, A. E., W. P. Heuschele, M. L. Schramke, and J. F. Dotson. 1985. Ultrastructure of cellular changes in the replication of the alcelaphine herpesvirus-1 of malignant catarrhal fever. Am. J. Vet. Res. 46:1231-1237.

11. Castro, A. E., G. G. Daley, M. A. Zimmer, D. L. Whitenack, and J. J. Jensen. 1982. Malignant catarrhal fever in an Indian gaur and greater kudu: Experimental transmission, isolation, and identification of a herpesvirus. Am. J. Vet. Res. 43:5-11.

12. Castro, A. E., M. L. Schramke, E. C. Ramsay, D. L. Whitenack, and J. F. Dotson. 1983. A diagnostic approach in the identification and isolation of malignant catarrhal fever virus from inapparent carriers in a wildebeest herd. Proc. III Int. Sym. Vet. Lab. Diag., pp. 715-721.

13. Castro, A. E., E. C. Ramsay, J. F. Dotson, M. L. Schramke, A. A. Kocan, and D. L. Whitenack. 1984. Characteristics of the herpesvirus of malignant catarrhal fever isolated from captive wildebeest calves. Am. J. Vet. Res. 45:409-415.

14. Clark, K. A. and L. G. Adams. 1976. Viral particals associated with malignant catarrhal fever in deer. Am. J. Vet. Res. 37:837-840.

15. Clarke, J. K., J. B. McFerran, and R. T. Nelson. 1973. The isolation of a strain of bovine syncytial virus in Northern Ireland. Res. Vet. Sci. 14:117-119.

16. Coulter, G. R., and J. Storz. 1979. Identification of a cell-associated morbillivirus from cattle affected with malignant catarrhal fever: Antigenic differentiation and cytologic characterization. Am. J. Vet. Res. 40:1671-1677.

17. Darlington, R. W., and L. H. Moss. 1968. Herpesvirus envelopment. J. Virol. 2:48-55.

18. Daubney, R., and J. R. Hudson. 1936. Transmission experiments with bovine malignant catarrhal. J. Comp. Path. 47:63-89.

19. de The, G., M. A. Epstein, and H. zur Hansen. 1975. Oncogenesis and herpesviruses II. Part 2, Epidemiology, Host Response, and Control. IARC Scientific Publication No. 11.

20. Denholm, L. J., and H. A. Westbury. 1982. Malignant catarrhal fever in farmed Rusa deer (Cervus timorensis). 1. Clinical-pathological observations. Aust. Vet. J. 58:81-87.

21. Edington, N., and J. R. Patel. 1981. The location of primary replication of the herpesvirus of bovine malignant catarrhal fever in rabbits. Vet. Micro. 6:107-112.

22. Edington, N., J. Patel, P. H. Russell, and W. Plowright. 1979. The nature of the acute lymphoid proliferation in rabbits infected with the herpesvirus of bovine malignant catarrhal fever. Europ. J. Cancer. 15:1515-1522.

23. Enders, J. F., T. H. Weller, and F. C. Robbins. 1949. Cultivation of the Lansing strain of poliomyelitis virus in cultures of various human embryonic tissues. Science 109:85-87.

24. Epstein, M. A., K. Hummeler, and A. Berkaloff. 1964. The entry and distribution of herpesvirus and colloidal gold in HeLa cells after contact in suspension. J. Exp. Med. 119:291.

25. Evermann, J. F. 1980. Etiology of malignant catarrhal fever. JAVMA 178:100-101.

26. Ferris, D. H., F. M. Hamdy, and A. H. Dardiri. 1976. Detection of African malignant catarrhal fever virus antigens in cell cultures by immunofluorescence. Vet. Micro. 1:437-448.

27. Goss, L. W., C. R. Cole, and R. E. Kissling. 1947. The pathology of malignant catarrhal fever (bovine epithelosis). Am. J. Path. 23:837-840.

28. Gray, A. P., and H. D. Anthony. 1969. Outbreak of malignant catarrhal fever in Kansas. Proc. U. S. Anim. Hlth. Assoc., 72nd Ann. Mtg., pp. 446-464.

29. Hamdy, F. M., A. H. Dardiri, C. Mebus, R. E. Pierson, and D. Johnson. 1979. Etiology of malignant catarrhal fever outbreak in Minnesota. Proc. U. A. Anim. Hlth. Assoc., 82nd Ann. Mtg., pp. 248-267.

30. Harkness, J. W., and D. M. Jessett. 1981. Influence of temperature on the growth in cell culture of malignant catarrhal fever virus. Res. Vet. Sci. 31:164-168.

31. Hatkin, J. 1980. Endemic malignant catarrhal fever at the San Diego Wild Animal Park. J. Wildlife Dis. 16:439-443.

32. Heuschele, W. P. 1982. Malignant catarrhal fever in wild ruminants--A review and current status report. Proc. U. S. Anim. Hlth. Assoc., 86th Ann. Mtg., pp. 552-570.

33. Heuschele, W. P. 1983. Diagnosis of malignant catarrhal fever due to alcelaphine herpesvirus-1. Proc. III Intl. Symp. Vet. Lab. Diag., pp. 707-713.

34. Heuschele, W. P. 1984. Malignant catarrhal fever, in Foreign Animal Disease Report. USDA Vet. Services, No. 12-4.

35. Heuschele, W. P., and A. E. Castro. 1984. Malignant catarrhal fever in Foreign Animal Diseases, their prevention, diagnosis and control, pp. 244-255. Comm. on Foreign Anim. Dis., U. S. Anim. Hlth. Assoc.

36. Heuschele, W. P., and A. E. Castro. 1985. Malignant catarrhal fever. In Comparative Pathobiology of Viral Diseases. Vol. 1, pp. 115-125. (R. G. Olson, S. Krakowka, J. R. Blakeslee, Jr., eds.). CRC Press, Boca Raton, Fl.

37. Heuschele, W. P., and H. R. Fletcher. 1984. Improved methods for the diagnosis of malignant catarrhal fever. Amer. Assn. Vet. Lab. Diag., 27th Ann. Proc., pp. 137-150.

38. Heuschele, W. P., M. Swansen, and H. R. Fletcher. 1983. Malignant catarrhal fever in U. S. Zoos. 1983 Ann. Mtg. Amer. Assoc. of Zoo Vets., pp. 67-72.

39. Heuschele, W. P., N. O. Nielsen, J. Oosterhuis, and A. E. Castro. 1985. Dexamethasone-induced recrudescence of malignant catarrhal fever and associated lymphosarcoma and granulomatous disease in a Formosan sika deer (Cervus nippon taiouanus). Am. J. Vet. Res. 46:1578-1583.

40. Hoffmann, D., S. Sobironingsih, B. C. Clarke, P. J. Young, and I. Sendow. 1984a. Transmission and virological studies of a malignant catarrhal fever syndrome in the Indonesian swamp buffalo (Bubalus bubalis). Aust. Vet. J. 61:113-116.

41. Hoffmann, D., S. Soeripto, S. Sobironingsih, R. S. F. Campbell, and B. C. Clarke. 1984b. The clinical-pathology of a malignant catarrhal fever syndrome in the Indonesian swamp buffalo (Bubalus bubalis). Aust. Vet. J. 61:108-112.

42. Holmes, I. H., and D. H. Watson. 1963. An electron microscope study of the attachment and penetration of herpesvirus in BHK-21 cells. Virology 21:112.

43. Horner, G. W., R. E. Oliver, and R. Hunter. 1975. An epizootic of malignant catarrhal fever. 2. Laboratory investigations. New Zealand Vet. J. 23:36-38.

44. Huck, R. A., A. Shand, P. J. Allsop, and A. B. Paterson. 1961. Malignant catarrhal of deer. Vet. Rec. 73:457-465.

45. Hunt, R. D., and L. H. Billups. 1979. Wildebeest-associated malignant catarrhal fever in Africa: A neoplastic disease of cattle caused by an oncogenic herpesvirus? Comp. Immun. Microbiol. Infect. Dis. 2:275-283.

46. James, M. P., F. J. A. Neilson, and W. J. Stewart. 1975. An epizootic of malignant catarrhal fever. 1. Clinical and pathological observations. New Zealand Vet. J. 23:9-12.

47. Jawetz, E., J. L. Melnick, and E. A. Adelberg. 1980. Review of Medical Microbiology, 14th ed., pp. 484-496. Lange Medical Publications, Los Altos, California.

48. Jubb, K. V. F., and P. C. Kennedy. 1985. Pathology of Domestic Animals. 3rd ed., Vol. 2, pp. 102-108, Academic Press, N.Y.

49. Kalunda, M. 1975. African malignant catarrhal fever virus: Its biologic properties and the response of American cattle. Ph.D. dissertation, Cornell University.

50. Leibermann, H., P. Schulze, R. Kokles, and H. H. Hantschel. 1967. Isolierung and identifizierung eines weiteren neuartigen bovinen herpesvirus. Arch. Vet. Med. 21:761-776.

51. Liggitt, H. D., and J. C. DeMartini. 1980a. The pathomorphology of malignant catarrhal fever. I. Generalized lymphoid vasculitis. Vet. Pathol. 17:58-72.

52. Liggitt, H. D., and J. C. DeMartini. 1980b. The pathomorphology of malignant catarrhal fever. II. Multisystemic epithelial lesions. Vet. Pathol. 17:73-83.

53. Liggitt, H. D., A. E. McChesney, and J. C. DeMartini. 1980. Experimental transmissions of bovine malignant catarrhal fever to a bison (Bison bison). J. Wildlife Dis. 16:299-304.

54. Liggitt, H. D., J. C. DeMartini, A. E. McChesney, R. E. Pierson, and J. Storz. 1978. Experimental transmission of malignant catarrhal fever in cattle and histopathologic changes. Am. J. Vet. Res. 39:1249-1257.

55. Ludwig, H. 1983. Bovine herpesviruses, in The Herpesviruses, Vol. 2 (B. Roizman ed.), pp. 135-145. Plenum Press, N.Y. and London.

56. Mare, C. J. 1977. Malignant catarrhal fever: An emerging disease of cattle in the USA. Proc. U. S. Anim. Hlth. Assoc., 81st Ann. Mtg., pp. 151-157.

57. Marshall, C. J., T. E. Munce, M. F. Barnes, and F. Boerner. 1919-1920. Malignant catarrhal fever. JAVMA 56:570-580.

58. Martin, W. B. 1976. The herpesviruses. Vet. Rec. 99:352-354.

59. Murray, R. B., and D. C. Blood. 1961. An outbreak of bovine malignant catarrhal in a dairy herd. I. Clinical and pathologic observations. Can. Vet. J. 2:277-281.

60. Mushi, E. Z., and L. Karstad. 1981. Prevalence of virus neutralizing antibodies to malignant catarrhal fever virus in Oryx (Oryx beisa callotis). J. Wildlife Dis. 17:467-470.

61. Mushi, E. Z., and W. Plowright. 1979. A microtiter technique for the assay of malignant catarrhal fever virus and neutralizing antibody. Res. Vet. Sci. 27:230-232.

62. Mushi, E. Z., and F. R. Rurangirwa. 1981a. Malignant catarrhal fever virus infectivity in rabbits macrophages and monocytes. Vet. Res. Communications 5:51-56.

63. Mushi, E. Z., and F. R. Rurangirwa. 1981b. Immunoglobulins, haemolytic complement and serum C3 in cattle infected with malignant catarrhal fever herpesvirus. Vet. Res. Communications 5:57-62.

64. Mushi, E. Z., and F. R. Rurangirwa. 1981c. Epidemiology of bovine malignant catarrhal fever: A review. Vet. Res. Communications 5:127-142.

65. Mushi, E. Z., and J. S. Wafula. 1983. Infectivity of cell-free malignant catarrhal fever in rabbits and cattle. Vet. Res. Communications 6:153-155.

66. Mushi, E. Z., L. Karstad, and D. M. Jessett. 1980. Isolation of bovine malignant catarrhal fever virus from ocular and nasal secretions of wildebeest calves. Res. Vet. Sci. 29:168-171.

67. Mushi, E. Z., F. R. Rurangirwa, and L. Karstad. 1981. Shedding of malignant catarrhal fever virus by wildebeest calves. Vet. Micro. 6:281-286.

68. Mushi, E. Z., P. B. Rossiter, L. Karstad, and D. M. Jessett. 1980. The demonstration of cell-free malignant catarrhal fever herpesvirus in wildebeest nasal secretions. J. Hgy. Comb. 85:175-179.

69. Mushi, E. Z., P. B. Rossiter, D. Jessett, and L. Karstad. 1981. Isolation and characterization of a herpesvirus from topi (Damaliscus korrigum, Ogilby). J. Comp. Path. 91:63-68.

70. Orsborn, J. S., C. J. Mare, J. L. Ayers, and R. E. Reed. 1977. Diagnostic features of malignant catarrhal fever outbreaks in the western United States. Amer. Assoc. Vet. Lab. Diag. 20th Ann. Proc., pp. 215-224.

71. Osorio, F. A., D. E. Reed, M. J. Van der Maaten, and C. A. Metz. 1985. Comparison of the herpesviruses of cattle by DNA restriction endonuclease analysis and serology. Am. J. Vet. Res. 46:2104-2109.

72. Patel, J. R., and N. Edington. 1980. The detection of the herpesvirus of bovine malignant catarrhal fever in rabbit lymphocytes in vivo and in vitro. J. Gen. Virol. 48:437-444.

73. Patel, J. F., and N. Edington. 1982. Immune complexes associated with infection of cattle by the herpesvirus of malignant catarrhal fever. Vet. Micro. 7:335-341.

74. Piercy, S. E. 1954a. Studies in bovine malignant catarrhal. IV. Immunity and vaccination. Brit. Vet. J. 110:87-96.

75. Piercy, S. E. 1954b. Studies in bovine malignant catarrhal. V. The role of sheep in the transmission of the disease. Brit. Vet. J. 110:508-516.

76. Pierson, R. E., J. Storz, A. E. McChesney, and D. Thake. 1974. Experimental transmission of malignant catarrhal fever. Am. J. Vet. Res. 35:523-525.

77. Pierson, R. E., D. Thake, A. E. McChesney, and J. Storz. 1973. An epizootic malignant catarrhal fever in feedlot cattle. JAVMA 163:349-350.

78. Pierson, R. E., F. M. Hamdy, A. H. Dardiri, D. H. Ferris, and G. M. Schloer. 1979. Comparison of African and American forms of malignant catarrhal fever: Transmission and clinical signs. Am. J. Vet. Res. 40:1091-1095.

79. Plowright, W. 1965. Malignant catarrhal fever in East Africa. I. Behavior of the virus in free-living populations of blue wildebeest (Gorgon taurinus taurinus, Burchell). Res. Vet. Sci. 6:56-68.

80. Plowright, W. 1967. Malignant catarrhal fever in East Africa. III. Neutralizing antibody in free-living wildebeest. Res. Vet. Sci. 8:129-136.

81. Plowright, W. 1968. Malignant catarrhal fever. JAVMA 152:795-804.

82. Plowright, W. 1981. Herpesviruses of wild ungulates, including malignant catarrhal fever virus. In Infectious Diseases of Wild Mammals, 2nd ed., pp. 126-146. (Davis, J. W., L. H. Karstad, and D. O. Trainer, eds.). Ames, Iowa State University Press, Ames, Iowa.

83. Plowright, W. 1984. Malignant catarrhal fever virus: A lymphotropic herpesvirus of ruminants. Curr. Top. Vet. Med. Anim. Sci., pp. 279-305.

84. Plowright, W., R. D. Ferris, and G. R. Scott. 1960. Blue wildebeest and the etiological agent of bovine malignant catarrhal fever. Nature 188:1167-1169.

85. Plowright, W., R. F. Macadam, and J. A. Armstrong. 1963. Growth and characterization of the virus of bovine malignant catarrhal fever in East Africa. J. Gen. Micro. 39:253-266.

86. Plowright, W., M. Kalunda, D. M. Jessett, and K. A. Herniman. 1972. Congenital infection of cattle with the herpesvirus causing malignant catarrhal fever. Res. Vet. Sci. 13:37-45.

87. Ramachandran, S., M. Malole, D. Rifuliadi, and T. Safriati. 1982. Experimental production of malignant catarrhal fever in Bali cattle (Bos sondaicus). Aust. Vet. J. 58:169-170.

88. Ramsay, E. C., A. E. Castro, and B. M. Baumeister. 1982. Investigations of malignant catarrhal fever in ruminants at the Oklahoma City Zoo. Proc. U. S. Anim. Hlth. Assoc., 86th Ann. Mtg, pp. 571-582.

89. Reid, H. W., and D. Buston. 1984. Malignant catarrhal fever of deer. Proc. Royal Society of Edinburgh 82B:261-293.

90. Reid, H. W., and L. Rowe. 1973. The attenuation of a herpesvirus (malignant catarrhal fever virus) isolated from hartebeest (Alcelaphus buselaphus cokei Gunther). Res. Vet. Sci. 15:144-146.

91. Reid, H. W., W. Plowright, and L. W. Rowe. 1975. Neutralizing antibody to herpesvirus derived from wildebeest and hartebeest wild animals in East Africa. Res. Vet. Sci. 18:269-273.

92. Reid, H. W., D. Buxton, E. Berrie, I. Pow, and J. Finlayson. 1984. Malignant catarrhal fever. Vet. Rec. 114:581-583.

93. Reid, H. W., D. Buxton, W. Corrigall, A. R. Hunter, D. A. McMartin, and R. Rushton. 1979. An outbreak of malignant catarrhal fever in red deer (Cervus elephus). Vet. Rec. 104:120-123.

94. Reid, H. W., D. Buxton, I. Pow, J. Finlayson, and E. L. Berrie. 1983. A cytotoxic T-lymphocyte line propagated from a rabbit infected with sheep associated malignant catarrhal fever. Res. Vet. Sci. 34:109-113.

95. Rines, M. P., and R. D. Barner. 1955. A prolonged case of malignant catarrhal fever. Michigan State College Vet. 15:108-109.

96. Roizman, B. 1978. The herpesviruses, in the Molecular Biology of Animal Viruses, Vol. 2, pp. 769-848. (D. P. Nayak, ed.). Marcel Dekker, N.Y., and Basel.

97. Roizman, B. 1982. The Herpesviruses, Vol. 1, pp. 1-23. (B. Roizman, ed.). Plenum Publishing, N.Y.

98. Roizman, B., and P. G. Spear. 1973. Herpesviruses: in Ultrastructure of Animal Viruses and Bacteriophage: An Atlas, Vol. 5, pp. 83-107. (A. J. Dalton, ed.). Academic Press, Inc., N.Y.

99. Rossiter, P. B. 1981a. Immunofluorescence and immunoperoxidase techniques for detecting antibodies to malignant catarrhal fever in infected cattle. Trop. Anim. Hlth. Prod. 13:189-192.

100. Rossiter, P. B. 1981b. Antibodies to malignant catarrhal fever virus in sheep sera. J. Comp. Path. 91:303-311.

101. Rossiter, P. B. 1983a. Antibodies to malignant catarrhal fever virus in cattle with non-wildebeest-associated malignant catarrhal fever. J. Comp. Path. 93:93-97.

102. Rossiter, P. B. 1983b. Proliferation of T-lymphoblasts in rabbits fatally infected with the herpesvirus of malignant catarrhal fever. Clin. Exp. Immunol. 54:547-553.

103. Rossiter, P. B., and D. M. Jessett. 1980. A complement fixation test for antigens of and antibodies to malignant catarrhal fever virus. Res. Vet. Sci. 28:228-233.

104. Rossiter, P. B., D. M. Jessett, and L. Karstad. 1983. Role of wildebeest fetal membranes and fluids in the transmission of malignant catarrhal fever virus. Vet. Rec. 113:150-152.

105. Rossiter, P. B., D. M. Jessett, and E. Z. Mushi. 1980. Antibodies to malignant catarrhal fever virus antigens in the sera of normal and naturally infected cattle in Kenya. Res. Vet. Sci. 29:235-239.

106. Rossiter, P. B., E. Z. Mushi, and W. Plowright. 1977. The development of antibodies in rabbits and cattle infected experimentally with an African strain of malignant catarrhal fever virus. Vet. Micro. 2:57-66.

107. Rossiter, P. B., E. Z. Mushi, and W. Plowright. 1978. Antibody response in cattle and rabbits to early antigens of malignant catarrhal fever virus in cultured cells. Res. Vet. Sci. 25:207-210.

108. Rossiter, P. B., D. M. Jessett, E. Z. Mushi, and L. Karstad. 1983. Antibodies in carrier wildebeest to the lymphoproliferative herpesvirus of malignant catarrhal fever. Comp. Immun. Microbiol. Infect. Dis. 6:39-43.

109. Rurangirwa, F. R., and E. Z. Mushi. 1981-1982. Target cells for malignant catarrhal fever virus in rabbits. Vet. Res. Communications 5:285-288.

110. Rurangirwa, F. R., and E. Z. Mushi. 1982. Effect of malignant catarrhal fever virus infection on the immune response of rabbits to sheep red blood cells. Comp. Immun. Microbiol. Infect. Dis. 5:423-427.

111. Rurangirwa, F. R., E. Z. Mushi, and L. Karstad. 1982. Malignant catarrhal fever virus specific secretory IgA in nasal secretions of wildebeest calves. Comp. Immun. Microbiol. Infect. Dis. 5:429-436.

112. Russell, P. H. 1980. The in vitro response to phytohaemagglutinin during malignant catarrhal fever of rabbits and cattle. Res. Vet. Sci. 28:39-43.

113. Ruth, G. R., D. E. Reed, C. A. Daley, M. W. Vorhies, K. Wohlgemuth, and H. Shave. 1977. Malignant catarrhal fever in bison. JAVMA 171:913-917.

114. Rweyemamu, M. M., E. Z. Mushi, L. Rowe, and L. Karstad. 1976. Persistent infection of cattle with the herpesvirus of malignant catarrhal fever and observation on the pathogenesis of the disease. Br. Vet. J. 132:393-400.

115. Rweyemamu, M. M., L. Karstad, E. Z. Mushi, J. C. Otema, D. M. Jessett, L. Rowe, S. Drevemo, and J. G. Grootenhuis. 1974. Malignant catarrhal fever virus in nasal secretions of wildebeest: A probable mechanism for virus transmission. J. Wildlife Dis. 10:478-487.

116. Selman, I. E., A. Wiseman, N. G. Wright, and M. Murray. 1978. Transmission studies with bovine malignant catarrhal fever. Vet. Rec. 102:252-257.

117. Senior, M., C. R. E. Halnan, and E. H. Tong. 1962. An outbreak of malignant catarrhal among the Pere David deer. Vet. Rec. 74:932-936.

118. Singh, G., B. Singh, P. P. Gupta, and D. S. Hothi. 1979. Epizootiological observation on malignant catarrhal fever and transmission of the disease in buffalo calves (Bubalus bulalis). Act. Vet. Brno. 48:95-103.

119. Stenius, P. I. 1952. Bovine malignant catarrhal. Institute of Pathology, Vet. Coll. Helsinki State Vet. Med. Institute, Helsinki, Finland.

120. Storz, J. 1968. Comments on malignant catarrhal fever. JAVMA 152:804-806.

121. Storz, J., N. Okuna, A. E. McChesney, and R. E. Pierson. 1976. Virologic studies on cattle with naturally occurring and experimentally induced malignant catarrhal fever. Am. J. Vet. Res. 37:875-878.

122. Todd, W. J., and J. Storz. 1983. Morphogenesis of a cytomegalovirus from an American bison affected with malignant catarrhal fever. J. Gen. Virol. 64:1025-1030.

123. Vanselow, B. A. 1980. An epizootic of bovine malignant catarrhal in Malaysia. Vet. Rec. 107:15-18.

124. Wan. S. K., and A. E. Castro. 1984. Development of an enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to MCF virus. (Abstract). The 65th Annual Meeting of Conference of Research Workers in Animal Disease, p. 13.

125. Weaver, L. D. 1979. Malignant catarrhal fever in two California dairy herds. Bovine Practitioner 14:121-124.

126. Westbury, H. A., and L. J. Denholm. 1982. Malignant catarrhal fever in farmed Rusa deer (Cervus timorensis). 2. Animal transmission and virological studies. Aust. Vet. J. 58:88-92.

127. Whitenack, D. L., A. E. Castro, and A. A. Kocan. 1981. Experimental malignant catarrhal fever (African form) in white-tailed deer. J. Wildlife Dis. 17:443-451.

128. Wibberley, G. 1976. Observation on two strains of bovine malignant catarrhal fever virus in tissue culture. Res. Vet. Sci. 21:105-107.

129. Wilks, C. R., and P. B. Rossiter. 1978. An immunosuppress factor in serum of rabbits lethally infected with the herpesvirus of bovine malignant catarrhal fever. J. Inf. Dis. 137:403-409.

130. Williams, E. S., E. T. Thonre, and H. A. Dawson. 1984. Malignant catarrhal fever in a Shira's moose (Alces alces shirasi Nelson). J. Wildlife Dis. 20:230-232.

131. Wilson, P. R., M. R. Alley, and A. C. Irving. 1983. Chronic malignant catarrhal fever: A case in a sika deer (Cervus nippon). New Zealand Vet. J. 31:7-9.

132. Wright, T. H. 1973. Herpesviruses, p. 353. (A. S. Kaplan, ed.). Academic Press, N.Y.

133. Wyand, D. S., C. F. Helmboldt, and S. W. Nielsen. 1971. Malignant catarrhal fever in white-tailed deer. JAVMA 159:605-610.

134. Zimmer, M. A., C. P. McCoy, and J. M. Jensen. 1981. Comparative pathology of African form of malignant catarrhal fever in captive Indian gaur and domestic cattle. JAVMA 179:1130-1135.

CHAPTER II

# MALIGNANT CATARRHAL FEVER: BIOLOGICAL PROPERTIES OF FOUR ISOLATES OF THE ALCELAPHINE HERPESVIRUS-1 IN BOVINE CELL CULTURES


## Summary

The temporal viral replication scheme was determined for each of 4 isolates (less than 40 passages) of cell-associated alcelaphine herpesvirus-1 in bovine fetal kidney (BFK) cells by counting immunofluorescent foci of cells containing viral antigens. The eclipse phase determined for each virus was 5 hours which was followed by a rise in the numbers of fluorescent foci. The cytopathic effects (CPE) observed in BFK cells infected with the gaur and the greater kudu viral isolates changed from syncytia formation to rounded cells with a release of cell-free virus after continuous subpassages of virus-infected cell cultures. In the development of intranuclear inclusions, Cowdry type A inclusions were not prominent until 4 days post-infection. By electron microscopy (EM), the size of mature virions were 138 to 156 nm and their morphology was icosahedral. Enveloped nucleocapsids were found which contained either complete DNA cores or were empty. At the advanced stage of infection numerous naked viral particles were present in the cytoplasm and nuclei of the viral-infected BFK cells. Large numbers of virions were seen by EM in virus-infected cells, but low levels (less than $10^2 TCID_{50}$/ml) of infectious cell-free virus were detected in the cultured medium.

Introduction

The herpesvirus of malignant catarrhal fever (MCF), a usually fatal disease of cattle and certain ruminant species, was isolated in Africa from a blue wildebeest (Gorgon taurinus taurinus) (1). The herpesvirus of MCF has been classified as bovid herpesvirus-3; however, recently a recommendation (2) was made that the virus should be classified to reflect the origin of the viral isolate as alcelaphine herpesvirus-1 (2). The morphologic and biologic characteristics of an alcelaphine herpesvirus labeled wildebeest calf 11 or WC-11, have been studied in bovine thyroid and BFK cells (1, 3). Certain biological manifestations of the alcelaphine herpesvirus-1 are similar to properties described for viruses in the family of gamma-herpesviruses (4) which produce syncytia and Cowdry type A inclusion bodies in cell cultures and are lymphotropic.

Herpesviruses have been isolated (5) from clinical cases of MCF at the Oklahoma City Zoo in an Indian gaur (Bos gaurus) and a greater kudu (Tragelaphus strepsiceros) and from 2 healthy wildebeest neonates (Connochaetes gnou) (6). Experimentally, each viral isolate produced clinical MCF when inoculated into either cattle (Bos taurus) or white-tailed deer (Odocoileus virginianus) (5, 7). Herpesviruses isolated from each animal were designated by the species of origin and specific morphologic and biologic properties for each isolate in bovine cell cultures (5, 6, 7, 8, 9). The preceding investigations demonstrated that each herpesviral isolate was serologically related to the WC-11 strain of alcelaphine herpesvirus-1. Subsequently, the Oklahoma strain (gaur-derived) of alcelaphine herpesvirus was shown to have similar DNA pattern as the WC-11 strain after restraction endonuclease treatment (10).

The objectives of this report are to describe the growth characteristics in bovine cell cultures of each of the 4 isolates as determined by the development of viral-specific immunofluorescent antigens in infected cells, to provide morphologic data on each viral isolate and present certain cytologic characteristics associated with these viruses.

## Materials and Methods

### History, storage and recovery of alcelaphine herpesviruses

Isolation and passage of each alcelaphine herpesvirus was in BFK cells. Herpesvirus isolates were stored as intact cells in Dulbecco's medium which contained 10 to 20% fetal bovine serum (FBS) and 5 to 10% dimethyl sulfoxide (DMSO) or glycerol in glass vials at -70 C.

For recovery of virus, each vial of intact cells was rapidly thawed and co-cultivated with a freshly trypsinized uninfected BFK cell suspension in a 75 $cm^2$ flask. The flask which contained the cell mixture was then placed in a $CO_2$ incubator at 36.5 C. The herpesviral-infected cells were subsequently passaged 3 to 5 times or until a CPE was present. At alternate passages, fresh uninfected BFK cell suspension was added. When viral CPE was visible, the virus was continuously subpassaged at 33 to 34 C and intact virus-infected cells for future use were frozen at -70 C with Dulbecco's medium supplemented with 10% FBS and 10% DMSO.

### Cell cultures

Primary BFK cells were propagated by methods previously described (7). For this study, the second to fifth passage of BFK cells which were grown in Dulbecco's medium supplemented with 10% FBS and 200 ug/ml

of gentamicin were used. Cells were propagated at 36.5 C in a $CO_2$ incubator. Bovine turbinate (BT) cells[a] (between passage 25 and 40) were propagated and maintained at 36.5 C in Eagle's minimum essential medium supplemented with 10% FBS and 200 ug/ml of gentamicin.

Viruses

Oklahoma strain. This herpesvirus was isolated from the lymphocytes of an Indian gaur with clinical MCF (5). Bovine kidney cells infected with virus were frozen at the 4th passage in January 1980. The herpesvirus was recovered by co-cultivation of these thawed virus-infected cells with the 3rd passage BFK cells. In this study, passage 17 through 40 of this herpesvirus were used.

Greater kudu isolate. This herpesvirus was isolated from a 10% homogenate of lymph nodes from a young greater kudu with clinical MCF (5). Cells infected with virus at the 4th passage were stored at -70 C. This herpesvirus was cell-associated from passage 15 to 40 but some cell-free virus was present at passage 25 to 43.

Wildebeest-1 isolate. This herpesvirus was isolated from an organ pool obtained from a 12-day old wildebeest calf (6). The virus-infected cells were stored at the 8th passage in a -70 C freezer. The virus used was cell-associated between passage 10 to 20.

Wildebeest-2 isolate. This virus was isolated from the buffy coat cells of a 1-day old wildebeest calf (6). The virus infected cells were stored at -70 C on the 6th passage. The virus used in this investigation was cell-associated between passage 10 to 20.

---

[a]Bovine turbinate cells were provided by Dr. C. Metz of the National Veterinary Service Laboratories, Ames, IA.

Indirect Immunofluorescent (IIF) test

The IIF test for the detection of immunofluorescing antigens of alcelaphine herpesvirus in BFK cells has been described (11). Reference antiserum to alcelaphine herpesvirus (IIF titer of 40) came from an Indian gaur with clinical MCF (5). Test slides were reacted for 30 minutes at 37 C with a 1:10 dilution of reference serum in FA buffer[b] (pH 7.4). Slides were then rinsed 3 times with FA buffer and air-dried. A 1:10 dilution of a rabbit antibovine immunoglobulin G fluoresceine-conjugate[c] was placed on each slide for 30 minutes at 37 C and then rinsed. Specific viral immunofluorescence was determined by observation, using a fluorescent microscope equipped with a mercury arc lamp (200 HBO), an exciter (KP 500), and barrier filter (KP 510).[d]

Development of immunofluorescent viral antigens as a determinant of virus replication

Early passages of the gaur, greater kudu and 2 wildebeest viral isolates were inoculated into BFK cells to determine the growth parameters for each virus. Because the viruses were highly cell-associated in the early passages, the input multiplicity of infections (m.o.i.) was done as follows: virus-infected BFK cells were trypsinized when CPE was observed and co-cultivated at a ratio of 1:3 with a suspension of uninfected BFK cells. The cell suspension for each virus was then dispensed onto 10 4-chamber slides. To determine the number of infected cells at 0 hour, one slide of each virus isolate was set

---

[b]DIFCO Laboratories, Detroit, MI.

[c]Miles Laboratories, Elkhart, IN.

[d]Dialux, Scientific Instrument Division, E. Leitz Inc., Rockleigh, NJ.

at 4 C for 1 hour to allow cells to settle on the slide surface and then was air-dried, fixed in cold acetone. The remaining slides were placed immediately into a $CO_2$ incubator at 33 C. Single cultures were harvested at the following time periods, 2, 4, 6, 12, 24, 30, 48, and 72 hours following planting of cell mixture. Subsequently, each slide was washed once in FA buffer, fixed in cold acetone (5 C) for 10 min and rinsed once in distilled water. The slides were air-dried and then stored at -70 C.

At passage 40 the greater kudu isolate produced infectious cell-free virus, therefore this passage was used to ascertain the amount of cell-free virus released from infected bovine cell culture. Cell cultures of BFK seeded onto 4-chamber slides were inoculated with $6.8x10^{4}$ $TCID_{50}$/ml of virus for a m.o.i. of $6.8x10^{-2}$ $TCID_{50}$/cell. Harvest fluids of 2 ml per slide collected at 0, 24, 48, 72, 96, 120, and 144 were inoculated (0.5 ml/well) immediately onto confluent monolayers of BFK cells of 4-chamber slides. After overnight adsorption of the inoculum, the fluids were removed and fresh medium was added. Slides for the detection of cell-free virus were incubated at 33 C for 7 days and fixed as previously described. Cell cultures from which the supernant fluids were obtained were also harvested and fixed as described. To determine virus titers for each cell-associated viral isolate, the number of fluorescent nuclei were counted for each well of the 4-chamber slides. The average fluorescent nuclei of 4 wells was then obtained. The titers were expressed as numbers of fluorescent focus unit (FFU)/well.

Cytology of cells infected with alcelaphine herpesvirus-1

To demonstrate the ability of each isolate to produce inclusion bodies in bovine cell cultures, cells infected with each viral isolate on 4-chamber slides were harvested sequentially at 0, 2, 4, 6, 8, 10, 12, 24, 48, 72, 96 and 144 hours post-inoculation. The harvested cell monolayers were then fixed in 95% ethanol and stained using a trichrome staining procedure (12).

Sensitivity of alcelaphine herpesvirus to ether

Cell-free virus preparations of the greater kudu isolate were tested for loss of infectivity after ether treatment by mixing 1.0 ml of virus with 0.25 ml of diethyl ether (13). The virus-ether mixtures and nontreated virus suspensions were incubated at 37 C for 1 hour and then stored overnight at 4 C. Residual ether was separated from the aqueous viral suspension by centrifugation at 1000 rpm for 10 minutes. The viral suspension was then diluted tenfold. A volume of 0.1 ml of each dilution was inoculated in duplicate onto confluent monolayers of BT cells in 4-chamber slides. The inoculated slides were incubated for 6 days at 33 C and then fixed and stained by IIF test for the presence of viral antigens. The number of FFU per well was determined for ether treated and nontreated virus suspensions. The difference in FFU between these values was used as a measure of the sensitivity of virus to ether.

Preparation of virus for electron microscopy (EM)

Cell cultures with CPE at either high or low passages of the gaur, greater kudu and wildebeest viral isolates were harvested, fixed and embedded for EM as previously described (8). The morphology of cell-free

alcelaphine herpesvirus was determined by clarifying supernatant fluids from BFK cells infected with fluids from 38 to 43 passages of the greater kudu isolates. Virus-containing fluids were concentrated and purified by the method of Schloer (14) in a 30 to 60% discontinuous sucrose gradient centrifuged at 77,000 x g. The density of each fraction collected was determined using a refractometer.[e] Sucrose was removed from each 0.5 ml fraction by overnight dialysis against 2 changes of 0.01 M Tris buffer (pH 7.4). Each of the 12 fractions collected from the gradient were screened for the presence of viral antigens by an enzyme-linked immunosorbent assay (ELISA) for alcelaphine herpesvirus (15). Fractions from the gradient with densities of 1.2241 and 1.2296 g/ml which were positive for viral antigens by ELISA were dispensed a drop onto a (200 mesh) carbon-coated grid with one minute. The grid was then stained for 30 seconds with 1% (w/v) potassium phosphotungstate (pH 6.8) solution. Specimens were immediately examined using a Phillips EM 200 transmission electron microscope.

## Results

### Cytopathic effects of alcelaphine herpesvirus in cell cultures

Viruses of the gaur, greater kudu and 2 wildebeest at the passage levels between 3 and 20 were highly cell-associated and the CPE of each virus was characterized by the formation of syncytia (Fig 1A). These polykaryocytes, which contained 10 to 50 nuclei and indicated the presence of virus were found after 4 to 6 subpassages of

---

[e] Bausch & Lomb, Rochester, NY.

co-cultivated cultures following removal of alcelaphine herpesvirus containing cells from the freezer.

In most syncytia, nuclei were usually randomly distributed, but in a few syncytia the nuclei formed clusters around a dark refractive center (Fig 1B). As the infected cell cultures aged, cytoplasmic vacuoles were evident and the number of syncytia gradually increased. At 7 to 10 days after the onset viral CPE, syncytia began detaching from the surrounding healthy cells which resulted in a refractile appearing masses (Fig 2A). Shrunken syncytia detached from the cell monolayer creating space which eventually replaced by the surrounding cells (Fig 2B). As retraction of the syncytia continued, the cytoplasmic masses shrunk with the formation of "pseudopodial-like" strands with cytoplasmic blebs (Fig 3).

Syncytia were present throughout 40 serial subpassages of BFK cells persistently infected with the gaur strain of alcelaphine herpesvirus-1; however, when the infected BFK cells were co-cultivated with BT cells, a cell rounding CPE occurred. Subsequently, cell-free at a concentration of less than $10^2$ $TCID_{50}$/ml was found after the 25th subpassage of gaur strain infected BT cells.

In contrast, the CPE produced in BFK cells by the greater kudu isolate of alcelaphine herpesvirus-1 gradually changed with each subpassage from syncytia to refractile, rounded cells by the 17th passage (Fig 4). Two types of CPE produced by the greater kudu isolate were observed in virus-infected BFK cells with subpassages greater than 40; namely, small syncytia with refractile, rounded cells and only rounded cells. Cell-free virus was detectable after the 25th subpassage in BFK cells infected with greater kudu isolate of alcelaphine

herpesvirus. Bovine turbinate cells which infected with the 30th subpassage of greater kudu isolate had a CPE characterized by rounded cells.

The CPE produced in BFK cells by the 2 wildebeest isolates were identical (syncytia) to that of the greater kudu and gaur isolates. Cell-free virus from 2 wildebeest was not detected in supernatant fluids up to 20 subpassages.

Replication of alcelaphine herpesvirus-1 in cell cultures as measured by immunofluorescence

The replication of each of 4 isolates of alcelaphine herpesvirus-1 in BFK cells as determined by the increase in FFU/well is presented in Figure 5. The m.o.i. in FFU was identical for each virus. The viral eclipse (no changing in the numbers of FFU) phase for each isolate was 5 hours. The 2 wildebeest isolates had the most FFU per well 24 and 48 hours post-infection. The FFU for the greater kudu isolate were occurred maximal at 72 hours but were less than the wildebeest isolate. The gaur strain replicated the slowest and an increase number of FFUs were counted at 72 hours post-infection.

The measurement of intracellular and extracellular virus by FFU in BFK cells was done with subpassages 40 and 43 using the greater kudu isolate (Fig 6). The viral eclipse phase was 48 hours and detection of cell-free virus corresponded over a period of 7 days with increases in FFU. Counts for FFU were discontinued at 7 days when cellular monolayers had over 60% fluorescing nuclear foci but release of cell-free virus was monitored for 8 days post-infection.

Observations of immunofluorescing foci in virus-infected BFK cells led to the identification of a diffuse fluorescent-staining antigen

in the cytoplasm (Fig 7A) and both solid and particulate intranuclear antigens (Fig 7B). In some syncytia, both types of intranuclear fluorescence and non-fluorescing nuclei were also observed (Fig 8).

Development of inclusion bodies in bovine kidney cell cultures infected with alcelaphine herpesvirus-1

Intranuclear inclusions were not present in co-cultivated BFK cells at 6 hours with low subpassages of the gaur, greater kudu and 2 wildebeest isolates. At 6 hours, the nuclei in the syncytia present were granular in appearance with eosinophilic nucleoli. As viral CPE progressed, inctranuclear inclusions at various stages of development were observed in the same syncytium (Fig 9A). In syncytia, enlargement of nuclei, beading of nuclear membranes and vacuolation of the cytoplasm were also observed. When virus-infected BFK cells were fixed at 96 hours, typical "Cowdry type A" intranuclear inclusions were visible (Fig 9B). A perinuclear halo was seen associated with the darker but clear intranuclear inclusions. Intranuclear inclusions were also seen within cells containing single nucleus. The greater kudu isolate at subpassage 38 produced type A intranuclear inclusions which were usually eccentric and irregular with a beading of the nuclear membrane by 24 hours after infection (Fig 9C).

Ether sensitivity

In each well inoculated with a dilution of virus treated with ether FFU was not observed but the untreated virus had a titer $2.1 \times 10^4$ FFU/ml. Therefore, exposure of the greater kudu isolate of alcelaphine herpesvirus-1 to ether decreased the infectivity more than 1000 fold.

Morphologic characteristics of strains of alcelaphine herpesvirus-1

By EM, examination of cell free subpassages 38 to 42 of the greater kudu isolate revealed herpesviral particles with most lacking envelopes. Several nucleocapsids had dark stained centers which indicated the absence of a DNA core (Fig 10A). The capsids of the virions were hexagonal or polygonal characteristic of an icosahedral morphology. The size of the dark central core of the virions ranged between 73 to 83 nm. The capsomeric surface was tubuler in morphology and the hollow capsomeric subunits measured 15.7 nm in diameter and 11.7 nm in length (Fig 10B). Enveloped virions were visible which were enclosed by 1 or more membranes (Fig 10C). The size range of the enveloped virions was 159 to 334 nm. Membranes surrounding the nucleocapsid has 2 distinct bands which ranged in thickness from 0.12 to 0.18 nm.

The diameter of enveloped capsids located within the cells infected with the gaur strain was 94 to 103 nm while the immature intranuclear capsids were 87 to 98 nm. The enveloped virions were located within the cytoplasm and with the double envelopes had a diameter of 138 to 156 nm. The inner viral envelope had a double membrane which was resolved by EM, however, the outer envelope appeared to be irregular. Envelopes surrounding 2 or more nucleocapsids were also seen. A tegument, an electron lucent area between the capsid and envelope was present in numerous complete virions (Fig 11).

Immature virions were seen in the nuclei of BFK cells infected with different passages of the gaur or greater kudu isolates (Fig 12) and empty viral capsids were found in the cytoplasm (Fig 13A).

Aggregations of nucleocapsids within a homogenous and dense-stained cytoplasmic bodies were also observed (Fig 13B). Complete virions had a dense central core which stained with different intensities. The core of these virions was composed of a "spool-like" structure with a "thread-like" appearance (Fig 13C).

Discussion

The replication in cell culture and biologic properties of 4 isolates of alcelaphine herpesvirus-1 from exotic ruminant species in Oklahoma were examined to provide an approach for the preparation of virus-specific antigens for use in an ELISA. Our findings indicated that either the viral isolate from a gaur or greater kudu had the biological characteristics previously determined (5, 7) of an alcelaphine herpesvirus and their replication in BFK cells provided sufficient antigens for the ELISA. Because of the basic similarities found between viral isolates, certain experiments were performed on only 1 of the 4 herpesviruses.

The alcelaphine herpesviruses of MCF have been previously studied in bovine thyroid cells (3, 16), BFK cells (3, 5, 6, 7, 8, 9) and fetal aoudad kidney (FAK) cells (11). Because cell-free virus is essentially non-existent in early subpassages, the replicative cycle of low passage alcelaphine herpesviruses has been examined for infectivity by the titration of suspensions of virus-infected cells onto healthy cell cultures. Our initial observations indicated that low passage virus did not exhibit CPE in cultures, however, healthy but infected cells contained viral antigens that were detectable by an IIF test. Thus, by counting FFU as indicators of virus replication, the sequence of the temporal replication in bovine cell cultures were

determined for 4 isolates of alcelaphine herpesvirus-1 which included the Oklahoma strain. Our findings showed that during the first 5 hours post-infection (eclipse phase) the number of FFU for each viral isolate remained unchanged. Subsequent to this 5 hour period, a rise in FFU was observed for the 2 wildebeest and greater kudu viral isolates. The Oklahoma strain also had an increase in FFU after 5 hours; however, the number of FFUs found at 72 hours suggested that the Oklahoma strain of alcelaphine herpesvirus-1 replicated slower or was not completely adapted to bovine cell cultures.

The CPE of the low subpassages of each isolate of alcelaphine herpesvirus-1 were characterized by syncytial formation. The viral CPE also consisted of foci of highly refractile, rounded cells and the production of cell-free virus occurred with both gaur and greater kudu isolates after 25 subpassages. The CPE observed with each isolate of alcelaphine herpesvirus-1 at higher subpassages were similar to that described (11) for herpesviral isolates of MCF from exotic ruminants at the San Diego Zoo.

A latent infection appeared to have been established by certain of the herpesviral isolates in BFK cells. During this viral latent phase, CPE was not observed and viral antigens were not detected in any of the virus-infected BFK cells by immunofluorescence. However, continuous subpassage of these virus-infected cells led to the formation of syncytia which became the predominant CPE. If the virus-infected cells were frozen with a cryopreservative and then recovered from -70 C, a delay of CPE frequently occurred. In several instances, virus was completely lost after serial passages of these latently infected cells. This latter phenomenon posed a restriction in the maintenance of the

infectivity of certain isolates of alcelaphine herpesvirus-1 in the frozen state. However, additional subpassage of fusion by co-cultivation with susceptible cells could release the virus from the latent state.

The replication of alcelaphine herpesvirus-1 in BFK cells was manifested by a focal CPE in the cell monolayer. These foci of CPE contained refractile masses which eventually detached from the flask leaving a cell-free space in the cell monolayer. These spaces were subsequently monolayered by the infiltration of adjacent healthy cells. Subsequently, viral CPE reappeared in other areas of the cell monolayer and the cycle of cytopathic changes was repeated which suggested a persistent viral infection. The pattern of CPE of isolates of alcelaphine herpesvirus-1 in BFK cells was similar to the persistent infection described in Chinese hamster and Earle's cell cultures infected by herpesvirus simplex (17). In this investigation, we were unable to determine whether the cycle of virus replication was augmented by cell to cell fusion, or subpassage of the cell cultures, or the production of low levels of infectious cell free virus, or by the reattachment of virus-containing refractile masses onto healthy cells.

Eosinophilic intranuclear inclusion bodies (Cowdry type A) were detected only after CPE occurred. The visible changes in the appearance of nuclei during the formation of intranuclear inclusions was as follows: a ground glass to granular texture first occurred followed by the formation of intranuclear inclusions. These changes were observed in nuclei within syncytia and in single cells. Similar sequential changes have been described in the formation of intranuclear inclusions in cell cultures infected by herpesvirus simplex (18). The intranuclear

inclusions produced by alcelaphine herpesvirus-1 were seen in the bovine cell cultures only during the late stage of virus infection similar to the findings with two African strains of alcelaphine herpesvirus (19). Therefore, formation of inclusions appeared to be a late replicative function or lesion of a cell infected with alcelaphine herpesvirus-1.

By EM, the size and morphology of herpesviral particles seen were identical to those in previous reports (3, 7, 8, 9). However, we found that many naked and empty herpesviral nucleocapsids were present in both the cytoplasm and nuclei of cells infected with higher passages (greater than 25 passages) of alcelaphine herpesvirus-1. Roizman and Furlong (20) suggested that the presence of unenveloped nucleocapsids may be due to the lack of an enzyme necessary for the biosynthesis of the viral envelope and may result in a low yield of infectious cell-free virus. Although the gaur and greater kudu isolates of alcelaphine herpesvirus did not produce high titers of infectious virions, the maximal infectivity ($10^4$ $TCID_{50}$/ml) were produced by the greater kudu isolate (at the passage 40 to 43) at 72 hours post-infection with input multiplicity of infection of $6.8 \times 10^{-2}$ $TCID_{50}$/cell. The viral antigen extract and semi-purified virus from the greater kudu virus isolate from BFK cells has been used successfully as the binding antigen in an ELISA which measures specific antibody to alcelaphine herpesvirus-1 (15).

References

1. Plowright W, Ferris RD, Scott GR: Blue wildebeest and the etiological agent of bovine malignant catarrhal fever. Nature 188: 1167-1169, 1960.

2. Castro AE, Heuschele WP: Conference on malignant catarrhal fever. Bovine Practitioner 20:162-168.

3. Plowright W, Macadam RF, Armstrong JA: Growth and characterization of the virus of bovine malignant catarrhal fever in East Africa. J Gen Microbiol 39:253-266, 1963.

4. Roizman B: The family Herpesviridae: General description, taxonomy and classification, in The Viruses, Vol. 1, Herpesviruses (B. Roizman, ed.) Plensun Press, N.Y., 1982.

5. Castro AE, Whitenack DL, Goodwin DE: Isolation and identification of malignant catarrhal fever from exotic ruminant species in a zoologic park in North America. Ameri Assn Vet Lab Diag pp. 67-78, 1981.

6. Castro AE, Schramke ML, Ramsay EC, et al: A diagnostic approach in the identification and isolation of malignant catarrhal fever virus from inapparent carries in a wildebeest herd. Proc III Intl Symp Vet Lab Diag pp. 715-721, 1983.

7. Castro AE, Daley GG, Zimmer MA, et al: Malignant catarrhal fever in an Indian gaur and greater kudu: Experimental transmission, isolation, and identification of a herpesvirus. Am J Vet Res 43:5-11, 1982.

8. Castro AE, Daley GG: Electron microscopic study of the African strain of malignant catarrhal fever virus in bovine cell cultures. Am J Vet Res 43:576-582, 1982.

9. Castro AE, Ramsay EC, Dotson JF, et al: Characteristics of the herpesvirus of malignant catarrhal fever isolated from captive wildebeest calves. Am J Vet Res 45:409-415, 1984.

10. Osorio FA, Reed DE, Van der Maaten MJ, et al: Comparison of the herpesviruses of cattle by DNA restriction endonuclease analysis and serology. Am J Vet Res 46:2104-2109, 1985.

11. Heuschele WP: Diagnosis of malignant catarrhal fever due to alcelaphine herpesvirus-1. Proc III Intl Symp Vet Lab Diag pp. 707-713, 1983.

12. Roszel JF: Membrane filtration of canine and feline cerebrospinal fluid for cytologic evaluation. J Am Vet Med Assoc 160:720-725, 1972.

13. Christensen M: Basic laboratory procedures in diagnostic virology. Charles C. Thomas Publisher, Springfield Ill, 1977.

14. Schloer GM, Breese SS: Purification of African malignant catarrhal fever virus using two-phase aqueous polymer system. J Gen Virol 58: 101-110, 1982.

15. Wan SK, Castro AE: Development of an enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to MCF virus. (Abstract) The 65th Ann Mtg Con Res Workers in Animal Diseases, p. 13, 1984.

16. Kalunda M: African malignant catarrhal fever virus: Its biologic properties and the response of American cattle. Ph.D. Dissertation. Cornell University, 1975.

17. Rapp F, Jerkofsky MA: Persistent and latent infections, in The Herpesviruses. (Kaplan AS, ed.) Academic Press, N.Y., pp. 271-289, 1973.

18. Teplitz RL, Valco Z, Rundall T: Comparative sequential cytologic changes following in vitro infection with herpesvirus type I and II. Acta Cytologica 15:455-459, 1971.

19. Wibberley G: Observations on two strains of bovine malignant catarrhal fever virus in tissue culture. Res Vet Sci 21:105-107, 1976.

20. Roizman B, Furlong D: The replication of herpesvirus. In: Comprehensive virology (Fraenkel-Conrat H, Wagnes RR, eds.), Vol. 3, Plenum Press, N.Y., pp. 229-403, 1974.

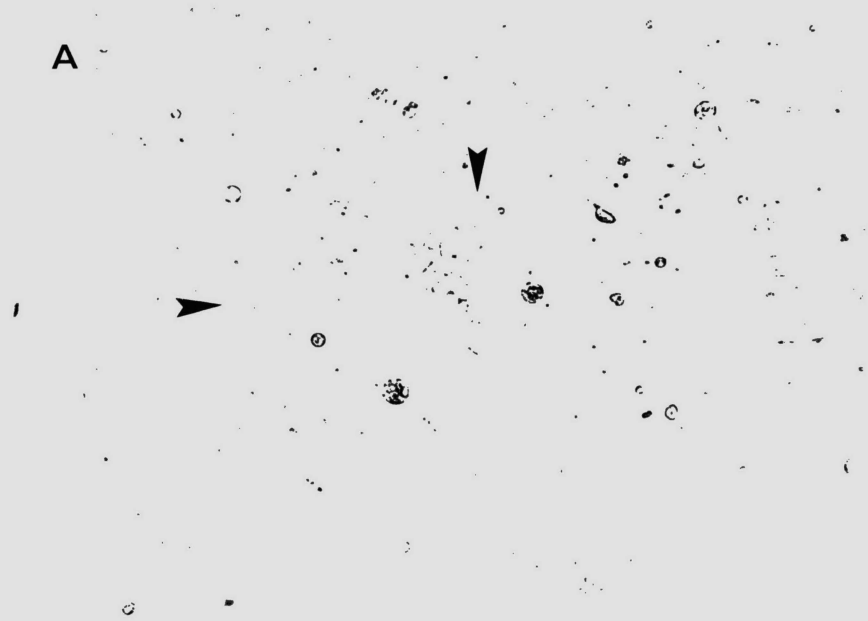
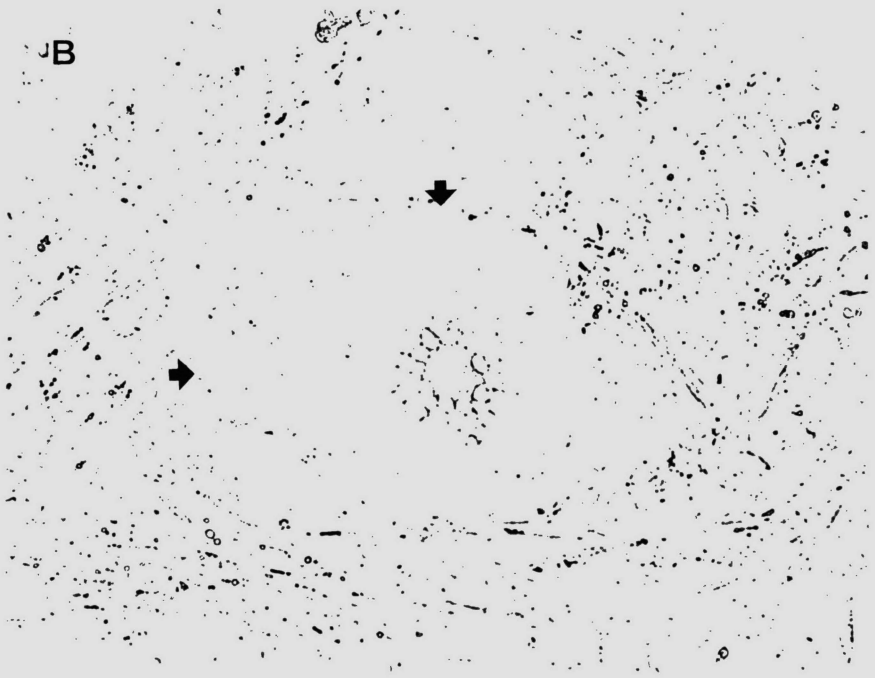



Fig 1A--Cytopathic effects of alcelaphine herpesvirus-1 in BFK cells. A syncytium (arrowheads) produced by the greater kudu isolate. x140

B--A syncytium (arrows) with rosette arrangement of nuclei produced by the gaur. x140



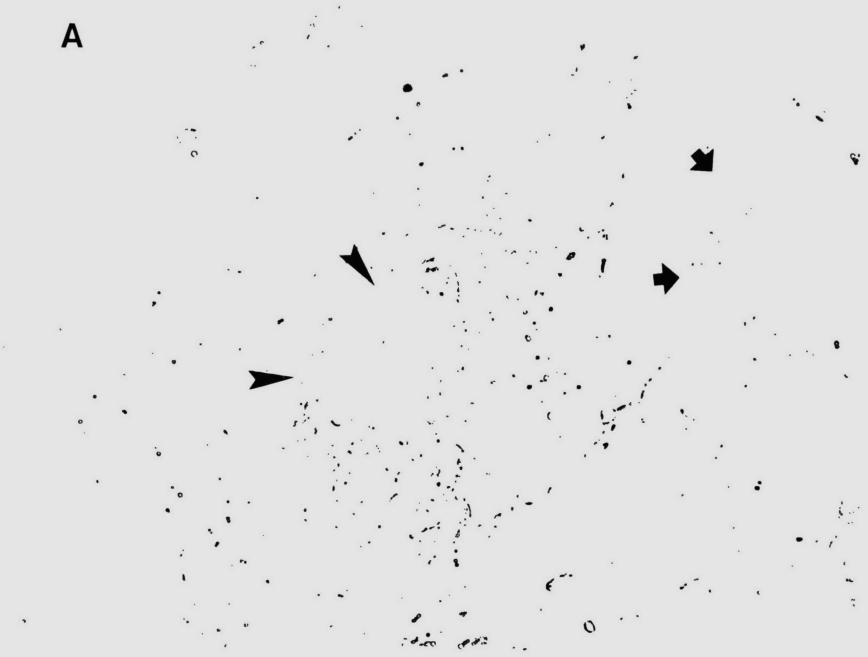
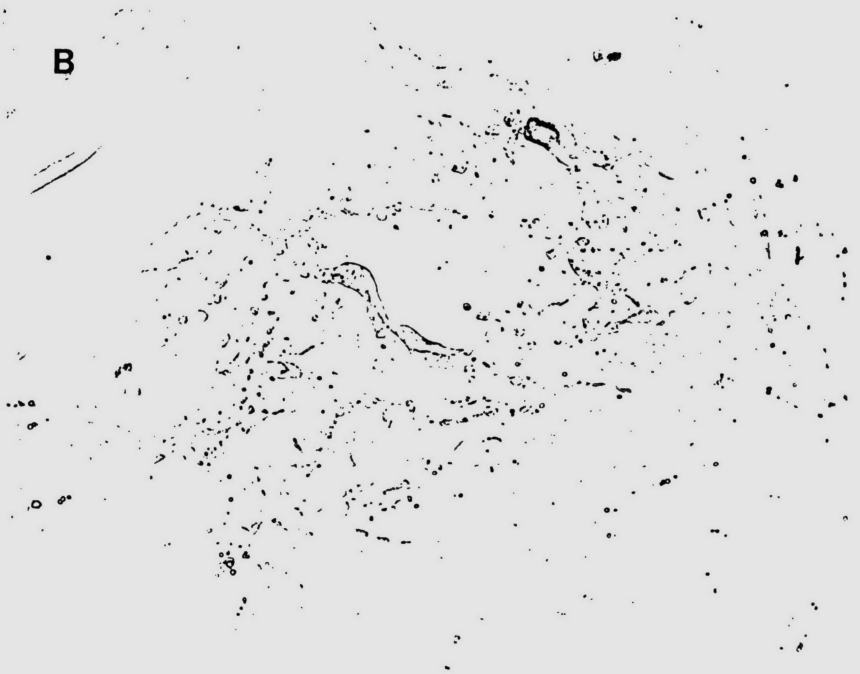



Fig 2A--Cytopathic effects of alcelaphine herpesvirus-1 in BFK cells. A contracted, refractile syncytium (arrowheads) and a syncytium (arrows) produced by the wildebeest-1 strain. x130

B--A cell-free space with cellular debris resulting in peeling of syncytium produced by the wildebeest-1 strain. x130



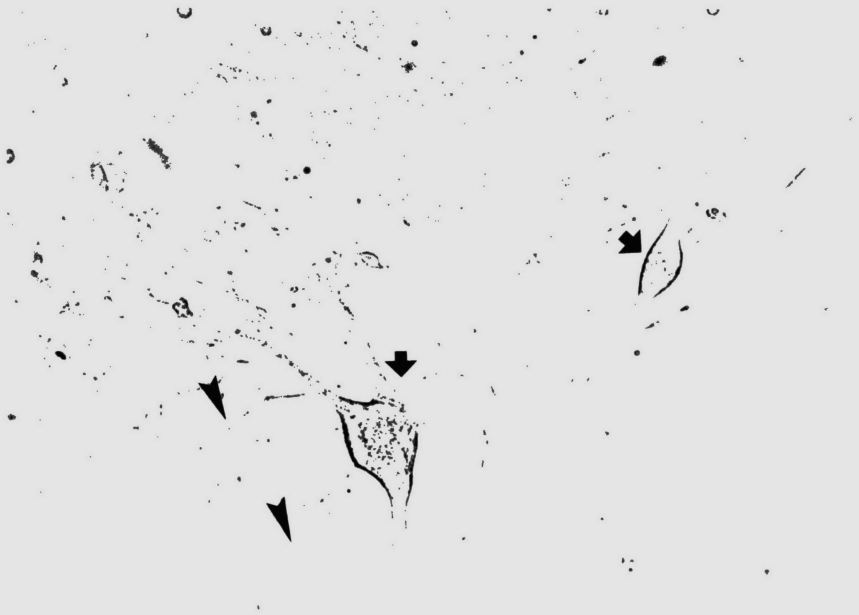
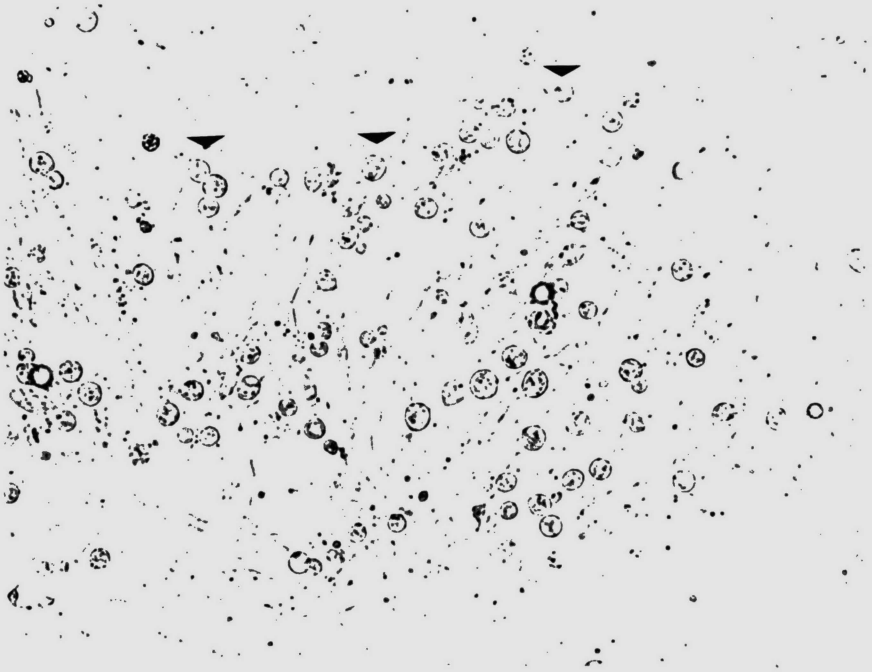


Fig 3--Cytoplasmic strands which contain blebs (arrowheads) in a retracted syncytia (arrows) in BFK cells infected with the gaur strain of alcelaphine herpesvirus-1. x130

Fig 4--Formation of 'baloon-like' rounded cells (arrowheads) in BFK cells infected with the 30th passage of the greater kudu isolate of alcelaphine herpesvirus-1. x160



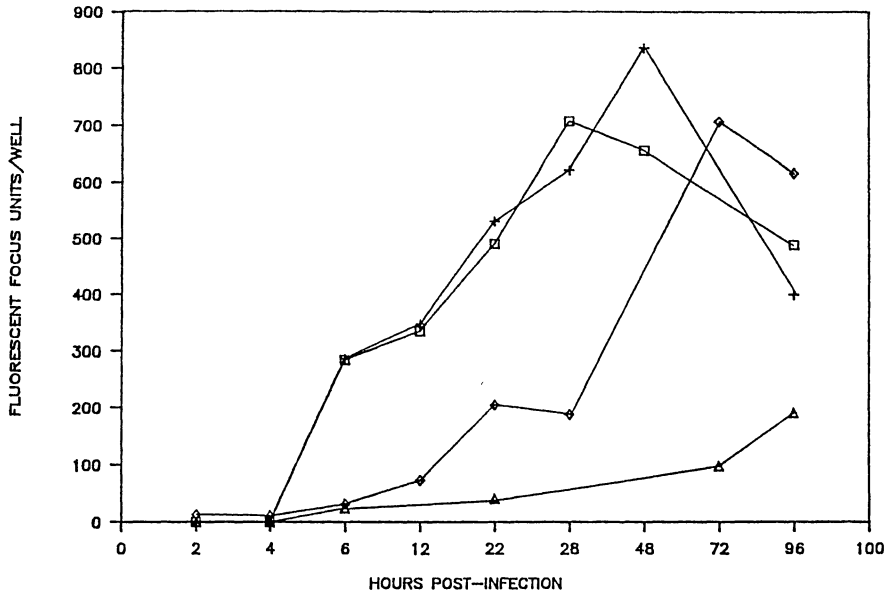
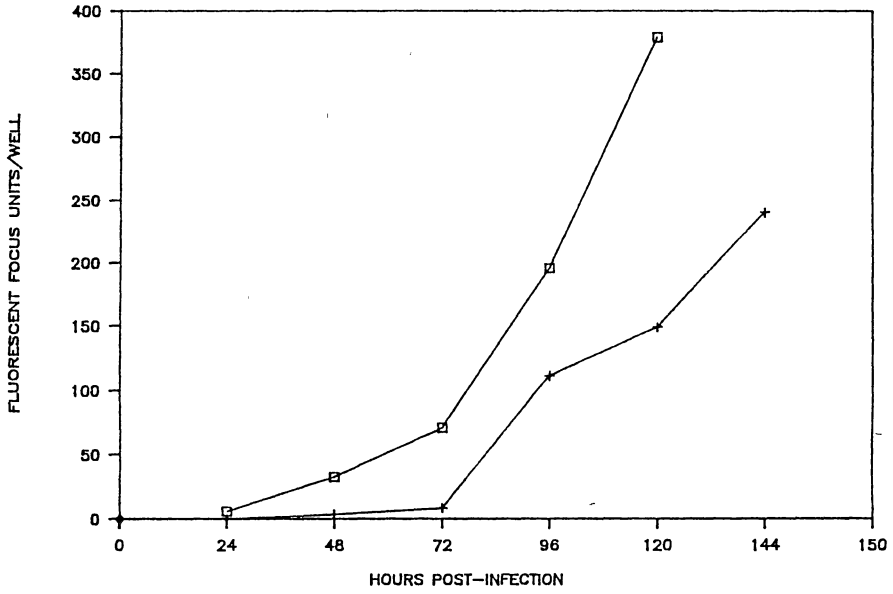



Fig 5--Replication of 4 isolates of alcelaphine herpesvirus-1 in BFK cells as measured by an indirect immunofluorescent assay. Δ:gaur; ◇:greater kudu; □:wildebeest-1; +:wildebeest-2.

Fig 6--Development of intracellular and extracellular viruses in bovine cell cultures infected with an alcelaphine herpesvirus-1. □:intracellular virus; +:extracellular virus.



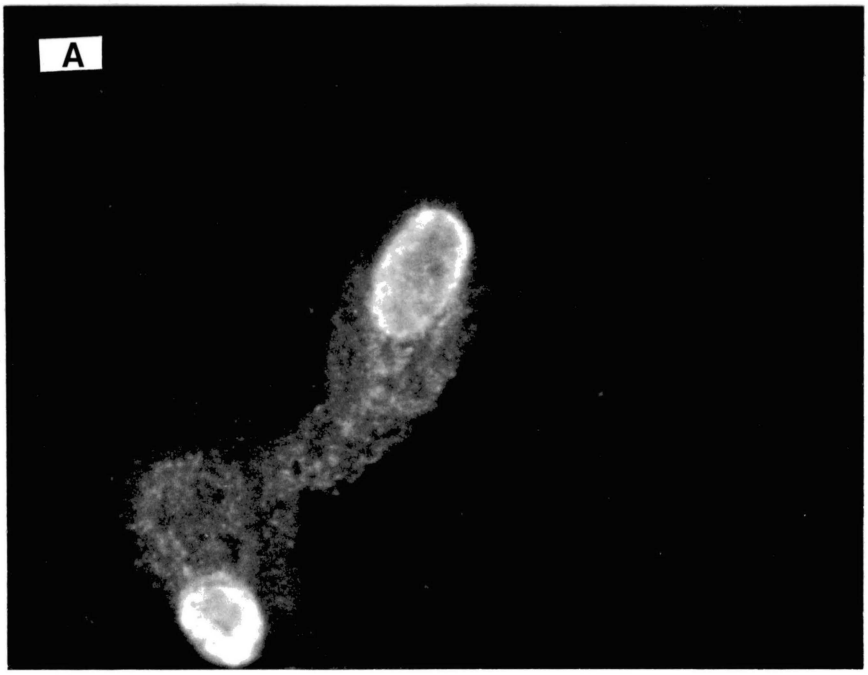
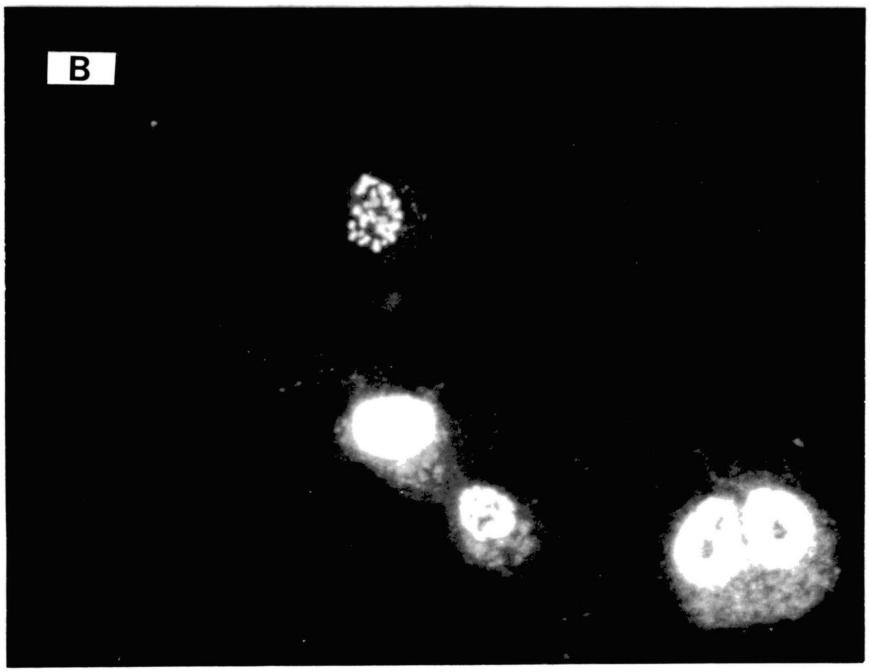



Fig 7A--Bovine kidney cells infected with the greater kudu isolate which contained both the diffuse and particulate antigens in the cytoplasm and intranuclear as detected by immunofluorescence. x600

B--Solid and particulate intranuclear antigens in virus-infected cells.



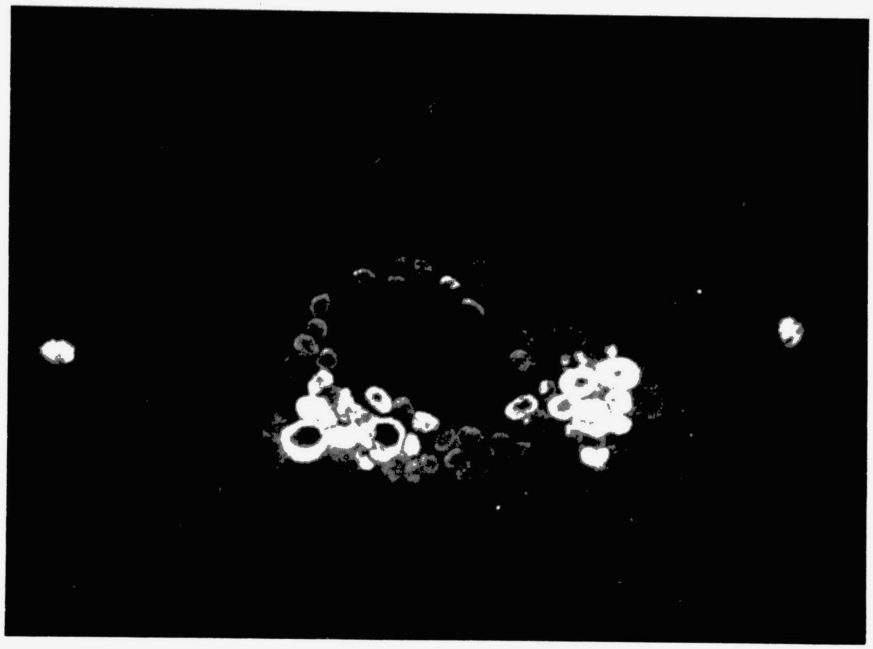
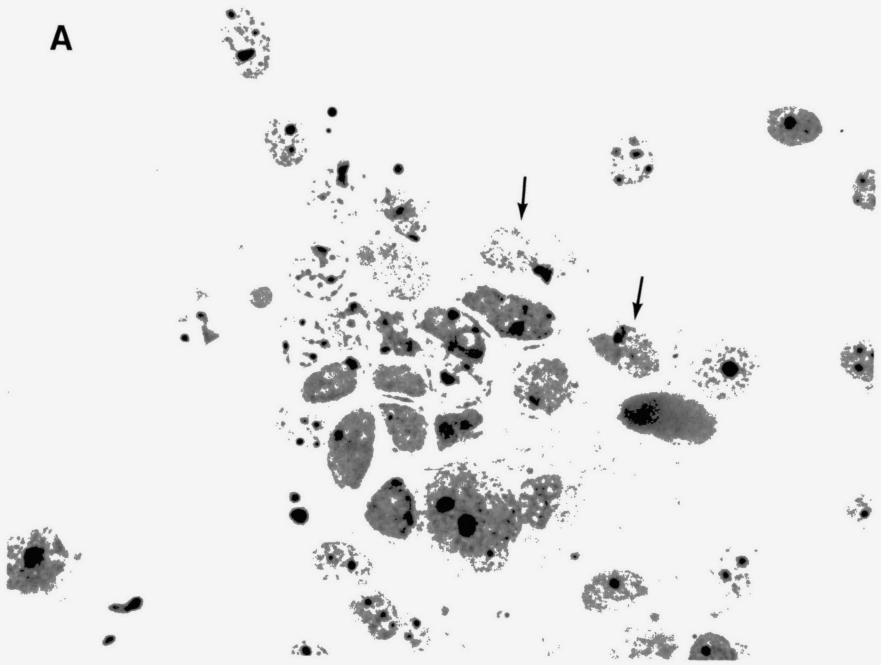


Fig 8--A fluorescent syncytium produced in BFK cells by the wildebeest-2 isolate of alcelaphine herpesvirus-1. x130

Fig 9A--Cytology in the development of intranuclear inclusion bodies by alcelaphine herpesvirus-1. A syncytium in which certain nuclei have ground-glass, granular texture of the nucleoplasm (arrows). Trichrome stain. x500



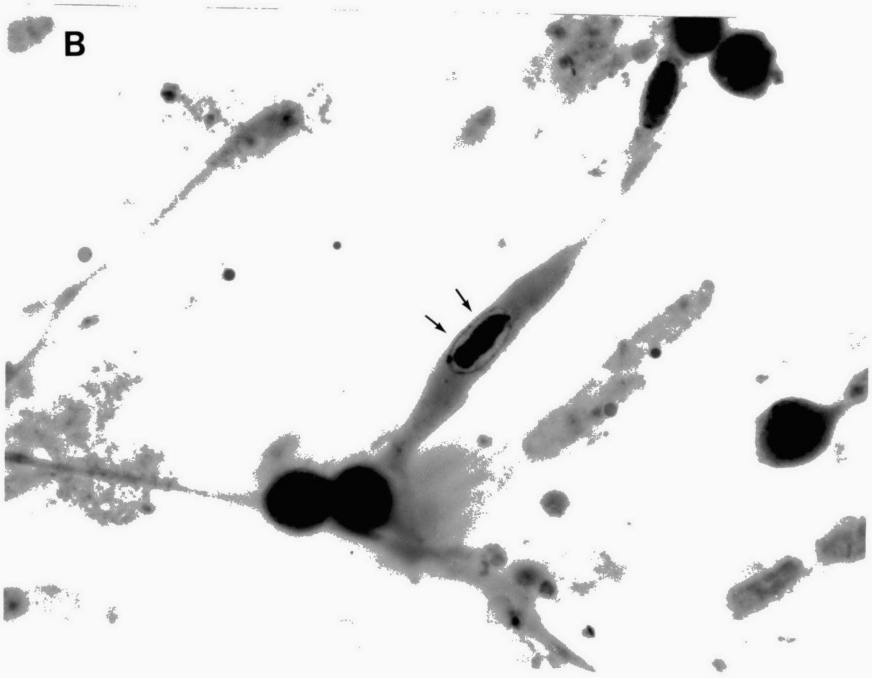
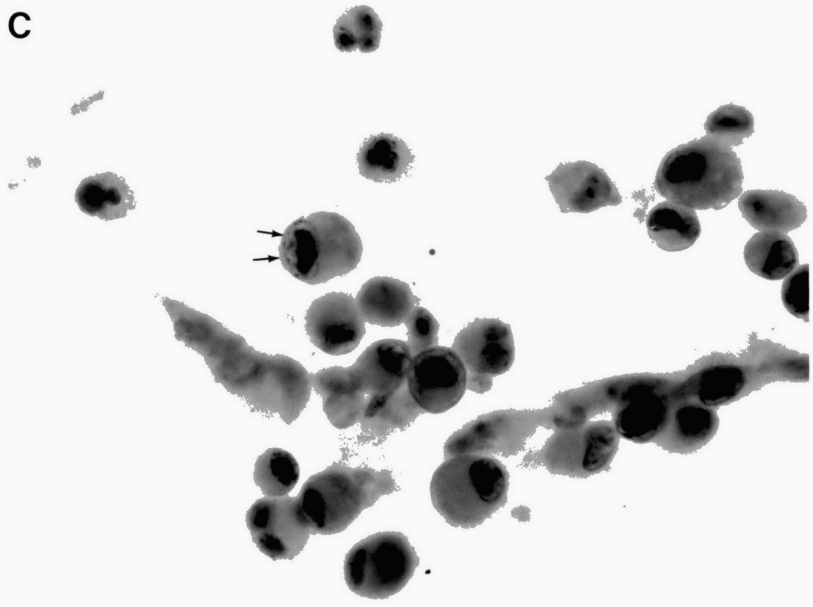



Fig 9B--A type A intranuclear inclusion body with clear halo (arrows). Trichrome stain. x600

C--Eccentric intranuclear inclusion bodies with beaded chromatin around nuclear membrane (arrows). Trichrome stain. x600



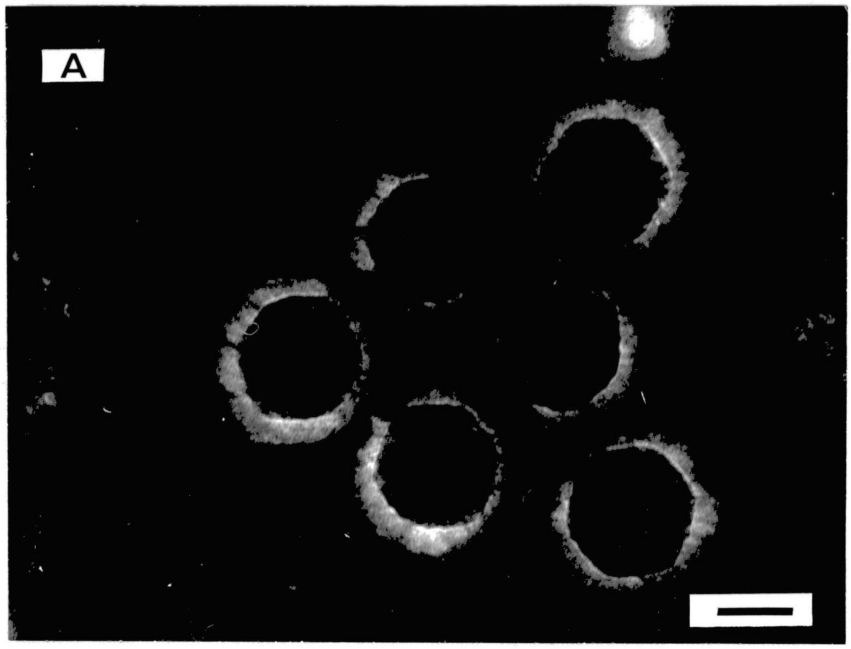
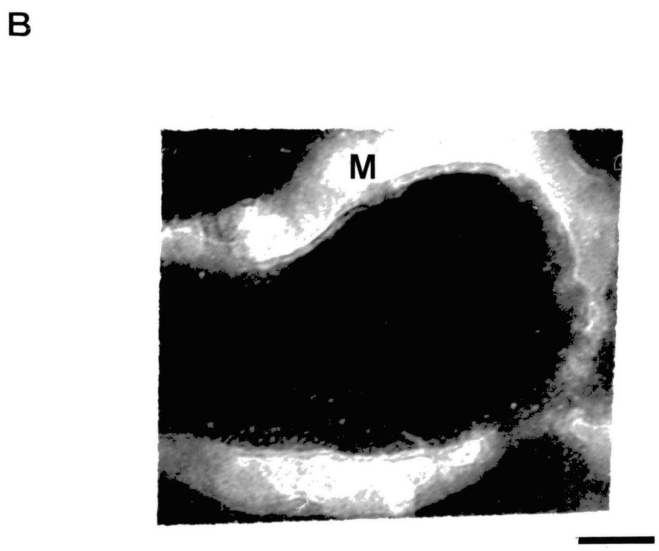



Fig 10A--Phosphotungstate negative-stain preparations of semi-purified the greater kudu isolate of alcelaphine herpesvirus-1. Hexagonal in shape of naked nucleocapsids with dark stained center indicating the absence of DNA. x167,000. Bar=50 nm.

B--Viral particles with tubular capsomeric structures (arrowheads) which are most prominent at surrounding surface of the particles. x168,000. Bar=50 nm. M=membrane.



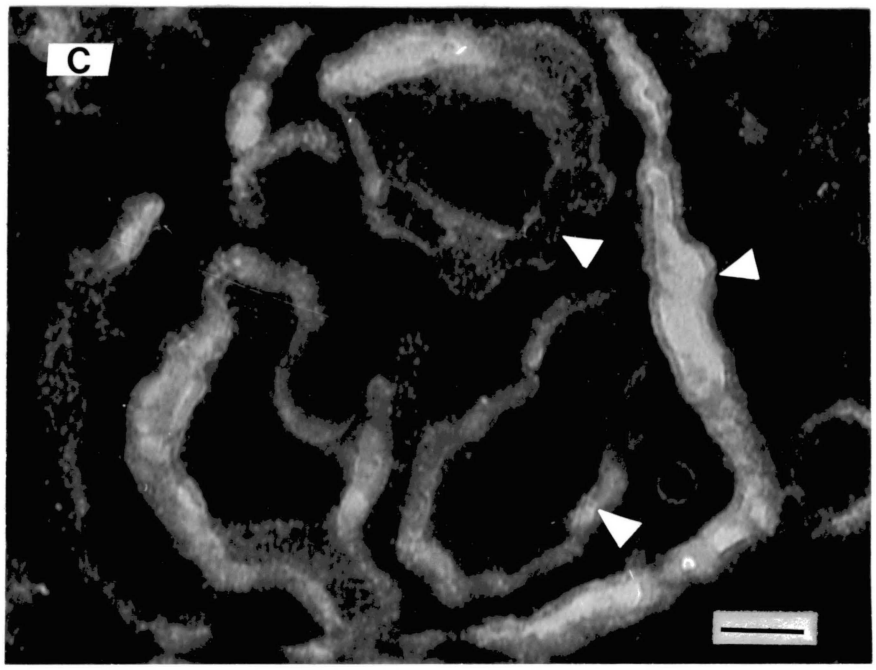
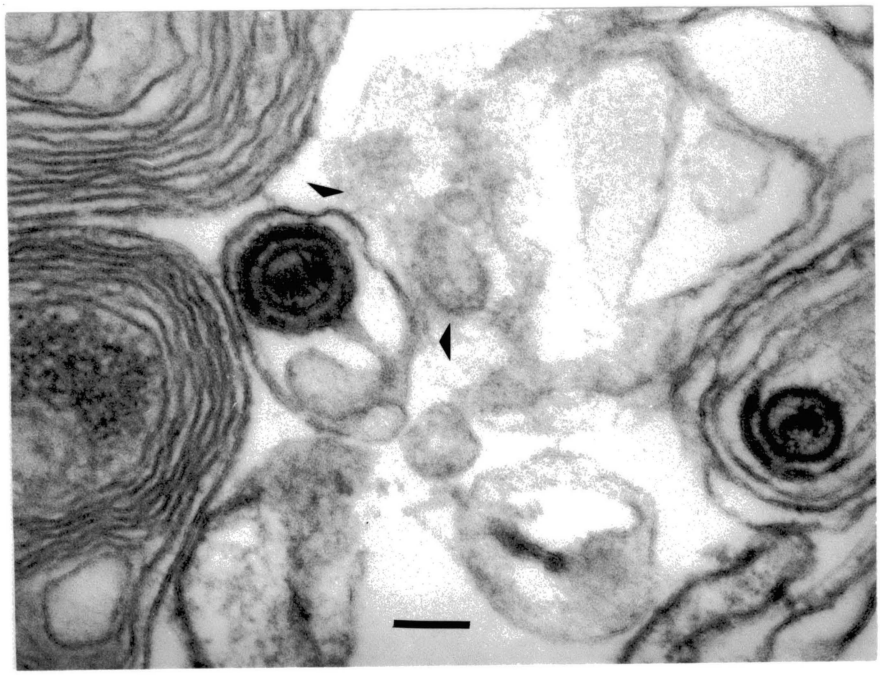



Fig 10C--Nucleocapsids surrounding by 2 membranes (arrowheads). x100,000. Bar=100 nm.

Fig 11--Enveloped nucleocapsid (arrowheads) in cytoplasm of BFK cells infected with subpassages 31 of the greater kudu isolate. x97,100. Bar=100 nm.



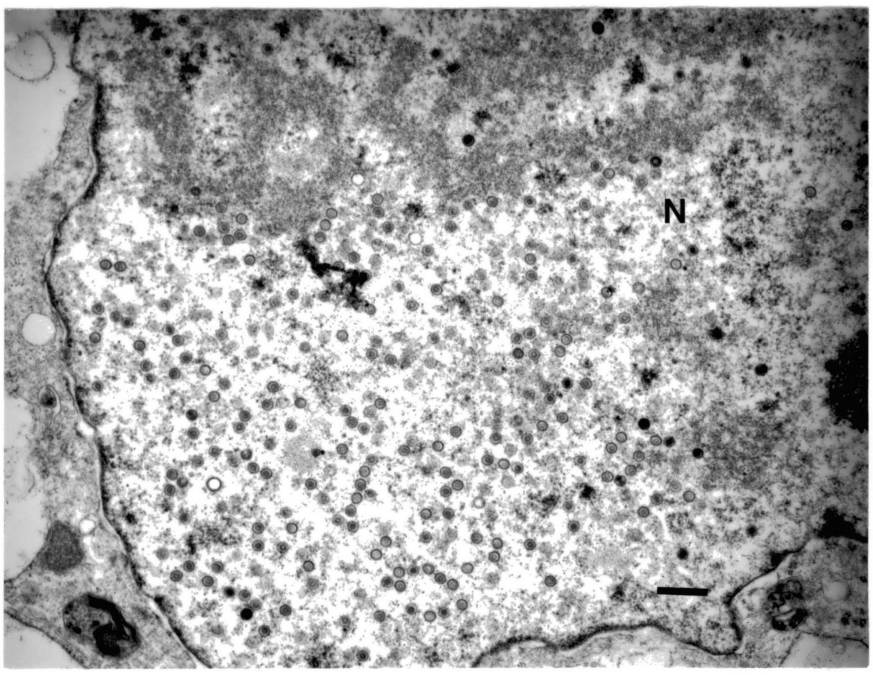
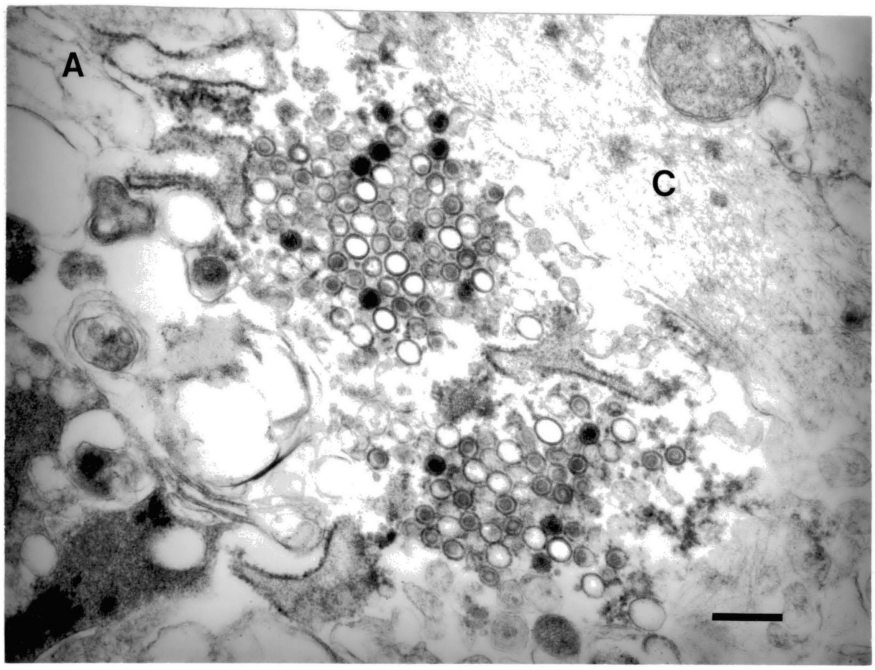



Fig 12--Immature herpesviral particles uniformily scattered within the nucleus of BFK cells infected with the greater kudu isolate of alcelaphine herpesvirus-1. x169,000. Bar=300 nm. N=nucleus.

Fig 13A--Subpassage 22 of the gaur isolate of alcelaphine herpesvirus in BFK cells. Presence of numerous nucleocapsids within the cytoplasm probably due to rupture in nuclear membrane. x29,800. Bar=300 nm. C=cytoplasm.



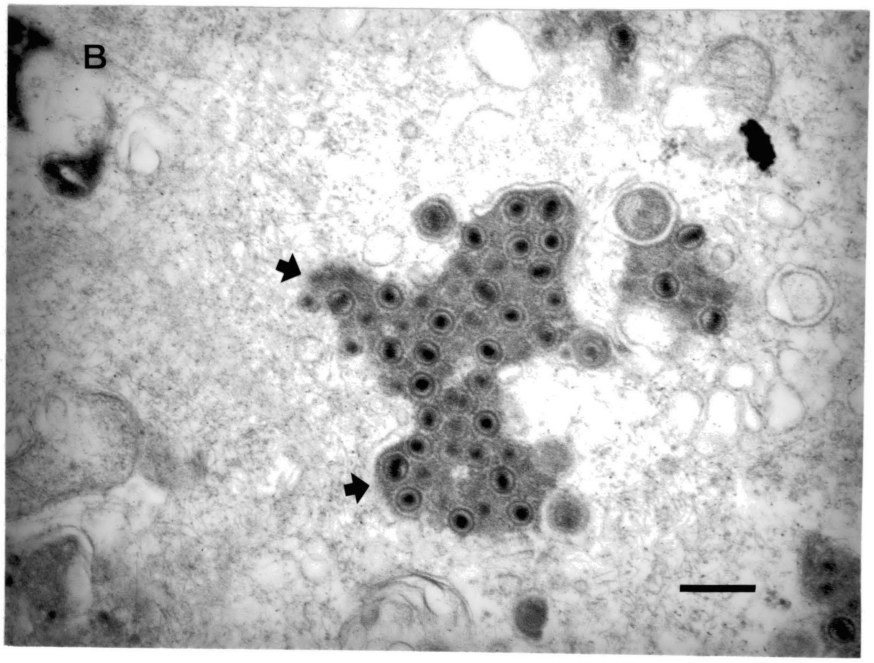
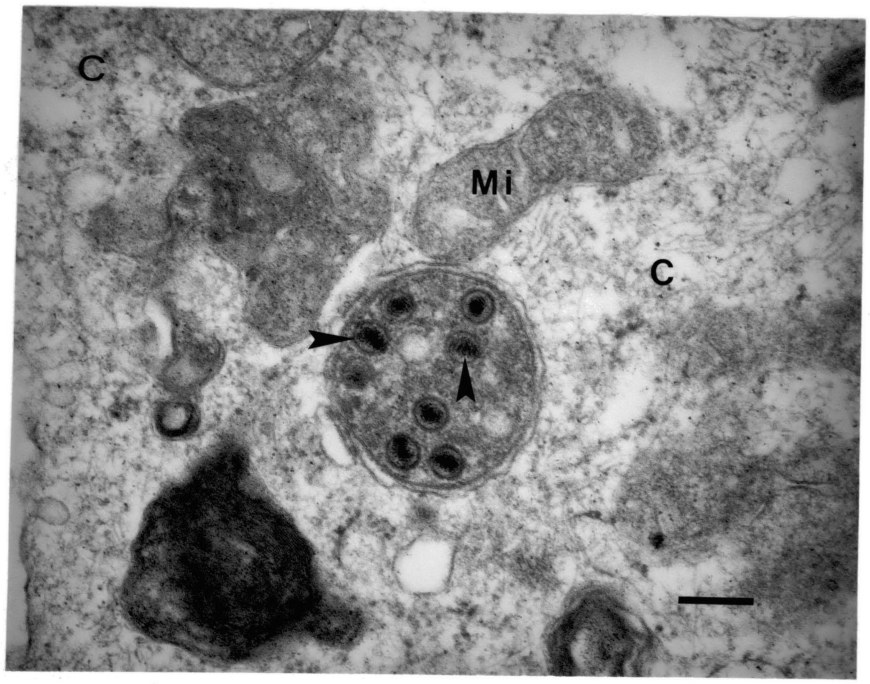



Fig 13B--An aggregation of viral nucleocapsids (arrows) within a dense-stain body in the cytoplasm. x38,000. Bar=300 nm.

C--Spool-like cores (arrowheads) composed of DNA which aggregated in a double membrane structure within the cytoplasm. x51,000. Bar=100 nm. C=cytoplasm. Mi=mitochondria.



## CHAPTER III

## EFFECT OF INTERFERONS ON THE REPLICATION OF THE ALCELAPHINE HERPESVIRUS-1 OF MALIGNANT CATARRHAL FEVER

Malignant catarrhal fever (MFC) is a fatal lymphoproliferative disease of cattle, deer and exotic ruminant species (7,16) and recently has been reported to be associated with tumors in a Formosan sike deer (8). The virus of MCF has been termed bovid herpesvirus 3 (17); however, recently a more appropriate classification as alcelaphine herpesvirus-1 has been proposed (1). The herpesvirus of MCF has certain biological characteristics which are similar to the herpesviruses of Epstein-Barr, Marek's disease, herpesvirus saimiri and ateles (9). Each of these herpesviruses is classified within the gamma herpesvirinae subfamily which as a group are primarily lymphotropic, highly cell-associated, usually produce latent infections in their natural hosts and may contain oncogenic properties (9,19).

In cattle of Africa, MCF occurs during the calving season of wildebeest. It is during this calving period that cell-free infectious virus is shed in both nasal and ocular secretions of neonates and wildebeest calves up to 3 months of age (14,17). Cell-free virus is not shed in adult wildebeest; however virus neutralizing (VN) antibody is present throughout their lives (18). The isolation of alcelaphine herpesvirus is accomplished by either direct cultivation of trypsinized cells from specific organs (i.e. spleen,

lung, lymph nodes, thymus) or by co-cultivation of buffy coat cells with bovine fetal kidney (BFK) or thyroid cells (16).

Although herpesviruses are considered relatively insensitive to interferons (IFN) and are themselves poor IFN inducers, recent experimental evidence has shown that specific herpesviruses are sensitive to IFN and may induce IFN production (5,10,11,13,20,21). Experiments with cell cultures have demonstrated that endogenous IFN does protect cells against infection by Epstein-Barr virus (13), Marek's disease virus (20) and pseudorabies virus (11). In the absence of specific antibody, interferon-like substances have produced persistent infections in cell cultures infected with herpesviruses (6).

Because information on the interaction between alcelaphine herpesvirus with IFN was lacking, we investigated whether isolates of alcelaphine herpesvirus-1 in cell cultures were sensitive to homologous and heterologous IFN and if isolates of alcelaphine herpesvirus-1 were inducers of IFN.

Viruses identified as alcelaphine herpesvirus-1 which had been isolated from a gaur (Bos gaurus), a greater kudu (Tragelaphus strepsiceros), (2) and two wildebeest calves (Connochaetes gnou), (3) were propagated in BFK cells. Passage of each virus was done by co-cultivation of MCF-infected cells with healthy BFK cells (2,3). Viral cytopathology (i.e. syncytia formation) was used as an indicator of virus replication. Culture fluids from the cellular monolayers infected with each of the four viral isolates were harvested at varying intervals between 0 to 120 hours. To assay for IFN production, supernatant fluids were initially diluted (1:5 v/v) in Dulbecco's medium and 0.5 ml was inoculated in duplicate onto confluent 48-hour

monolayers of BFK cells in a 24-well plate. The inocula were allowed to adsorb for 24 hours and removed by aspiration and then the cell monolayers were washed once with Dulbecco's medium. The activity of each harvest for IFN was determined by the IFN assay previously described by Epstein (4). The Indiana strain of vesicular stomatitis virus (VSV) was used at challenge concentration of 40 to 60 PFU/well in our IFN assay. The IFN titers of the test fluids were calculated by the method described by Epstein (4). In duplicate assays with each of the test fluids, we were unable to detect any IFN activity as determined by a greater than 50% reduction in PFUs of VSV. However, a reduction in the PFUs of VSV of less than 10% was measured in the supernantant fluids collected at 96 and 120 hours from cell cultures infected with wildebeest-1 viral isolate. Nevertheless, the level of inhibition of PFUs of VSV was considered to be nonspecific and not indicative of IFN. The fetal bovine serum (FBS) used in each assay had been previously tested for the presence of adventitious agents and no endogenous IFN was detected in fluids from uninfected BFK cell cultures.

The IFN used in this investigation were of bovine (BoIFN) and human (HuIFN) origin. The BoIFN were prepared by the infection of either cell line (MDBK) of bovine kidney or bovine alveolar macrophages (BAM) with Newcastle disease virus (NDV): these bovine-derived IFN were labeled BoIFN-NDV-MDBK and BoIFN-NDV-BAM, respectively. The characteristics of the BoIFN-NDV-MDBK and BoIFN-NDV-BAM IFN were reported by Fulton and Burge (5). The bovine IFN (BoIFN-$\alpha$-1) was

produced in E. coli cultures by recombinant DNA technology.[a] The human IFN (HuIFN-αA/D) was a recombinant produced hybrid IFN.[b]

Since low passages of cells infected by alcelaphine herpesvirus-1 contained mostly cell-associated virus and of the viral isolates used most were below 20 passages in cell culture, the measurement of the effect of each IFN preparation on alcelaphine herpesvirus-1 in BFK and bovine turbinate (BT) cells was done by counting virus produced fluorescent foci. Each focus of fluorescence was delineated as a fluorescent focus units (FFU). By this procedure, we compared cells infected with the 4 isolates of alcelaphine herpesvirus to similarly virus-infected cells treated with different IFN.

To conduct the IFN assay, cell monolayers of BFK infected by alcelaphine herpesvirus-1 were trypsinized and dispersed as single cells in Dulbecco's medium supplemented with 10% FBS and 200 ug/ml of gentamicin. The cells were then dispensed at 0.6 ml/well into a 4-chamber Lab-Tek slide. A 0.05 ml inoculum of 14 units of BoIFN-NDV-MDBK or 7.6 IFN units of BoIFN-NDV-BAM was simultaneously added into duplicate wells. Subsequently, the cells were incubated at 33 C in a humidified $CO_2$ incubator for 7 days. Following incubation, cell monolayers on each slide were washed with a fluorescent antibody (FA) buffer,[c] fixed for 10 minutes in cold acetone and rinsed once in distilled water. The fixed slides were air-dried and stored at -70 C until stained for immunofluorescence.

---

[a] BoIFN-α-1 was kindly supplied by Genetech, Inc., S. San Francisco, CA.

[b] HuIFN-αA/D was provided by Hoffman LaRoche, Nutley, NJ.

[c] DIFCO Laboratories, Inc., Detroit, MI.

The isolates of alcelaphine herpesvirus-1 tested for sensitivity to IFN included: the prototype strain of alcelaphine herpesvirus (WC-11) (16), virus isolates from a gaur and greater kudu (2) and 2 wildebeest neonates (3). The antiserum to alcelaphine herpesvirus-1 used in the indirect immunofluorescent antibody (IIF) assay was obtained from a gaur which died with clinical MCF (2). A rabbit antibovine immunoglobulin G (IgG) fluorescein-conjugate used in the IIF was obtained commercially.[d] The methodology used for the fluorescent staining and examination of immunofluorescent slides containing cells infected with alcelaphine herpesviruses have been described (3).

The effects of different IFN (BoIFN-NDV-MDBK and BoIFN-NDV-BAM) on the replication of alcelaphine herpesvirus-1 following treatment of BFK cells infected with each of 4 virus isolates are presented in Table 1. For each viral isolate, decrease of 62 to 81% in FFU were found after treatment of cells by BoIFN-NDV-MDBK at a concentration of 14 IFN units. Decrease in FFU of the 4 viruses from 50 to 80% were found after treatment of cells with BoIFN-NDV-BAM at a concentration of 7.6 IFN units (Table 1). These preceding results indicated that replication of each of the 4 isolates of alcelaphine herpesvirus-1 was partially inhibited by each IFN tested, however, the level of inhibition varied between viral isolates. The decreases in FFU for each virus following treatment by BoIFN-NDV-MDBK was greater than BoIFN-NDV-BAM but the concentration of BoIFN-NDV-BAM was 1/2 that used for BoIFN-NDV-MDBK. This finding suggests a greater sensitivity to BoIFN-NDV-BAM during the replication of each of the 4 alcelaphine herpesviruses.

---

[d]Miles Laboratories, Elkhart, IN.

The dose response of 3 of the alcelaphine herpesvirus with 2 IFN produced by recombinant DNA were also compared. Various concentrations of homologous (bovine) BoIFN-α-1 and a heterologous (human) HuIFN-αA/D were assayed against the WC-11 strain, the gaur and greater kudu isolates of the alcelaphine herpesvirus-1. At a concentration of 64 IFN units of either BoIFN-α-1 or HuIFN-αA/D, a 50% decrease in FFU of WC-11 strain was found in virus-infected BT cells (Fig. 1a). A 50% decrease in FFU of the greater kudu virus isolate occurred when infected cells treated with 64 IFN of BoINF-α-1. However, 480 IFN units of HuIFN-αA/D were required for a 50% reduction in FFU of this isolate (Fig. 1b). A concentration of 6.4 IFN units of BoIFN-α-1 produced a 50% decrease in FFU of the gaur isolate in BFK cells, but 16 IFN units of HuIFN-αA/D were required to produce the same 50% decrease in FFU (Fig. 1c).

Previously, we found (Wan, unpublished data) that the multiplicity of infection (m.o.i.) of the gaur isolate was less than those of the 3 other alcelaphine herpesviruses. This difference in m.o.i. could explain the variation in sensitivity of the isolates to the 2 recombinant IFN tested. Both low passage virus (gaur, greater kudu, and 2 wildebeest) and high passage of cell-free virus (WC-11 strain) were sensitive to IFN which suggested that cell-associated virus present at low passages was as susceptible to the effects of IFN as cell-free virus. In contrast, the cell-free murine cytomegalovirus, a herpesvirus, was found to be susceptible to inhibition by IFN but cell-associated virus was not (15).

Our findings indicated that 4 virus isolates classified as alcelaphine herpesvirus-1 did not induce measurable levels of IFN in BFK cells. However, the replication of each of the 4 isolates of

alcelaphine herpesvirus-1 in cell cultures was significantly inhibited by homologous (bovine) cell culture derived IFN and also by homologous (bovine) and heterologous (human) recombinant IFN. Although the successful inhibition of alcelaphine herpesviruses *in vitro* by these IFN is encouraging, we cannot presently equate this finding as to the potential *in vivo* activity of IFN. Nevertheless, because at present an effective method of control of MCF in ruminants in not available, the emergency treatment of an endangered exotic ruminant species with clinical MCF with either bovine or human-derived recombinant IFN should be considered.

References:


1. Castro, A.E., and W.P. Heuschele. 1985. Conference on malignant catarrhal fever. Bovine Practitioner 20: 162-168.

2. Castro, A.E., D.L. Whitenack and D.E. Goodwin. 1981. Isolation and identification of the herpesvirus of malignant catarrhal fever from exotic ruminant species in a zoologic park in North America. Am. Ass. Vet. Lab. Diag. 24th Ann. Proc., pp. 66-78.

3. Castro, A.E., E.C. Ramsay, J.F. Dotson, M.L. Schramke, A.A. Kocan and D.L. Whitenack. 1984. Characteristics of the herpesvirus of malignant catarrhal fever isolated from captive wildebeest calves. Am. J. Vet. Res. 45: 409-415.

4. Epstein, L.B. 1976. Assay of human immune interferon from lymphocyte-macrophage cultures by a virus plaque reduction method. In Manual of Clinical Immunology, pp. 120-128. N.R. Rose and H. Friedman Eds. Washington, D.C.: American Society for Microbiology.

5. Fulton, R.W. and L. J. Burge. 1985. Susceptibility of feline herpesvirus-1 and a feline calicivirus to feline interferon and recombinant human leukocyte interferons. Antimicrobiol Agents and Chemotherapy 28:698-699.

6. Glasgow, L. and K. Hable. 1963. Role of polyoma virus and interferon in a herpes simplex virus infection in vitro. Virology 19:328-339.

7. Heuschele, W.P. and A.E. Castro. 1985. Malignant catarrhal fever. In Comparative Pathobiology of Viral Diseases, Vol. 1, pp. 115-125. R.G. Olson, S. Krakowka, J.R. Blakeslee, Jr. Eds. CRC Press. Boca Raton, FL.

8. Heuschele, W.P., N.O. Nielsen, J.E. Oosterhuis and A.E. Castro. 1985. Dexamethasone-induced recrudescence of malignant catarrhal fever and associated lymphosarcoma and granulomatous disease in a Formosan sika deer (Cervus nippon taiouanus). Am. J. Vet. Res. 46: 1578-1583.

9. Hunt, R.E. and L.H. Billup. 1979. Wildebeest-associated malignant catarrhal fever in Africa: A neoplastic disease of cattle caused by an oncogenic herpesvirus. Comp. Imm., Micro. and Inf. Dis. 2: 275-283.

10. Letchworth, G.J., III and L.E. Carmichael. 1983. Production of interferon by bovine peripheral blood monocytes infected with bovid herpesvirus 2. Vet. Micro. 8: 69-80.

11. Libikova, H. 1973. Interferon in young and aged chick embryo cell cultures infected with herpes simplex and pseudorabies viruses. Acta Virologica 17: 464-471.

12. Ludwig, H. 1983. Bovine herpesviruses, in The Herpesviruses, Vol. 2, B. Roizman Ed. pp. 135-145. Plenum Press, NY.

13. Lvovsky, E., P. H. Levine, D. Fuccillo, D.V. Ablashi, Z.H. Bengall, G.R. Armstrong and H.B. Levy. 1981. Epstein-Barr virus and herpesvirus saimiri: Sensitivity to interferons and interferon inducers. J. Natl. Cancer Inst. 66: 1013-1019.

14. Mushi, E.Z., L. Karstad and D.M. Jessett. 1980. Isolation of malignant catarrhal fever virus from ocular and nasal secretion of wildebeest calves. Res. Vet. Sci. 29: 168-171.

15. Oie, H.K., J.M. Easton, D.V. Ablaski and S. Baron. 1975. Murine cytomegalovirus: Induction of and sensitivity to interferon in vitro. Inf. and Imm. 12: 1012-1017.

16. Plowright, W., R.D. Ferris and G.R. Scott. 1960. Blue wildebeest and the etiological agent of bovine malignant catarrhal fever. Nature 188: 1167-1169.

17. Plowright, W. 1965. Malignant catarrhal fever in East Africa. I. Behavior of the virus in free-living population of blue wildebeest (Grogon taurinus taurinus, Burchell). Res. Vet. Sci. 6: 56-68.

18. Plowright, W. 1967. Malignant catarrhal fever in East Africa. III. Neutralizing antibody in free-living wildebeest. Res. Vet. Sci. 8: 129-136.

19. Roizman, B. 1982. The Herpesviruses, Vol. 1, pp. 1-23. B. Roizman Ed. Plenum Press, NY.

20. Schat, K.A., J. Gonzalez and A. Solorzano. 1974. Interferon production by strains of herpesvirus associated with Marek's disease. Avian Disease 18: 531-535.

21. Vaczi, L., E. Horvath and G. Hadhazy. 1965/1966. Studies on the condition of interferon production by cells infected with herpesviruses. Acta Microbiologica Academiae Scientiarum Hungaricas 12: 345-349.

Table 1. Effect of Two Different Interferons on the Replication of Alcelaphine Herpesvirus-1 in BFK Cells

| Alcelaphine Herpesviruses Tested | Interferons Tested | | | | | |
|---|---|---|---|---|---|---|
| | BoIFN-NDV-MDBK[a] | | | BoIFN-NDV-BAM[b] | | |
| | Fluorescent Focus Unit (FFU) in BFK | | % Reduction in FFU | Fluorescent Focus Unit (FFU) in BFK | | % Reduction in FFU |
| | IFN Treated | Untreated | | IFN Treated | Untreated | |
| Gaur | 43.5[c] | 231.2[c] | 81.2 | 40.5[c] | 204.5[c] | 80.2 |
| Greater Kudu | 263.5 | 697.0 | 62.2 | 314.5 | 640.0 | 50.9 |
| Wildebeest-1 | 54.0 | 227.5 | 76.3 | 89.5 | 240.0 | 62.7 |
| Wildebeest-2 | 119.0 | 426.5 | 72.1 | 199.0 | 442.0 | 55.0 |

IFN=Interferons
a:14 IFN units/0.05 ml/well
b:7.6 IFN units/0.05 ml/well
c:mean of two IFN assays

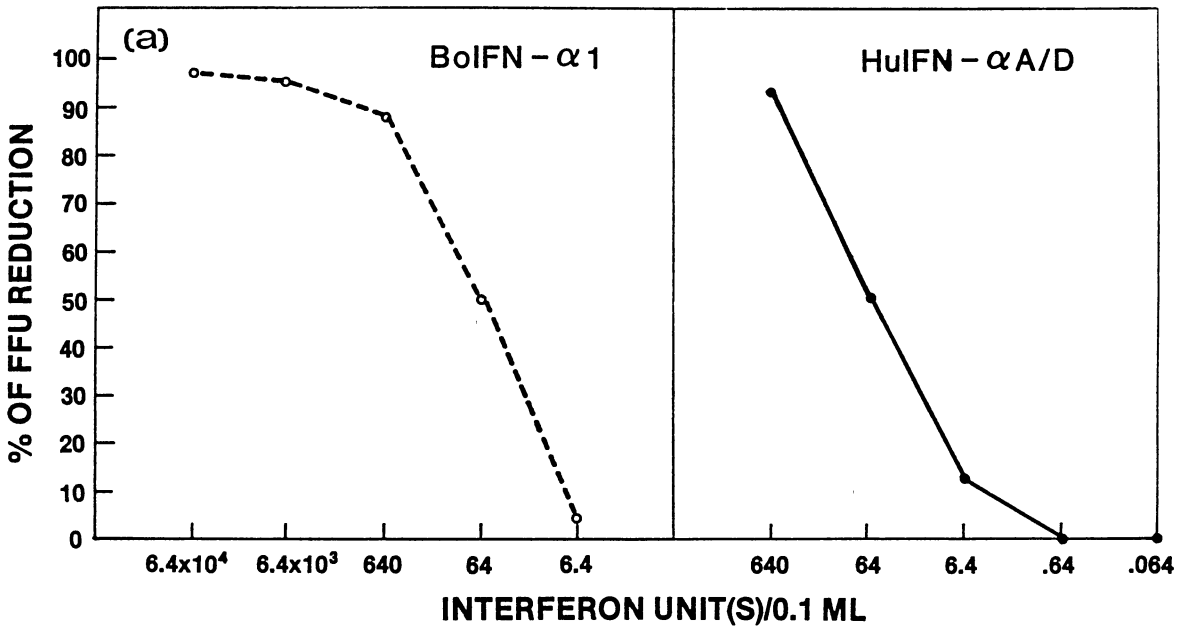
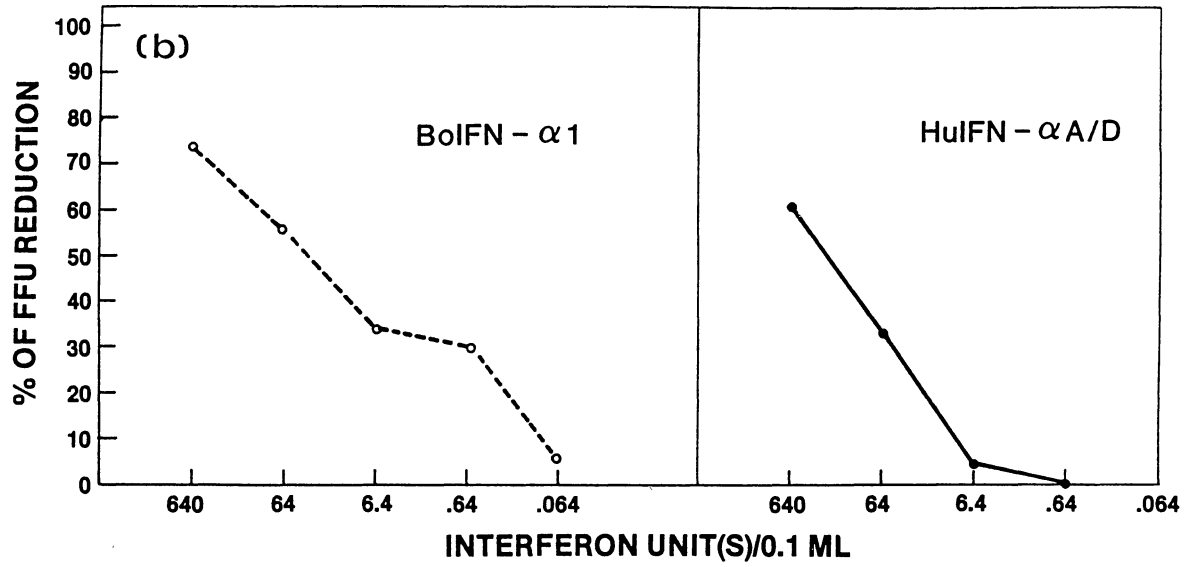
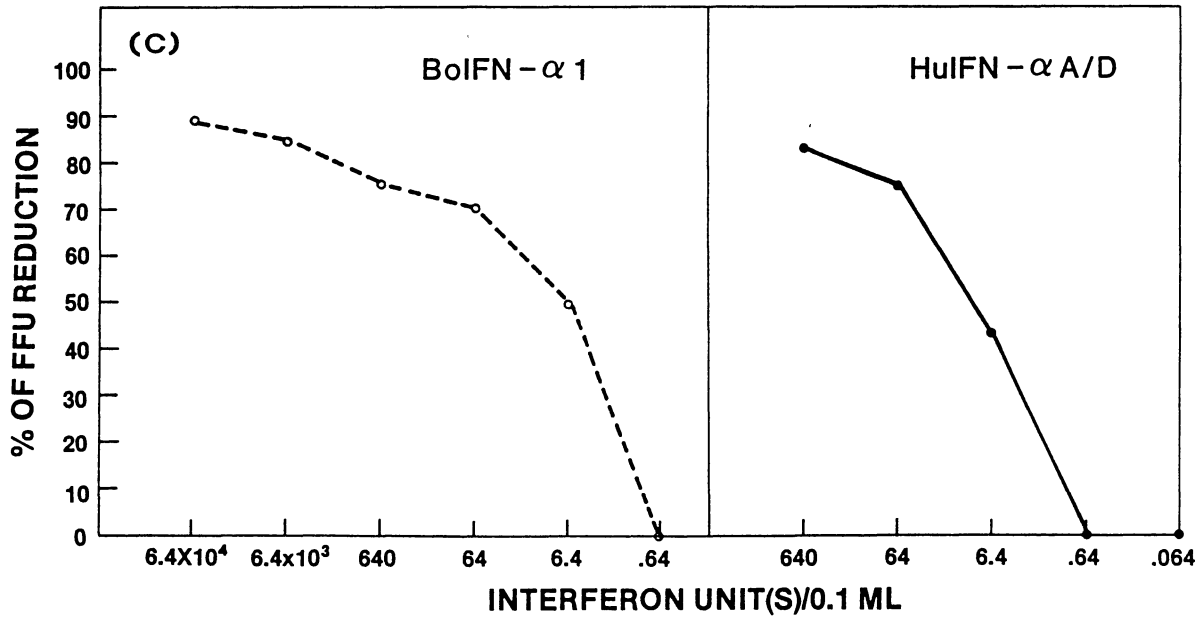



Fig 1. Effect of homologous and heterologous recombinant interferons on fluorescent focus formation in BFK cells infected with alcelaphine herpesviruses. (a) WC-11 strain, (b) greater kudu isolate, (c) gaur isolate.


(b)
BoIFN – α 1
HuIFN – α A/D
% OF FFU REDUCTION
100
90
80
70
60
50
40
30
20
10
0
640
64
6.4
.64
.064
INTERFERON UNIT(S)/0.1 ML


(c)
BoIFN – α 1
HuIFN – α A/D
% OF FFU REDUCTION
100
90
80
70
60
50
40
30
20
10
0
6.4X10⁴
6.4x10³
640
64
6.4
.64
.064
INTERFERON UNIT(S)/0.1 ML

CHAPTER IV

# SEROLOGIC EVIDENCE IMPLICATING DOMESTIC SHEEP AND GOATS AS CARRIERS OF THE ALCELAPHINE HERPESVIRUS-1 OF MALIGNANT CATARRHAL FEVER


## ABSTRACT

Sera from 403 domestic cattle, 260 sheep and 33 goats were examined by an enzyme-linked immunosorbent assay (ELISA) for antibodies to the alcelaphine herpesvirus-1 of malignant catarrhal fever (MCF). Of 173 sera from cattle documented as clinical cases of sheep-associated MCF, 40 (23.1%) were seropositive. In sera from clinically healthy sheep and goats, 41% of the sera tested contained antibodies to alcelaphine herpesvirus-1. A temporal rise in antibody to alcelaphine herpesvirus-1 in a calf inoculated with the WC-11 strain of alcelaphine herpesvirus was demonstrated by both ELISA and a virus neutralization (VN) test. By ELISA, a rise in antibody to alcelaphine herpesvirus-1 was also detected in each of the 2 lambs inoculated with an isolate of alcelaphine herpesvirus but not in the contact uninoculated lamb. The specificity of the ELISA was determined by antibody absorption experiments with alcelaphine herpesvirus-1. The findings of antibodies reactive with alcelaphine herpesvirus-1 of MCF in sera of domestic sheep, goats and MCF-affected cattle implicates this herpesvirus is the etiology of the sheep-associated form of MCF.

Malignant catarrhal fever (MCF) is a severe and highly fatal systemic herpesviral disease of several ruminant species which is characterized by a generalized vasculitis with lymphoproliferation in affected tissues (1). The disease has been described as wildebeest or sheep-associated MCF based on contact of susceptible ruminants with either of these carrier species (1). The disease rarely manifests itself clinically in either wildebeest or sheep.

The causative agent of wildebeest-associated MCF is an alcelaphine herpesvirus which was isolated from the blood of an asymptomatic wildebeest (2). The virulent cell-free alcelaphine herpesvirus has been detected in both nasal and ocular secretions of wildebeest neonates and calves less than 3 months of age which accounts for the transmission of MCF during calving in wildebeest herds (3).

In the United States, alcelaphine herpesvirus-1 has been isolated from an Indian gaur and a greater kudu which had clinical MCF and from 2 clinically healthy wildebeest calves located at the Oklahoma City Zoo (4). Subsequently, several herpesviruses which were serologically related to the alcelaphine herpesvirus-1 were isolated from clinical MCF cases and from apparently healthy ruminants (wildebeest, topi, and oryx) at the San Diego Zoo (5).

In outbreaks of MCF in England (6), the United States (7) and Australia (8), where wildebeest were not present lambing ewes have been implicated as carriers of the disease. However, the etiologic agent of sheep-associated MCF has not been isolated. By indirect immunofluorescence (IIF), antibodies to late viral antigens (IIF(L)) of MCF were detected in the sera of several flocks of sheep associated with clinical episodes of MCF in cattle and deer (9). Additionally,

antibodies to viral antigens (IIF(L)) were also demonstrated in sera of cattle which had experienced non-wildebeest-associated MCF (10).

In recent studies on sheep-associated MCF, an infectious cell line of T-lymphocytes has been established from a rabbit inoculated with specimens from red deer with clinical MCF (11). In the study on the pathogenesis of MCF in rabbits, the rabbit-derived T-lymphocyte cell line produced clinical signs and histopathological lesions similar to those seen in susceptible ruminants (1, 6, 7, 8). However, the infectious agent could not be found in the T-lymphocyte cell line by electron microscopy. Therefore, Reid et al. (11) hypothesized that the virus exists as an episomal DNA in a subpopulation of T-lymphocytes and causes a profound dysfunction of these cells. Furthermore, the viral-caused dysfunction then results in the production of a benign T-lymphocyte hyperplasia with an associated necrotizing vasculitis which arises from the indiscriminate natural killer activity of the altered lymphocytes.

We report on the development of an enzyme-linked immunosorbent assay (ELISA) for the measurement of specific antibodies to alcelaphine herpesvirus-1 in sera of domestic cattle, sheep and goats. We further describe the specificity of the ELISA for antibodies to alcelaphine herpesvirus-1 by absorption with uninfected and virus-infected bovine cells of sera from seropositive sheep, goats, a gaur and 2 experimental lambs.

The ELISA has been used to measure antibodies to the herpesviruses of pseudorabies (12), varicella-zoster (13), and Epstein-Barr (14). A modification (15) of the ELISA protocol developed for pseudorabies virus at the National Veterinary Service Laboratory (NVSL), Ames,

Iowa (12) was used (15) for the alcelaphine herpesvirus. To prepare the ELISA antigens (16), a modification of a procedure (17) for the extraction of nuclei from mammalian cells was used.

To determine the specificity of the ELISA for alcelaphine herpesvirus, serial bleedings from a calf inoculated at the NVSL with the WC-11 strain of alcelaphine herpesvirus (2) were examined by the ELISA and VN test (Fig. 1). An increasing antibody response to alcelaphine herpesvirus which began at day 9 was measured by each assay. To determine the immunologic response of sheep to the nuclei-extracted viral antigens and to infectious alcelaphine herpesvirus, 3 lambs (18) were inoculated after a preinoculation bleeding as follows: lamb A was inoculated in the prescapular lymph nodes with 1 ml of the nuclear extract from a greater kudu herpesvirus isolate (4) which was mixed with an equal volume of incomplete Freund's adjuvant; lamb B was inoculated intravenously with 1 ml washed BFK cells ($10^6$ cells/ml) which contained intracellular infectious herpesvirus from a greater kudu (4); lamb C was an uninfected contact control held in isolation with only lamb B (19).

During the 33 day observation period following the inoculations, each lamb was bled every 2 days. All lambs remained healthy without any demonstrable clinical signs or elevation in daily rectal temperature. Attempts at isolation of virus from each lamb from buffy coat cells, organ pools, the cornea, lymph nodes and the brain were uniformly negative. Examination by histopathology of selective tissues failed to reveal any lesions typical of MCF; however, lamb A had enlarged mesenteric lymph nodes. Lambs A and B had a measurable immunologic response and lamb A demonstrated an anamnestic reaction

after a booster injection at day 18. Lamb C in contact with lamb B, which had received infectious virus, remained seronegative. The conjugate used in the initial ELISA was antibovine IgG (Fig. 2A); however, when the lamb sera were assayed using an antisheep IgG conjugate the ELISA results were similar (Fig. 2B). Neutralizing antibodies to alcelaphine herpesvirus (WC-11 strain) were found in the 33 day sera from lamb A. In contrast, the other 2 lambs had no measurable VN antibody to alcelaphine herpesvirus.

To determine the specificity (20) of the ELISA, sera positive by ELISA were absorbed with either an alcelaphine herpesvirus-infected cell suspension or uninfected cells. Seropositive sera from 2 sheep, 2 goats, an Indian gaur with clinical MCF (4) and sera from the 2 experimentally inoculated lambs were used in the absorption experiments (21). The treated and untreated serum from each animal was diluted 1:20 with serum-conjugate dilution buffer prior to assay.

In Table 1, the specificity of antibody detected in sera of sheep and goats to alcelaphine herpesvirus-1 as shown by antigen absorption is presented. The percent reduction of antibody reaction in the ELISA was calculated by the following formula: (mean absolute absorbance of nonabsorbed serum - mean absolute absorbance of absorbed serum)/mean absolute absorbance of nonabsorbed serum x 100%. The ELISA reaction of the gaur serum was reduced 91% and 65% following absorption with cell pellets infected with the greater kudu and WC-11 isolate, respectively whereas sheep 1 serum was reduced 47% and 61% with the same treatment. Sera of sheep 2, goat 1, and 2 and experimental lambs A, B were also reduced 67%, 28%, 41%, 37%, and 51%, respectively after absorption with WC-11 infected cell. Because

herpesviruses contain host cell antigens in their surface (22), the reductions in the ELISA reactions seen in sera following absorption with uninfected cells was expected. However, these reductions were in all cases less than in sera treated with virus-containing cells.

A random sample of 260 sheep, 33 goats, and 403 cattle sera from field specimens were screened by ELISA for the antibody to alcelaphine herpesvirus (Table 2). Forty-one to 42 percent of the sera examined from sheep and goats had measurable antibody to alcelaphine herpesvirus. The 47 seropositive cattle sera, which had positive absorbances which ranged from 0.161 to 0.300 were from documented clinical cases of sheep-associated MCF (1, 7). A scatter plot of age versus the ELISA reaction on sera which represented 4 breeds of sheep is shown in Fig. 3. Based on the positive absolute threshold (0.150) established for cattle sera (23), sheep less than a year old were all negative by ELISA. Among the other age groups tested, a linear relationship was not found between age and a positive ELISA reaction. The sheep sera that had absorbance values above the positive threshold were in the age groups 1 to 8. This finding implied the horizontal infection of sheep with a herpesvirus that is immunologically related to alcelaphine herpesvirus-1. The affinity-purified commercial antibovine IgG used for the measurement of antibodies in sera of sheep and goats may not bind to sheep IgG as it does to bovine IgG and thus could account for false seronegative animals or miss animals with low antibody. In this study, the number of sera from sheep less than 1 year old were few, therefore, we could not conclude that all sheep in this age group are seronegative. Because sheep and goats do not develop clinical disease (24), a clinically negative criteria is not useful in the

establishment of positive ELISA threshold for sheep or goats. Therefore, based on prior experimental data, the positive threshold (0.150 absorbance units) established for sera from cattle with clinical MCF provided a reliable value to measure by ELISA an antibody response to alcelaphine herpesvirus in sheep and goats.

Historically, lambing ewes have been implicated as viral carriers of MCF in outbreaks of cattle or deer (1, 7). A high prevalence of antibodies to alcelaphine herpesvirus in sera from 10 flocks of sheep has been detected by an IIF test; but most of these sera were negative by the VN test (9). Our finding that sera from domestic sheep, and now in domestic goats, contained antibodies which bind specifically to antigens of the alcelaphine herpesvirus-1 of MCF in the ELISA further implies that both species may be infected with a herpesvirus similar to alcelaphine herpesvirus-1. Lamb B which received infectious virus was positive by ELISA but negative by VN test. This result was similar to the IIF findings (9). However, lamb A which was inoculated with nuclei extracted from infected cells developed VN antibody titer of 128 against 100-300 $TCID_{50}$ of alcelaphine herpesvirus (WC-11 strain). This result suggests that the neutralizing antigens of the virus may be enhanced by the extraction procedure. Lamb C remained seronegative throughout its contact with lamb B by the ELISA and VN test. This suggests the absence of horizontal transmission of MCF between the lambs. Nevertheless, VN antibody to alcelaphine hespesvirus has been detected in sera of 2 lambs experimentally inoculated (IV) with materials infective for MCF (9).

Alcelaphine herpesvirus-1 could not be isolated from sheep experimentally-inoculated with the WC-11 strain of alcelaphine

herpesvirus (9) which was verified by our findings. However, when blood from such experimentally-infected sheep was inoculated into a steer fatal MCF was produced and alcelaphine herpesvirus was isolated (9). The reduced immune response as determined by ELISA and the absence of an anamnestic response in lamb B suggests that alcelaphine herpesvirus may not replicate in sheep. Thus nonpermissive cells in hosts of sheep and goats may undergo a latent infection which are common with herpesviruses (22) and alcelaphine herpesvirus may exist in an unexpressed form.

The reported ELISA has been shown to be specific for the detection of antibodies to alcelaphine herpesvirus-1 infected cells as opposed to noninfected cells. Because herpesviruses can carry host cell antigens on their surface due to budding through cellular membranes (22), further purification of the plate binding antigens will be necessary. However, detergents that commonly are employed for the extraction (17) may alter the binding characteristics of either ELISA antigen onto the plates or antibody to antigen. To establish the relationship between alcelaphine herpesvirus and the virus of sheep-associated MCF, molecular procedures such as a DNA probe prepared from alcelaphine herpesvirus will be required.

References and Notes:


1. R. Daubney, J. R. Hudson, J. Comp. Path. 49, 63 (1936); S. E. Piercy, Brit. Vet. J. 108, 35 (1952); W. Plowright, JAVMA 152, 795 (1968); R. E. Pierson, J. Storz, A. E. McChesney, D. Thake, Am. J. Vet. Res. 40, 1091 (1979).
2. W. Plowright, Nature 188, 1167 (1960).
3. E. Z. Mushi, L. Karstad, D. M. Jessett, Res. Vet. Sci. 29, 168 (1980).
4. A. E. Castro, D. L. Whitenack, D. E. Goodwin, Amer. Ass. Vet. Lab. Diag. 24th Ann. Proc. p. 67 (1981); A. E. Castro, G. G. Daley, M. A. Zimmer, D. L. Whitenack, J. J. Jensen, Am. J. Vet. Res. 43, 5 (1982); A. E. Castro, E. C. Ramsay, J. F. Dotson, M. L. Schramke, A. A. Kocan, Am. J. Vet. Res. 45, 409 (1984).
5. W. P. Heuschele, the United States Animal Health Association, Proc. 86, 552 (1982), and Ibid. III. International Symp. Vet. Lab. Diagnostician, pp. 707-713 (1983).
6. R. A. Huck, A. Shand, P. J. Allsop, A. B. Paterson, Vet. Rec. 73, 457 (1961).
7. R. B. Murray, D. C. Blood, Can. Vet. J. 8, 277 (1961); C. J. Mare, The United States Animal Health Association, Proc. 81, 152 (1977).
8. W. A. Snowdon, Annual reports of the Division of Animal Health, SCIRO, Melbourne, Australia (1967-1973).
9. M. Kalunda, Ph.D. Thesis. Cornell University, p. 41 (1975); P. B. Rossiter, J. Comp. Path. 91, 303 (1981).
10. P. B. Rossiter, J. Comp. Path. 93, 93 (1983).
11. H. W. Reid, D. Buxton, I. Pow, J. Finlayson, E. L. Berrie, Res. Vet. Sci. 34, 109 (1983); H. W. Reid, D. Buxton, Proc. Royal Society of Edinburgh 82B, 261 (1984).

12. M. L. Snyder and G. A. Erickson. A procedural guide. USDA, APHIS, NVSL, P. O. Box 844, Ames, Iowa 50010 (1982).

13. J. Shanley, M. Myers, B. Edmond, R. Steele, J. Clinical Micro. 15, 208 (1982).

14. R. F. Hopkins, III, T. J. Witmer, R. H. Neubauer, H. Rabin. J. Inf. Dis. 146, 734, (1982).

15. In each ELISA, 2 preparations of antigens (alcelaphine herpesvirus and uninfected BFK cells) were used. By titration, the optimal concentration of each antigen and conjugate used were determined. To standardize the ELISA, a seropositive gaur serum was used which had an absolute absorbance of 0.900 to 1.200. Sera from healthy cattle which had an absorbance of less than 0.150 were used as negative control. To each of 96 wells of an Immunlon I plate (Dynatech Laboratories Inc.), 100 ul of coating antigen was added as follows: the nuclei-virus extract positive antigen was added vertically to all odd numbered rows and negative host cell antigen to all even numbered rows. The coated plates were then sealed in plastic bags and placed at 37 C for 3 hours on a Dynatech rotational rocker (Tek-Tato-V) at a speed setting of 60 rpm. Prior to their use, the coated plates were transferred to a 4 C refrigerator overnight. To assay for antibody to alcelaphine herpesvirus in each sera, sera were diluted 1:20 in a serum-conjugated diluent (0.15 M NaCl, 0.001 M EDTA, 0.05 M Tris base) which contained 1% gelatin and 0.05% Tween 20 (pH 7.4). For the assay, coated plates were washed 3 times in an automatic washer (Dynatech Dynawasher I) with a wash buffer (0.01 M phosphate buffer, 0.05% Tween 20,

pH 7.2). After removal of residual buffer, 100 ul of each test serum was added into each of 4 wells of positive and negative antigens on the same plate. On each plate, a positive, negative and diluent control was similarly added. The mixtures were reacted for 30 minutes at 37 C on a rocker set at 60 rpm. Following incubation, each test plate was washed 3 times followed by at room temperature rocking (setting 90 rpm) for 3 minutes. The residual buffer was removed, then 50 ul of a titrated dilution of conjugate (horseradish peroxidase-labeled affinity purified goat antibovine IgG, Kirkegaard & Perry Lab., Inc.) was added. The conjugate was prepared in serum-conjugate buffer (pH 7.4) which contained 1% melt gelatin and 0.05% Tween 20. The reaction mixture was rocked for 30 minutes at 37 C (60 rpm), then the plates were washed 3 times. To each well was then added 50 ul of a peroxidase substrate system ABTS (Kirkegaard and Perry Lab. Inc.) and plates were placed at room temperature for 20 minutes. The enzyme substrate reaction (color) was stopped with 50 ul of 1:400 dilution of hydrofluoric acid. Wells were read immediately on an automatic reader (MR580, Micro ELISA Auto Reader, Dynatech) at dual wave length of 405 nm with reference of 450 nm. The differences between the absolute absorbance values between adjacent 4 wells per serum reacted with positive and negative antigens were collected, and the mean and standard deviation of each serum were determined. The presence of antibody in a serum was determined by the mean for each unknown serum divided by the mean of a positive MCF-serum. Based on previous experimental data of the mean of 88 sera from healthy cattle, a

positive threshold of 0.150 was derived which was 1 standard deviation of the mean of the absolute absorbance of the 88 cattle sera.

16. Bovine fetal kidney (BFK) cells grown in 75 $cm^2$ flasks which contained a multiplicity of infection of $4.8x10^{-2}$/cell of a greater kudu isolate of alcelaphine herpesvirus were harvested by trypsinized and washed twice with Dulbecco's medium. Cells were pelleted at 500 x g for 2 minutes in a non-refrigerated centrifuge. To the pellet of cells, 10 ml of hypotonic buffer (0.01 M NaCl, 0.01 M Tris HCl, pH 7.4, 0.001 M $MgCl_2$) was added. The pellet was kept in the hypotonic buffer for 5 to 10 minutes and then spun at 500 x g for 2 minutes. The pellet was then added 2 ml of hypotonic buffer and the cell suspension was transferred into a Dounce homogenizer. Cells were ruptured by 30 strokes with the pestle. The homogenate of cells was transferred into a 15 ml centrifuge tube and centrifuged for 2 minutes at 500 x g. The upper layer was gently removed using a Pasteur pipette and the pellet was resuspended in 10 ml of hypotonic buffer and centrifuged twice at 500 x g for 2 minutes. The extracted preparation which consisted primarily of nuclei as determined by light microscopy was sonicated for 1.5 minutes in a Biosonik IV at the maximum setting. Maximal disruption of nuclei and release of virions was determined by electron microscopy of the sonicated preparation.
17. S. Penman, In Fundamental Techniques in Virology, K. Habel, N. P. Salzman, Eds, p. 35 (1969).
18. Dorset lambs (3 wethers) 3 to 4 months of age which were kept on a high protein diet were purchased from the Department of Animal

Science at the Oklahoma State University and confined in 2 animal isolation rooms of a $P_3$ isolation facility located in the OADDL.

19. $P_3$ facility. Richardson, J. H., Barkley, W. E.: Proposed Biosafety Guidelines for Microbiological and Biomedical Laboratories. Atlanta, GA, Manual of the U.S. Department of Health and Human Services, Public Health Service, Center for Disease Control, Office of Biosafety, 1983.
20. B. B. Gerstman, D. T. Cappucci, JAVMA 188, 248 (1986).
21. Bovine fetal kidney cells infected with greater kudu isolate and bovine turbinate (BT) cells infected with WC-11 strain of alcelaphine herpesvirus-1 and uninfected BFK and BT cells were trypsinized and washed twice with PBS (pH 7.4). Each cell preparation which contained approximately $4x10^6$ cells was pelleted in a glass test tube and 50 ul of a test serum was added. Sera and cells were mixed then incubated at 37 C on a rocker for 1 hour followed by an overnight absorption at 4 C.
22. H. Ludwig, In: The Herpesviruses, Vol. 2, B. Roizman, Ed., p. 135 (1983); B. Roizman, In: The Herpesviruses, Vol. 1, B. Roizman, Ed., p. 1 (1982).
23. S. K. Wan, A. E. Castro, The 65th Conference of Research Workers in Animal Disease, p. 13 (1984).
24. W. P. Heuschele, A. E. Castro, In: Comparative Pathobiology of Viral Diseases, Vol. 1, R. G. Olson, S. Krakowka, J. R. Blakeslee, Jr., Eds., p. 155 (1985).
25. This research which is a portion of S. K. Wan's PhD thesis was partially supported through a grant from the Oklahoma Zoological Society of the Oklahoma City Zoo. The author thanks

Mr. L. Curtis, Director and Dr. Ed. Ramsay for making this award possible and Dr. D. E. Goodwin, Director of the Oklahoma Animal Disease Diagnostic Laboratory for providing the isolation facility.

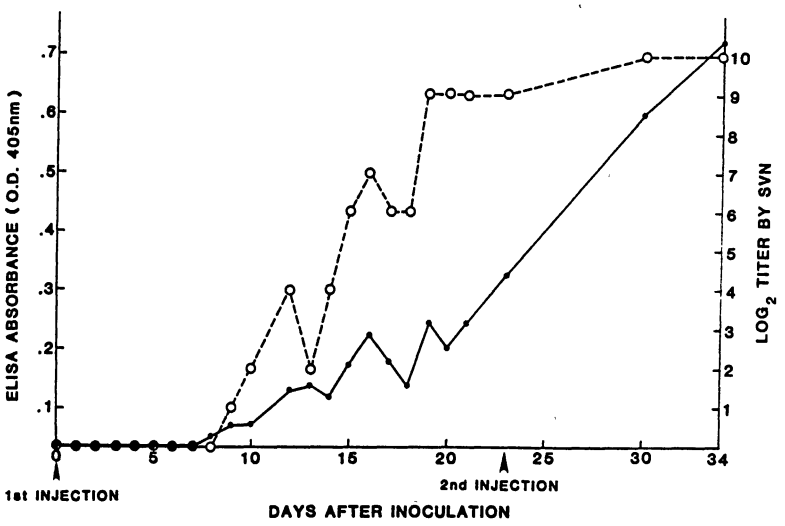



Fig 1. Comparison of the immunologic response as measured by ELISA and SVN test of a calf inoculated intravenously with cell-free WC-11 strain of alcelaphine herpesvirus-1. Serial bleedings and SVN data were provided by Dr. C. Metz of the National Veterinary Services Laboratories, Ames, Iowa. (●) ELISA results, (○) SVN results.

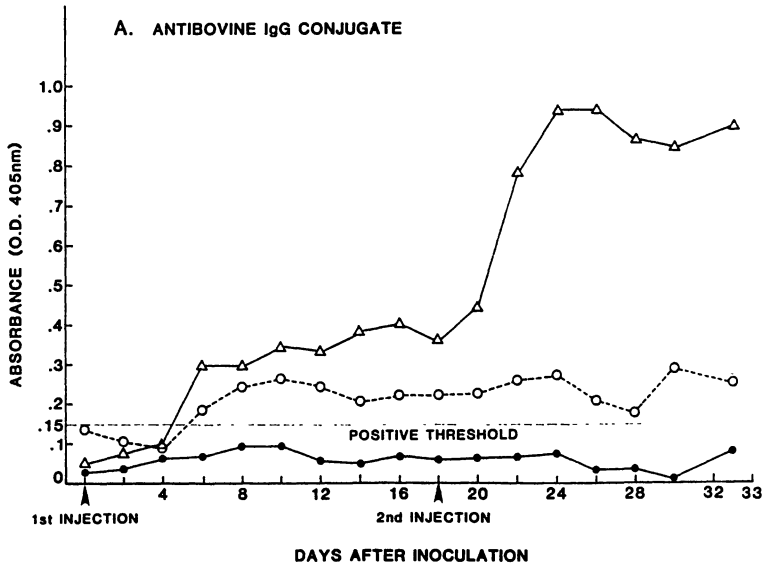



Fig 2. Comparison of the ELISA reaction with sheep sera using antibovine and antisheep IgG conjugates.

A. Serial immunologic response in lambs inoculated with infectious and extracted alcelaphine herpesvirus-1 (greater kudu isolate) as measured by ELISA. Lamb A (△) was inoculated with a nuclei extract from virus-infected-BFK cells. Lamb B (○) was inoculated with BFK cells containing infectious alcelaphine herpesvirus. Lamb C (●) was an uninfected animal housed with lamb B.

The conjugate used at a 1:1700 dilution was a peroxidase-labeled goat antibovine IgG (Kirkegaard & Perry Laboratories Inc.). An anamnestic response is seen at 22 days post-inoculation in lamb A after 2nd identical dose at day 20 (arrowhead).

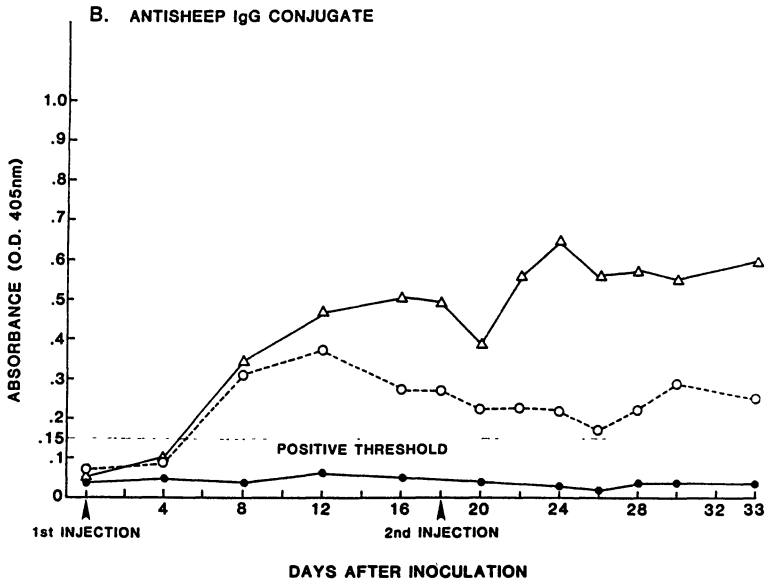



Fig 2B. Determination of immunologic response in lambs A, B, C as measured by ELISA using peroxidase-labeled rabbit antisheep IgG (Kirkegaard & Perry Laboratories Inc.) at dilution of 1:100. The calculated working concentration of commercial peroxidase-labeled antisheep IgG was higher than antibovine IgG. The pattern of reaction by ELISA was similar to that found with antibovine IgG conjugate; however, higher absolute absorbances were found between days 8 to 16 with sheep conjugate. An anamnestic response was also demonstrated in lamb A within 2 days following 2nd inoculation.

Table 1. Specificity of antibody as determined by ELISA to alcelaphine herpesvirus-1 following absorption of sheep and goat sera

| Species | Treatment of Sera | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | None | WC-11-infected BT Cells | | BT Cells Uninfected | | Greater kudu-Infected BFK Cells | | BFK Cells Uninfected | |
| | AA* | AA | % | AA | % | AA | % | AA | % |
| Gaur | 0.970 | 0.339 | 65 | 0.824 | 15 | 0.086 | 91 | 0.684 | 29 |
| Sheep-1 | 0.399 | 0.148 | 61 | 0.356 | 11 | 0.212 | 47 | 0.397 | 1 |
| Sheep-2 | 0.169 | 0.055 | 67 | 0.112 | 33 | | ND | | |
| Goat-1 | 0.443 | 0.318 | 28 | 0.378 | 15 | | ND | | |
| Goat-2 | 0.234 | 0.139 | 41 | 0.222 | 5 | | ND | | |
| Lamb A | 0.590 | 0.487 | 37 | 0.702 | 0 | | ND | | |
| Lamb B | 0.248 | 0.121 | 51 | 0.248 | 0 | | ND | | |

*Mean of 4 replicate wells of absolute absorbance from positive antigen well minus absolute absorbance from negative well.

%=percent reduction in AA by ELISA as calculated by $\frac{\bar{X} \text{ AA nonabsorbed} - \bar{X} \text{ AA absorbed}}{\bar{X} \text{ AA nonabsorbed}} \times 100\%$

Conjugate used in this experiment was peroxidase-labeled goat antibovine IgG.

ND:Not Determined.

Table 2. Antibodies to alcelaphine herpesvirus-1 in domestic ruminant species as determined by ELISA

| Species | Diagnosis of MCF | No. Animals | ELISA Results | | |
|---|---|---|---|---|---|
| | | | No. Positive | No. Negative | % Positive of Total |
| Cattle | No | 230 | 11 | 219 | 4.8 |
| | Yes | 173 | 40 | 133 | 23.1 |
| Sheep | No | 260 | 108 | 152 | 41.5 |
| Goat | No | 33 | 14 | 19 | 42.4 |

Sera Assay by ELISA against antigens of greater kudu herpesvirus.

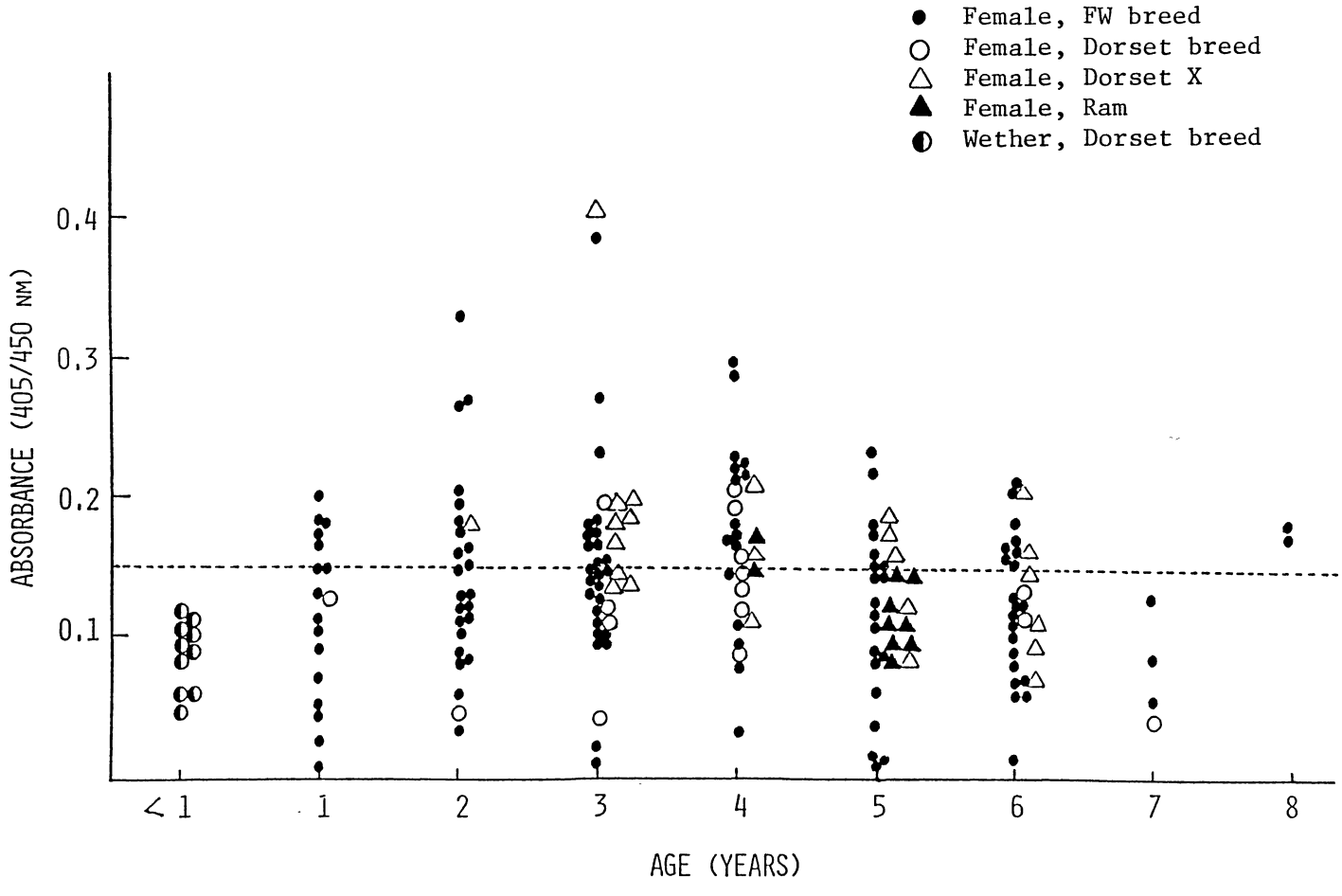



Fig 3. A scatter plot of the relationship of age of sheep to a positive ELISA for antibody to alcelaphine herpesvirus.

CHAPTER V

# AN ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE DETECTION OF ANTIBODIES TO THE ALCELAPHINE HERPESVIRUS OF MALIGNANT CATARRHAL FEVER IN EXOTIC RUMINANTS


## Summary

An enzyme-linked immunosorbent assay (ELISA) for the measurement of antibodies to the alcelaphine herpesvirus-1 of malignant catarrhal fever (MCF) was developed. A total of 216 sera which represented 42 exotic ruminant species were each assayed by the ELISA and a serum-virus neutralization (SVN) test. A statistically significant correlation ($r=0.564$, $p < 0.001$, $n=216$) between the ELISA and SVN was found. Of the sera tested positively by SVN, 86.1% were positive by the ELISA, and all the sera tested negatively by SVN, 83.9% were negative by the ELISA. The antibody detected by the ELISA was further correlated with clinical signs of MCF and the isolation of herpesviruses. The ELISA in conjunction with the SVN assay should enable zoos to conduct large scale seroepidemiologic surveys for MCF to ascertain the serological status of exotic ruminant species prior to their breeding or shipment.


## Introduction

Malignant catarrhal fever (MCF) is a highly fatal herpesviral disease of domestic and exotic ruminant species (1). The disease has been classified as wildebeest-associated or sheep-associated MCF

based on the suspected source of infection (2). Clinical MCF has been reported in 32 different zoological species within the United States (3, 4). The disease has also been reported in exotic ruminants in Canada (5), England (6), New Zealand (7), Africa (8), Malaysia (9), India (10), and Indonesia (11). Clinical outbreaks of MCF have usually coincided with the calving or lambing season of wildebeest or sheep, respectively (2). At the San Diego Zoo (SDZ) and the San Diego Wild Animal Park (SDWAP) from 1974 to 1983, 70 clinical cases of MCF have been documented (12). An outbreak of MCF was also documented at the Oklahoma City Zoo (OKZ) between 1979 and 1981 (13, 14).

To ascertain the MCF carrier status in an animal and to confirm a MCF infection in susceptible species, immunofluorescence and SVN assays have been used (15). The SVN test for antibodies to alcelaphine herpesvirus is virus specific (15); however, the SVN test requires a 10-to-12 day incubation period and requires the use of a cytopathic viral strain of MCF. An immunofluorescent serological assay has been used to measure antibody to alcelaphine herpesvirus in zoological ruminants, but the results have shown that antibodies to other herpesviruses of the bovine (i.e., infectious bovine rhinotracheitis, IBR; bovine herpes mammilitis, BHM; and DN599) cross-reacted with antigens of alcelaphine herpesvirus (3). Other serological assays such as complement fixation test (16), immunodiffusion and counter immunoelectrophoresis (17), or immunoperoxidase (18) have been used with moderate success to measure specific antibodies to alcelaphine herpesvirus.

In this report we present data on the development of an ELISA to alcelaphine herpesvirus-1 and a comparative study of the efficiency of the ELISA and SVN tests in the measurement of antibodies to alcelaphine herpesvirus-1 in 42 zoological species.

## Materials and Methods

Sera. Coded samples representing 216 sera from a variety of ruminant species housed at the SDZ, the SDWAP, the OKZ, and other zoological parks within the Eastern part of the United States were screened by ELISA. Among them, 9 speciments were collected from ruminants which exhibited clinical signs of MCF and herpesviruses were subsequently isolated from these animals. Hyperimmune sera[a] to IBR (bovine herpesvirus-1), BHM (bovine herpesvirus-2), and DN599 (bovine herpesvirus-4) were used for evaluation of cross-reactivity of alcelaphine herpesvirus antigens with antibodies specific for other bovine herpesviruses. Hyperimmune serum[a] was also prepared in a calf against the WC-11 strain of alcelaphine herpesvirus. Two serum samples positive by the SVN test to alcelaphine herpesvirus-1 which were collected from ruminants with clinical MCF were used to examine by the ELISA the reactivity of antibodies to the plate-coated viral antigens. To establish a negative reaction threshold for the ELISA, 88 domestic cattle sera were randomly selected from sera submitted to Oklahoma Animal Disease Diagnostic Laboratory (OADDL) for a respiratory serologic profile.

---

[a]Samples were kindly provided by Dr. C. Metz of the National Veterinary Service Laboratory, Ames, IA.

Preparation of antigen for ELISA. A modification of a procedure by Penman (19) for the extraction of nuclei was used in the preparation of the viral antigens for the ELISA. Bovine fetal kidney cells (BFK) infected with the greater kudu isolate (13) of alcelaphine herpesvirus-1 at a multiplicity of infection of $6.8 \times 10^{-2}$ $TCID_{50}$/cell were trypsinized and washed twice in Dulbecco's medium. The BFK cells which were free of bovine diarrhea virus (BVD), were then pelleted at 500 x g for 2 minutes at ambient temperature. The cell pellets were resuspended in hypotonic buffer (0.01 M NaCl, 0.01 M Tris NCl, pH 7.4, 0.01 M $MgCl_2$) and then incubated for 5 to 10 minutes at ambient temperature prior to centrifugation at 500 x g for 2 minutes. Pellets were resuspended in 2 ml of hypotonic buffer and immediately transferred into a Dounce homogenizer. Cells were ruptured by 30 stroked with the pestle. The cell homogenate was diluted with 10 ml of hypotonic buffer and centrifuged at 500 x g for 2 minutes. The upper layer was discarded and the cell pellets were washed with hypotonic buffer once and then pelleted at 500 x g for 2 minutes. The purity of the nuclear preparation was determined by microscopic examination of stained slides[b] of smears of the pellets (19). The preparation was then sonicated for 1.5 to 2 minutes in a Biosonik II sonifier at the maximal setting. The homogeneity of the sonicated preparation was determined by electron microscopy of negative stained (i.e., phosphotungstic acid) carbon-coated grids of the sonicated specimens. Uninfected BFK cells which served as negative antigen control were prepared concurrently by the preceding procedure.

---

[b]DIFCO Laboratories Inc., Detroit, MI.

ELISA conjugate and substrate. For the ELISA, a commercial conjugate[c] of horseradish peroxidase-labeled affinity-purified goat anti-bovine immunoglobulin G (IgG) (heavy and light chains-specific) was diluted in buffer (0.15 M NaCl, 0.001 M EDTA, 0.05 M Tris base, pH 7.4, containing 1% gelatin and 0.05% Tween 20) prior to its use. The substrate used in the ELISA was a commercial ABTS chromogen system.[c] Sera from all ruminant species were tested using the preceding anti-bovine conjugate.

ELISA for MCF. The ELISA for MCF was a modification of the protocol developed for detection of antibodies to pseudorabies virus (20). The test plates were prepared as follows: 100 ul of virus antigen (positive Ag) in carbonate buffer (pH 9.6) was added vertically to all odd numbered rows and uninfected cell (negative Ag) to all even numbered rows of a 96-well Immunlon I plate.[d] The coated plates were then sealed in plastic bags and placed on a rotational rocker (Tek-Tato-V)[d] at a speed setting of 60 and incubated at 37 C for 3 hours followed by overnight incubation at 4 C.

Each serum sample was diluted 1:20 with the serum-conjugate buffer prior to assaying. For the ELISA, coated plates were washed three times for 3 minute intervals with a wash buffer (0.01 M phosphate buffer, 0.05% Tween 20, pH 7.2) using a Dynawasher.[d] Excess wash solution was removed and 100 ul of each test serum was then added into each of four adjacent wells of positive and negative Ags. On each plate a reference positive and negative serum and a

---

[c]Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD.

[d]Dynatech Laboratories Inc., Alexandria, VA.

diluent control were added and all plates were incubated on a rocker (60 rpm) for 30 minutes at 37 C. Following incubation, wells were washed, and 50 ul of diluted (1:3000) conjugate was added to each well. The plates were incubated for 30 minutes at 37 C (60 rpm) and then washed. To each well, 100 ul of substrate (ABTS) was added and the plates were placed on a rocker (60 rpm) at ambient temperature for 30 minutes. The colorimetric reaction of the enzyme-substrate was stopped by the addition of 50 ul/well of a 1:400 dilution of 48% hydrofluoric acid. After blanking an automatic reader[d] with the diluent control at wavelength of 405/450 nm, the reaction mixtures were immediately read. For each serum, the absorbance obtained at 405/450 nm for each positive Ag well was subtracted from the absorbance obtained for the complimentary negative Ag well. The mean and its standard deviation ($\bar{X} \pm$ S.D.) of these absorbances were then determined for each serum. The absolute absorbance for each test serum was calculated by the following formula:

$$\text{absolute absorbance} = \frac{\bar{X} \text{ of net absorbance of test serum}}{\bar{X} \text{ of net absorbance of positive serum}}$$

The preceding formula provided standardized absorbance values from plate to plate since the same reference positive serum was run on each plate.

The optimal antigen and conjugate concentrations to be used in the ELISA were determined by checker-board titration of both antigens and conjugate against a constant diluation (1:20) of positive and negative sera. Briefly, antigens were diluted at 1:100, 1:150, 1:200, and 1:300 in carbonate buffer, and 100 ul of each antigen dilution was added to 8 wells of Immunlon I plate which was then

incubated at 37 C for 3 hours. This was followed by the addition of 100 ul/well of either a positive or negative serum and then the conjugate at various dilutions and substrate as previously described. The positive serum was obtained from an Indian gaur who was diagnosed as a case of clinical MCF by histopathology and virus isolation (13). The negative sera were from healthy heifers.

To establish the baseline absorbance for a positive serum, the mean absorbance of 88 cattle sera which were submitted to OADDL for respiratory serologic profile were used. The mean absorbance value for the 88 cattle sera was 0.085 ± 0.067. The mean absorbance for the positive serum was 1.125 ± 0.140. Therefore, an absorbance value of greater than 0.150 (mean plus 1 S.D. from the negative sera) was considered the positive threshold absorbance for sera containing antibodies to alcelaphine herpesvirus.

Other serological assays. The SVN tests were performed at either the Research Department of the SDZ or the National Veterinary Service Laboratory (NVSL) at Ames, Iowa.

Statistical methods. The correlation coefficient of results obtained by the 2 serologic assays was obtained by linear regression analysis. The specificity and sensitivity of the ELISA were calculated (21) as follows:

$$\text{sensitivity} = \frac{\text{positive tests actually obtained by ELISA}}{\text{all true positive tests by SVN}}$$

$$\text{specificity} = \frac{\text{negative tests actually obtained by ELISA}}{\text{all true negative tests by SVN}}$$

Results

Sera from 3 ruminants naturally or experimentally exposed to alcelaphine herpesvirus were each serially diluted and the dilutions were assayed by the ELISA (Fig 1). Each of the sera previously positive for antibodies to alcelaphine herpesvirus by the SVN test demonstrated a decreasing linear absorbance with dilution. A slight "tailing" effect was observed when the absorbance at 405/450 nm fell below 0.150. Sera from the sika deer and hyperimmune calf still produced an intense absorbance at a serum dilution of 1:320 (Fig 1). The sera from the Indian gaur also had a linear decrease in absorbance with dilution which was measurable at a serum dilution of 1:160. The absorbance values for a serum from a healthy heifer and negative by the SVN remained below the established 0.150 threshold at all dilutions examined. Based on the preceding results and also that a positive serum dilution of less than 1:20 produced an absorbance value beyond the range of the reader, the 1:20 dilution was selected as the test dilution for all serum samples to be assayed.

The specificity of the ELISA for the determination of antibodies to antigens of alcelaphine herpesvirus-1 was also demonstrated by the absence of a positive reaction with hyperimmune sera prepared against other bovine herpesviruses (Table 1). At a 1:20 dilution, none of 8 hyperimmune sera prepared against different bovine herpesviruses had an absolute absorbance greater than 0.128. Each of these sera, however, had moderate to high titers to their respective herpesviral antigens as determined by an indirect immunofluorescent (IIF) test.

In a comparative study of 216 sera from 42 exotic ruminant species for antibodies to the alcelaphine herpesvirus by ELISA and

SVN, a correlation coefficient of 0.564 ($p < 0.001$, n=216) was calculated in a comparison of the absorbances obtained by ELISA and SVN titers (Fig 2). Of the sera examined, 73 were negative by both assays and 110 contained antibodies by both assays (Table 2). Fourteen different sera were negative by SVN but positive by ELISA, and 19 others were ELISA negative but positive by SVN. The sensitivity of the ELISA, compared with the SVN, was 85.3%. The specificity of the ELISA was 83.9%.

Of the 9 different ruminants with clinical signs of MCF, 8 were positive by ELISA and an alcelaphine herpesvirus was subsequently isolated from each (Table 3). The ankole cow from which a virus was not isolated was positive by ELISA, but negative by SVN. The ELISA absorbance values on the sera ranged between 0.284 to 1.534 and SVN titers to MCF ranged between 4 and 516.

## Discussion

Low passages of alcelaphine herpesvirus are highly cell-associated (22). Furthermore, the maturation of alcelaphine herpesvirus is accomplished by budding through cellular membrances (23). Because of the acquisition of host cell antigens via the budding process by the alcelaphine herpesvirus similar to other herpesviruses (24), the high nonspecific background found in certain sera by ELISA may reflect a reaction with host cell membrane proteins. When semi-purified, cell-free virus was used as bound antigen, a strong color reaction occurred which rendered impossible the differentiation of a weak positive from a negative serum. However, when a homogenate of nuclei extracted from herpesvirus-infected cells were bound to the ELISA plate,

a low absorbance background occurred. Because of the nature of the nuclei-derived antigen preparations which contained some cellular debris, an uninfected host cell antigen extracted similarly was included to subtract the nonspecific reaction of antibody to host cell proteins in each assay.

For each preparation of viral antigen, a titration was made against a known positive and negative serum to MCF. Initially we attempted to quantify viral antigen bound to the plate by the concentration of protein in each viral preparation, however, this quantitation method yielded inconsistent results for each bound viral preparation. One explanation for this finding may be that virus absorption and replication rates in cell cultures may vary even when similar multiplicity of infection were made. Furthermore, because the method used in quantifying protein was based on total protein, the concentration of specific viral protein was unknown. Since viral to total proteins probably changed with each viral preparation, we standardized the ELISA by the titration of each viral antigen preparation against a known positive and negative serum. Alcelaphine herpesvirus is similar to other herpesviruses, such as Varicella-zoster (25) and Epstein-barr (26), in which viral-infected and non-infected cells have been applied simultaneously in an ELISA for the measurement of specific antibody with satisfactory results.

In the United States, a high prevalence of antibodies to alcelaphine herpesvirus as detected by SVN and immunofluorescence tests was reported (27) in species housed at the SDWAP of the subfamilies Alcelaphinae (i.e., wildebeest, hartebeest, and topi), Hippotraginae (i.e., oryx, gemsbok, addax), and Caprinae (i.e., wild goat, markhor,

pygmy goat, and European sheep). In a serologic survey which coincided with outbreaks of MCF between 1979-1981 at the OKZ, antibodies to alcelaphine herpesvirus-1 were found in wildebeest, hartebeest, muntjac, eland, addax, Grant's gazelle, nubian ibex, and chamois (14). In most serologic studies, either viruses have not been isolated from seropositive animals or herpesviruses which have been isolated (i.e., topi) were not virulent to susceptible cattle (28). Nevertheless, a hartebeest herpesvirus has been isolated and which has produced clinical disease in cattle similar to MCF (29). Therefore, these serologic surveys indicated that either exotic ruminants have antibodies which cross-react with antigens of alcelaphine herpesvirus-1 suggestive of a previous exposure or such exotic species harbor endogenous viruses which are antigenically related to alcelaphine herpesvirus-1.

The positive serologic results correlated with the clinical signs of MCF and herpesvirus isolation from 8 of 9 exotic ruminants. Although an ankole cow was the only ruminant of the 9 tested which was negative for virus isolation, the positive ELISA for this cow was significant. Recent serologic investigations have shown that the etiologic agent of sheep-associated MCF may be serologically related to the alcelaphine herpesvirus (30). Because a history on the ankole cow was not available, one can conjecture that this cow probably was exposed to either a wildebeest calf or to lambing ewes. The positive ELISA further suggests that the ankole cow may have been infected with sheep-associated MCF. Therefore, the ELISA may serve as a confirmatory diagnostic assay in conjunction with the

histopathologic lesions and anammesis in the diagnosis of sheep-associated MCF.

The majority of positive sera detected by the ELISA were from clinically healthy exotic ruminants which included: wildebeest, topi, hartebeest which are carriers of alcelaphine herpesvirus, and other non-alcelaphine species such as: oryx, addax, domestic and wild sheep and goats (3). Our findings were consistent with reports from Africa where antibodies to alcelaphine herpesvirus were detected in all wildebeest tested which were older than 7 months of age (31). Reid et al. (32) found that 60% of hartebeest and 40% of topi tested by the SVN test also had neutralizing antibodies to alcelaphine herpesvirus. A high percentage of domestic fringe-eared oryx in 2 herds have been found to contain neutralizing antibodies to alcelaphine herpesvirus (27). Therefore, antibody to alcelaphine herpesvirus appears to be widespread among exotic ruminant species.

Our results demonstrated that ELISA is a useful method for the determination of antibodies to alcelaphine herpesvirus-1 in exotic ruminant species. When compared to the SVN test, the ELISA showed a significant level ($p < 0.001$, $r=0.564$, $n=216$) of correlation to neutralizing titers. In this survey, we examined sera from over 42 different ruminant species of which the binding efficiency of immunoglobulin G (IgG) of each species to antibovine IgG is unknown. Therefore, prior to the ELISA, an immunodiffusion (Ouchterlony technique) assay was done on certain sera of species within subfamilies to determine the cross-reactivity of IgG of these ruminants with antibovine IgG (H+L) conjugate. From the few selected samples, a line of identity to bovine IgG was demonstrated

(data not shown) for all the species tested. Nevertheless, the binding affinity of antibovine IgGs (conjugate) to IgGs of each species probably influences the absorbance readings of ELISA reaction. This is suggested by the finding that certain sera had high neutralizing titers to alcelaphine herpesvirus but produced low absorbances in the ELISA.

The sensitivity and specificity of the ELISA were above 80% which suggests a high level of reproducibility of the ELISA for antibody to alcelaphine herpesvirus-1. Furthermore, the dose response of hyperimmune serum to alcelaphine herpesvirus and the two sera from clinical cases demonstrated a linear decrease with dilution of sera but the normal serum demonstrated no reactivity above the threshold of the ELISA. Hyperimmune sera to other bovine herpesviruses did not react with the viral antigens in the ELISA which further argues for the specificity of the present assay. In contrast, cross-reactions of different sera with alcelaphine herpesvirus has been reported when the immunofluorescence test was used (3). The data obtained by the described ELISA indicates its value as a rapid and specific serologic assay which can be used on large herd samplings of exotic species to ascertain their serological status to MCF.

References

1. Heuschele WP, Castro AE. In: Olson RG, Krakowka S, Blakeslee JR, Jr. eds. Comparative Pathobiology of Viral Disease. Vol. 1, Boca Raton: CRC Press, 1985;115-125.

2. Mushi EZ, Rurangiraw FR. Epidemiology of bovine malignant catarrhal fever, a review. Vet Res Communications 1981;5:127-142.

3. Heuschele WP. Malignant catarrhal fever in wild ruminants--a review and current status report. USAHA Proc 1982;86:552-570.

4. Hatkin J. Endemic malignant catarrhal fever at the San Diego Wild Animal Part. J Wildlife Dis 1980;16:439-443.

5. Sanford SE, Little PB, Rapley WA. The gross and histopathologic lesion of malignant catarrhal fever in three captive sika deer (Cervus nippon) in Southern Ontario. J Wildelife Dis 1977; 13:29-32.

6. Selman IE, Wiseman A, Murray M, et al. A clinical-pathological study of bovine malignant catarrhal fever in Great Britain. Vet Rec 1978;94:483-490.

7. Horner GW, Oliver RE, Hunter R. An epizootic of malignant catarrhal fever. V. Laboratory Investigation. New Zealand Vet J 1975;23:36-38.

8. Colley L. Malignant catarrhal. Vet Med at the Johannesburg Zoo. Prox AAZV Ann Mtg 1977;11-12.

9. Vanselow BA. An epizootic of bovine malignant catarrhal in Malaysia. Vet Rec 1980;107:15-18.

10. Singh G, Singh B, Gupta PP, et al. Epizootiological observation on malignant catarrhal fever and transmission of the disease in buffalo calves (Bubalus bubulis). Acta Vet Brno 1979; 48:95-103.

11. Hoffmann D, Soeipto S, Sobironingsih S, et al. The clinical-pathology of a malignant catarrhal fever syndrome in the Indonesian swamp buffalo (Bubalus bubalis). Australian Vet J 1984;61:108-112.

12. Heuschele WP, Swansen M, Fletcher HR. Malignant catarrhal fever in U.S. Zoos. Proc AAZV 1983;67-72.

13. Castro AE, Whitenack DL, Goodwin DE. Isolation and identification of the herpesvirus of malignant catarrhal fever from exotic ruminant species in a zoologic park in North America. Proc Amer Assn Vet Lab Diag 1981;24:66-78.

14. Ramsay EC, Castro AE, Baumeister M. Investigations of malignant catarrhal fever in ruminants at the Oklahoma City Zoo. USAHA Proc 1982;86:571-582.

15. Heuschele WP, Fletcher HR. Improved methods for the diagnosis of malignant catarrhal fever. Proc Amer Assn Vet Lab Diag 1984;27: 137-150.

16. Rossiter PB, Jessett DM. A complement fixation test for antigens of and antibodies to malignant catarrhal fever virus. Res Vet Sci 1980;28:228-233.

17. Rossiter PB. Antigens and antibodies of malignant catarrhal fever herpesvirus detected by immunodiffusion and counterimmuno-electrophoresis. Vet Micro 1980;5:205-213.

18. Rossiter PB. Immunofluorescence and immunoperoxidase techniques for detecting antibodies to malignant catarrhal fever in infected cattle. Trop Anim Hlth Proc 1981;13:189-192.

19. Penman S. In:Habel K and Salzman NP, eds. Fundamental Techniques in Virology. New York:Academic Press Inc., 1969;35-48.

20. Snyder ML, Erickson GA. Pseudorabies serodiagnosis using the enzyme-linked immunosorbent assay (ELISA). A procedural guide. USDA, APHIS, NVSL, P.O. Box 844, Ames, Iowa 50010. 1982.

21. Tizard I. Serologic assays. JAVMA 1982;181:1162-1165.

22. Plowright W, Ferris RD, Scott GR. Blue wildebeest and the etiological agent of bovine malignant catarrhal fever. Nature 1960; 118:1167-1169.

23. Castro AE, Heuschele WP, Schramke ML, et al. Ultrastructure of cellular changes in the replication of the alcelaphine herpesvirus-1 of malignant catarrhal fever. Am J Vet Res 1985;46:1231-1237.

24. Roizman B. In:Roizman B ed. The Herpesviruses. Vol. 1 1982;1-15.

25. Shanley JM, Myers BE, Steele R. Enzyme-linked immunosorbent assay for detection of antibody to varicella-zoster virus. J Clinic Micro 1982;15:208-211.

26. Hopkins RF, Witmer TJ, Russell HN, et al. Detection of antibodies to Epstein-Barr virus antigens by enzyme-linked immunosorbent assay. J Inf Dis 1980;146:734-740.

27. Heuschele WP. Malignant catarrhal fever, in Foreign Animal Disease Report. USDA Vet Service 1984; No. 12-4.

28. Mushi EZ, Rossiter PB, Jessett D, Karstad L. Isolation and characterization of a herpesvirus from Topi (Damaliscus Korrigum, agilly). J Comp Path 1981;91:63-68.

29. Reid HW, Rowe L. The attenuation of a herpesvirus (malignant catarrhal fever virus) isolated from hartebeest (Alcelaphus buselaphus cokei Gunther). Res Vet Sci 1973;15:144-146.

30. Rossiter PB. Antibodies to malignant catarrhal fever virus in sheep sera. J Comp Path 1981;91:303-311.

31. Plowright W. Malignant catarrhal fever in East Africa. III. Neutralizing antibody in free-living wildebeest. Res Vet Sci 1967; 8:129-136.

32. Reid HW, Plowright W, Rowe LW. Neutralizing antibody to herpesvirus derived from wildebeest and hartebeest wild animals in East Africa. Res Vet Sci 1975;18:269-273.

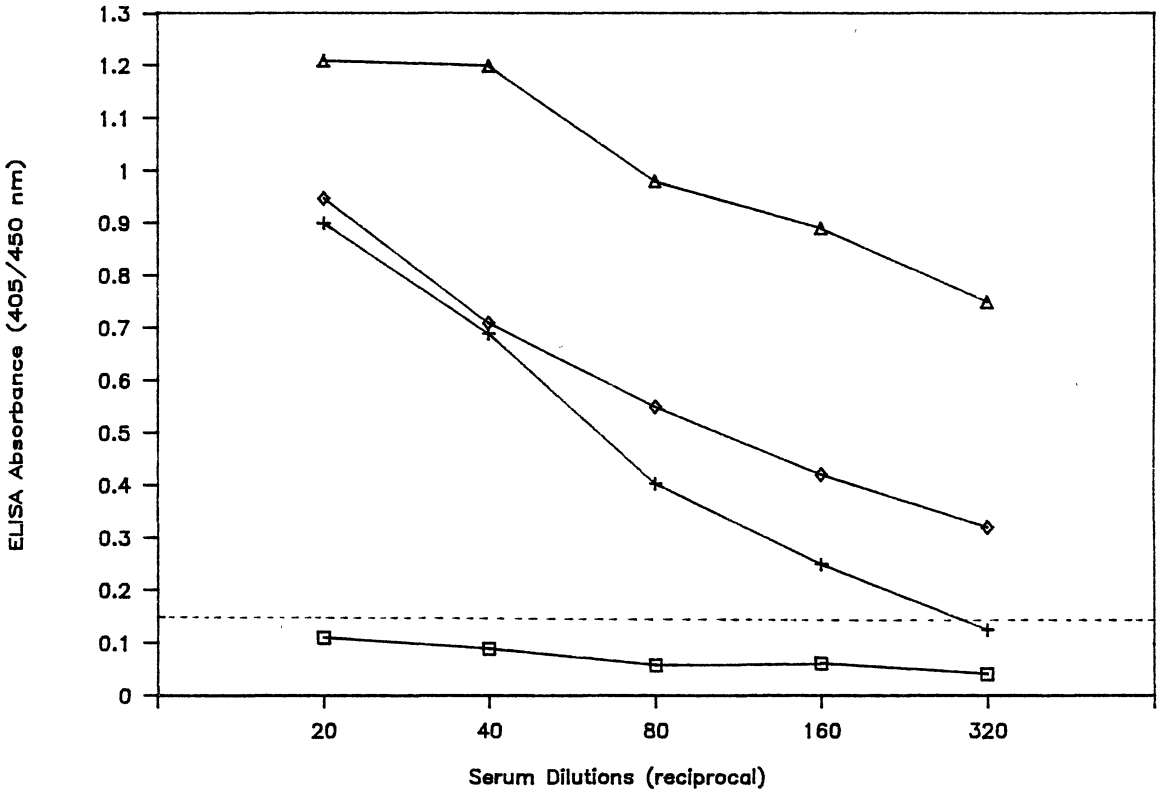



FIGURE 1. Titration in the ELISA of sera from 3 ruminants naturally or experimentally exposed to alcelaphine herpesvirus and a clinically healthy cow against the antigens of the greater kudu isolate of alcelaphine herpesvirus. Indian gaur (+) and sika deer (△) with clinical MCF; hyperimmune calf (◇); and clinically healthy cow (□).

TABLE 1. Specificity of the ELISA for Antibodies to Alcelaphine Herpesvirus-1

| Hyperimmune Serum Prepared Against[a] | Titers by IIF Test[a] | Absorbance at 405/450 nm[b] | ELISA Results |
|---|---|---|---|
| Bovine Herpesvirus-1 (IBR) | 20,480 | 0.027 ± 0.058 | Neg |
| Bovine Herpesvirus-1 (IBR) | 5,120 | 0.128 ± 0.064 | Neg |
| Bovine Herpesvirus-1 (IBR) | 5,120 | -0.068 ± 0.049 | Neg |
| Bovine Herpesvirus-2 (Mammalitis) | 20,480 | -0.018 ± 0.053 | Neg |
| Bovine Herpesvirus-2 (Mammalitis) | ND | 0.128 ± 0.064 | Neg |
| DN 599 | 20,480 | -0.009 ± 0.054 | Neg |
| DN 599 | ND | 0.062 ± 0.058 | Neg |
| DN 599 | 20,480 | -0.014 ± 0.084 | Neg |
| Healthy Heifer (Neg Control) | | -0.078 ± 0.077 | Neg |
| Indian Gaur (pos control) | 40 | 1.280 ± 0.075 | Pos |

[a]Titer against homologous antigen. Sera and data were provided by the National Veterinary Service Laboratories at Ames, Iowa 50010.

[b]Positive threshold was experimentally established at an absorbance (405/450 nm) of 0.150.

IIF Test: Indirect Immunofluorescent test.

ND: Not Determined.

Neg: Negative.

Pos: Positive.

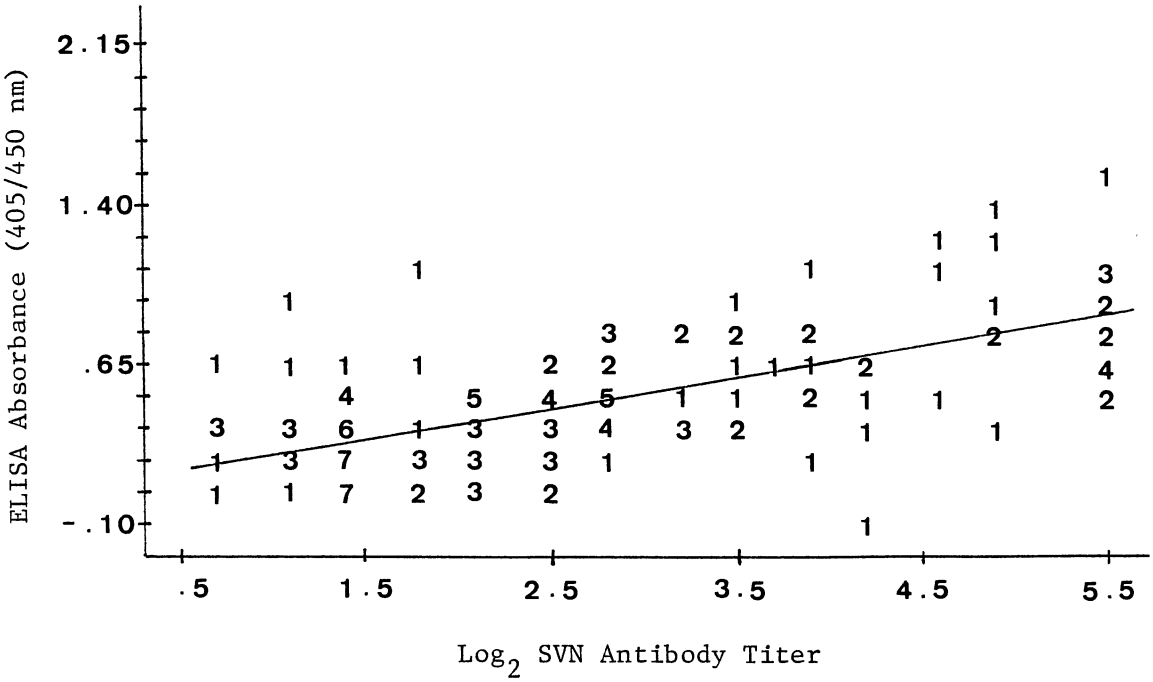



FIGURE 2. Correlation of ruminant sera for antibody to alcelaphine herpesvirus-1 by the ELISA and SVN test. The linear regression line determined for values obtained by each test gave a correlation coefficient of $r=0.564$ ($p < 0.001$, $n=216$). Each number along the regression line represents the number of sera at that titer with the corresponding absorbance value.

TABLE 2. Comparison of the SVN and ELISA in the Detection of Antibodies to Alcelaphine Herpesvirus-1

| ELISA | No. of Sera Tested by SVN | |
|---|---|---|
| | Positive[a] (129) | Negative (87) |
| Positive[b] (124) | 110 | 14 |
| Negative (92) | 19 | 73 |

[a]Titer $\geq$ 1:4. Total number of sera is shown within parentheses.

[b]Positive threshold was experimentally established at an absorbance (405/450 nm) of 0.150.

TABLE 3. Relationship of the Serologic Assays to Clinical MCF and Virus Isolation

| Species | Clinical Signs of MCF | Herpesvirus Isolation | Absorbance at 405/450 nm | SVN Titers |
|---|---|---|---|---|
| Formosan Sika Deer (A) | + | + | 0.654 ± 0.055(+)[a] | 4 |
| Formosan Sika Deer (B) | + | + | 1.534 ± 0.030(+) | >516 |
| Formosan Sika Deer (C) | + | + | 1.325 ± 0.016(+) | 4 |
| Axis Deer | + | + | 0.721 ± 0.054(+) | ND |
| Gaur | + | + | 1.200 ± 0.085(+) | 4 |
| Nilgai (A) | + | + | 0.471 ± 0.029(+) | ND |
| Nilgai (B) | + | + | 0.799 ± 0.047(+) | 16 |
| Ankole Cow | + | - | 0.557 ± 0.034(+) | <4 |
| Heifer | + | + | 0.284 ± 0.062(+) | 8 |

[a]Positive threshold = 0.150 at 405/450 nm.

A, B, C represent different animals.

ND: Not Determined.

# CHAPTER VI

## DETECTION OF ANTIBODY TO ALCELAPHINE HERPESVIRUS-1 BY ELISA IN CATTLE WITH SHEEP-ASSOCIATED MALIGNANT CATARRHAL FEVER


### Summary

An enzyme-linked immunosorbent assay (ELISA) and a serum-virus neutralization (SVN) test were used to measure antibody specific for the alcelaphine herpesvirus-1 of malignant catarrhal fever (MCF) in sera from healthy cattle and cattle with clinical signs of sheep-associated MCF. Of 128 cattle associated with outbreaks of MCF in California and Colorado, 30 were seropositive for MCF by ELISA but seronegative by the SVN test. In cattle sera from 47 experimental and clinical cases of MCF in Colorado, 11 were seropositive for MCF by ELISA and 4 of these 11 had low levels of virus neutralizing (VN) antibody. Seroconversion was demonstrated in 3 paired sera from cattle experimentally infected with inocula from clinical cases of sheep-associated MCF. By ELISA, 11 of 35 cattle sera from Indiana which had elevated levels of antibody to bovine herpesvirus-1 (infectious bovine rhinotracheitis virus) were seropositive for MCF. Of 88 tested sera from cattle in Oklahoma which had antibody to IBR virus, all were seronegative by ELISA. The significance of the finding of antibodies to alcelaphine herpesvirus-1 by the ELISA and SVN test in sera of domestic cattle with sheep-associated MCF is discussed.


### Introduction

The sheep-associated form of MCF is similar clinically to the

wildebeest-associated or African form of MCF (5, 13, 17). The etiological agent for wildebeest-associated MCF has been characterized as an alcelaphine herpesvirus (1, 2, 10, 17). However, the etiological agent of sheep-associated MCF has yet to be identified. Outbreaks of sheep-associated MCF in the United States, which have led to major losses in domestic cattle, have been reported in California and Arizona (11), Colorado (12), and Minnesota (3). The diagnosis of sheep-associated MCF in cattle or red deer is usually based on a history of contact with sheep, the clinical signs in the affected animal, and the histopathologic lesions (3, 11, 12, 16). The characteristic lesions in the tissues of cattle infected with alcelaphine herpesvirus-1 have been described as a vaculitis with lymphoproliferation associated with a monocytic infiltrate (5, 31) and these described lesions are identical to those reported in cattle with sheep-associated MCF (8, 9, 10, 11, 12).

In a series of investigations (20-22) on sera from different sheep flocks associated with outbreaks of MCF, specific antibodies to alcelaphine herpesvirus were detected using an indirect immunofluorescence (IIF) procedure. Furthermore, antibodies to alcelaphine herpesvirus were identified by the IIF test in cattle with sheep-associated MCF (20), and also in cattle infected with alcelaphine herpesvirus (21). However, VN antibody to alcelaphine herpesvirus was not detected in sera from either sheep (22) or cattle with sheep-associated MCF (20). In contrast, VN antibodies of low titer were detected in sera of cattle infected with alcelaphine herpesvirus (21).

Previously, we reported on the development of an enzyme-linked immunosorbent assay (ELISA) for the detection of specific antibody to alcelaphine herpesvirus-1 in various zoological species of ruminants

(29). The purpose of this report is to present the data of a serologic study for antibody to alcelaphine herpesvirus-1 as determined by the ELISA and the SVN test on domestic cattle sera obtained from various geographical areas of the United States. The sera assayed included specimens from documented clinical cases in cattle of sheep-associated MCF and cattle sera from non-MCF cases.

## Materials and Methods

### Sera

The first group of specimens comprised 128 sera obtained from cattle with a diagnosis of MCF as determined by clinical histories and histopathologic lesions. These sera were provided by Dr. J. Store of the Department of Veterinary Microbiology and Parasitology, Louisiana State University and represented cattle involved in MCF outbreaks in California and Colorado and also cattle experimentally infected with inocula from clinical cases in cattle of sheep-associated MCF.

In the second group, 69 sera from 47 cattle in Colorado were provided by Dr. J. C. DeMartini of the Department of Pathology, Colorado State University. These cattle sera included 24 preinoculation and 21 post-inoculation specimens which were collected from cattle experimentally inoculated with clinical materials from suspected cases of sheep-associated MCF (7) and 24 sera from clinical cases of cattle with MCF. In addition, 3 specimens from synovial fluids of hock joints and 3 cerebrospinal fluids (CSF) from the clinical cases of MCF were also examined.

In the third group, 35 sera with moderate to high titers of VN antibody to bovine herpesvirus-1 were supplied by Dr. C. Kanitz of the Veterinary Diagnostic Laboratory, Purdue University.

In the fourth group, 88 sera randomly selected from cattle sera submitted to the Oklahoma Animal Disease Diagnostic Laboratory (OADDL) for a serologic profile of bovine respiratory disease were examined.

Enzyme-Linked Immunosorbent Assay (ELISA)

The method used for the preparation of the viral antigens for the ELISA has been previously described (29). Briefly, trypsinized bovine fetal kidney cells infected with the greater kudu strain of alcelaphine herpesvirus-1 (1) were allowed to swell in a hypotonic buffer (0.01M NaCl, 0.01M Tris HCl, 0.001M $MgCl_2$, pH 7.4) for 5 to 10 minutes and then ruptured by homogenization using a Dounce homogenizer. The intact nuclei were pelleted by centrifugation[a] at 500 X g for 2 minutes. The nuclei, which were essentially free of cytoplasmic membranes as determined by staining (29), were then sonicated in a sonicator[b] for 1.5 to 2 minutes at maximal setting. The sonicated nuclear homogenates were then diluted in the coating buffer (carbonate buffer, pH 9.6) and added to microtiter plates[c] which then were incubated for 3 hours at 37 C. The concentrations for the viral antigens (positive) and the host cell antigens (negative) to be used in the ELISA were determined by the titration of each antigen on each batch of coated plates against a 1:20 dilution of a positive and negative serum for MCF. All test sera were non-heat inactivated and were assayed at a 1:20 dilution in the ELISA. The procedures for the ELISA for the detection of antibodies to alcelaphine herpesvirus-1 were similar to those described for pseudorabies virus (27).

---

[a]IEC HN-SII Centrifuge, DAMON/IEC Division, Needham Heights, MA 02194.

[b]Biosonik II.

[c]Immunlon I microtiter plates, Dynatech Lab., Inc., Alexandria, VA 22314.

To standardize the absorbance values obtained for each test serum in the microtiter plate at a wavelength of 405/450[d], the formula below was developed:

$$\text{absolute absorbance} = \frac{\bar{X} \text{ of net absorbance of test serum}}{\bar{X} \text{ of net absorbance of positive serum}}$$

The mean ($\bar{X}$) for each serum assayed was composed of the absorbance readings of 4 wells. A positive threshold for absolute absorbance of a serum positive for antibodies to alcelaphine herpesvirus was previously established as 0.150 absorbance units (29). Therefore, a test serum with an absorbance value greater than 0.150 was considered seropositive for alcelaphine herpesvirus-1.

Serum-Virus Neutralization (SVN) Test

The SVN test on the cattle sera was performed by a microtiter procedure (4). The procedure was as follows: serial 2-fold (25 ul) dilutions of heat-inactivated (56 C for 30 minutes) serum were made in a 96-well microtiter plate using growth medium containing 10% fetal bovine serum (FBS) and 200 ug/ml of gentamicin. To each serum dilution was added 25 ul which contained 100 to 300 $TCID_{50}$ of a cell-free preparation of the WC-11 strain (14) of alcelaphine herpesvirus-1. Serum-virus mixtures were allowed to react for one hour at 37 C in a $CO_2$ incubator. Following incubation of the serum-virus mixtures, a $10^6$ cells/ml suspension of bovine turbinate (BT) cells in growth medium was prepared and 150 ul was added to each well on the plate. The inoculated plates were then placed at 33 C in an incubator with 5% $CO_2$. The titer of each serum was determined by the inhibition of viral cytopathology at the 10th day of incubation and serum titers were calculated by the method of

---

[d]MR580, MicroELISA Auto Reader, Dynatech Lab., Inc., Alexandria, VA 22314.

Karber (6). A serum titer of 4 or greater was considered to be seropositive for alcelaphine herpesvirus-1.

## Results

Of 128 serum samples in group 1 from cattle associated with MCF outbreaks in California and Colorado and assayed by ELISA, 30 sera (23%) were positive for antibody to alcelaphine herpesvirus-1 (Table 1). Sixty-nine of the 128 sera which were received at a 1:4 dilution in phosphate buffer were tested at a 1:8 dilution for VN antibody to alcelaphine herpesvirus (WC-11 strain). Thirty-six cattle sera were negative for VN antibody and 33 were toxic to the BT cells. Clinical histories were not available for any of the 30 seropositive cattle.

In the cattle from group 2 which were experimentally infected with MCF, 11 of 69 tested cattle sera were seropositive for MCF by ELISA. Seroconversion was detected by ELISA in 3 of 16 paired sera but in only one paried serum by the SVN test. Of sera collected from cattle when clinical signs of MCF appeared, only 1 of 6 sera was seropositive. One of the preinoculation serum from the experimentally inoculated cattle was detected as positive by ELISA, but the post-inoculation serum from this animal was seronegative; however, all other preinoculation sera were negative by ELISA. Of 5 post-inoculation cattle sera which were unpaired, only 1 was seropositive by ELISA.

Sera from 18 cattle which developed clinical signs of MCF after receiving an inoculum of clinical materials from cattle with sheep-associated MCF or had been naturally-infected were tested by ELISA and 5 were seropositive. Of these 5 positive sera, only 1 had a VN titer of 4; all the others were negative by SVN test. However, from

these 18 cattle sera, 2 which were negative by ELISA had a VN titer of 4 and 8 respectively.

In sera from group 3 which were from cattle in Indiana and contained VN titers to IBR virus of 128 to 256, 11 of 35 sera were positive for antibody to alcelaphine herpesvirus-1 by ELISA. Clinical histories available on these cattle did not indicate a prior infection with alcelaphine herpesvirus-1 but did document a respiratory problem. Due to insufficient volume, sera were not assayed by the SVN test for antibodies to alcelaphine herpesvirus-1.

In group 4, cattle sera selected at random and representing 88 different cattle which had been submitted to OADDL for a virus respiratory profile were all negative for antibody to alcelaphine herpesvirus-1 by ELISA. Most of these sera contained VN titers to IBR virus which ranged in titer from 4 to 32. Furthermore, 23 of these sera contained antibody to parainfluenza virus type 3 and 50 of the sera contained antibody to bovine viral diarrhea virus.

## Discussion

In this serologic study, antibodies to alcelaphine herpesvirus-1 were detected by ELISA in a portion of cattle sera with clinical MCF. The low percent of seropositive sera for MCF as detected by the ELISA and SVN test in our serologic survey was in agreement with previous assays on MCF in naturally and experimentally infected cattle as measured by the IIF and SVN tests (18, 19, 20, 21). Previous serologic surveys on MCF have shown that VN antibody was detectable only in cattle which had recovered following clinical MCF (14) and also in cattle that were hyperimmunized with either inactivated or attentuated alcelaphine herpesvirus-1 (15).

The absence of demonstrable humoral antibody to alcelaphine herpesvirus-1 may be related to the tropism of alcelaphine herpesvirus-1 for the cells of the immune system. A recent investigation of rabbits infected with alcelaphine herpesvirus-1 has shown that the target cells for MCF virus were thymus-derived "T" lymphoblasts (24). Therefore, a dysfunction of either B or T-lymphocytes in MCF-affected ruminants has been cited as a possible reason for the absence of antibody production (25). Additionally, immunosuppressive factors have been found in acute sera from rabbits infected with alcelaphine herpesvirus (30). This immunosuppressive factor in acute rabbit sera was measured by the inhibition of blastogenic transformation in lymphocytes following mitogen stimulation (30). In contrast in another study, lymphocytes from cattle and rabbits infected with alcelaphine herpesvirus were found to have an enhanced activity to mitogens in the presence of acute-phase rabbit sera to alcelaphine herpesvirus (26). This finding provided no evidence to indicate a dysfunction of B or T-lymphocytes in MCF-affected cattle or rabbits. Furthermore, a 4-fold or greater increase in the concentrations of immunoglobulins G and M have been demonstrated in sera of rabbits following their infection with alcelaphine herpesvirus, but VN antibodies were not detected in either whole or fractionated rabbit sera (23). This finding suggests that certain antibodies produced by the host following an infection with alcelaphine herpesvirus may not be measurable by SVN test.

The ELISA detected more seropositive sera than did the SVN test which suggested that the ELISA was more sensitive in the detection of antibodies to alcelaphine herpesvirus-1. However, the SVN test was done on only a portion of the sera because of insufficient amounts of

sera and thus, no correlation between assays could be done. Nevertheless, we found that 2 seropositive sera by the SVN test from group 2 were ELISA negative. One explanation for this conundrum may be the use of an affinity purified peroxidase-labeled antibovine IgG (H+L) conjugate in the ELISA since early antibody belongs to IgM class (28). Thus, the affinity purified antibody to bovine IgG used in the ELISA might not recognize this early IgM and might account for a negative ELISA.

In group 2, although 1 serum labeled preinoculation was positive by ELISA, post-inoculation serum from the same animal was negative. This may be a result of mislabeling since the sera had been collected, coded and stored for several years prior to their use in the ELISA. Nevertheless, in certain paired sera from cattle receiving an inoculum of sheep-associated materials, the preinoculation sera were negative by ELISA and the post-inoculation sera were seropositive by ELISA. This finding implies that the etiological agent of sheep-associated MCF is serologically related to alcelaphine herpesvirus-1.

Because the complete histories for the California and Colorado serum samples in group 1 and 2 were not available or the time of collection of sera or the duration of the clinical disease, these serological findings will require further confirmation. However, a bovine cytomegalovirus and a bovine leukemia virus had been isolated from some of these cattle in group 1[e] and the possibility that these viruses have epitopes similar to alcelaphine herpesvirus-1 which are detected by the ELISA has not been investigated.

---

[e]Dr. J. Storz, personal communication, 1985.

Detection of seropositive cattle for MCF by ELISA in the sera from group 3 was unexpected. Based on our previous findings (Wan, unpublished results) and on 88 sera from group 4, we found no evidence of a cross-reaction of the alcelaphine herpesvirus antigens with cattle sera containing high, moderate, or low levels of antibody to IBR virus. As further evidence of the absence of cross-reaction in the ELISA of bovine herpesvirus-1 positive sera with alcelaphine herpesvirus-1, all the ELISA negative sera from the Indiana cattle also had VN titers of 128 to 256 to IBR virus.

Nevertheless, our present findings do not eliminate the possibility that an antibody response to a previous infection to other bovine herpesviruses (e.g., cytomegaloviruses and a variety of orphan bovine herpesviruses) may have cross-reacting antibodies for antigens of alcelaphine herpesvirus-1. Another explanation for these MCF-seropositive cattle may have been a previous exposure to sheep-associated MCF by association with lambing ewes but such information was not available.

Our serologic findings supported the hypothesis (17) and findings (20-22) that the putative virus of sheep-associated MCF is serologically related to alcelaphine herpesvirus-1. Therefore, the ELISA provides a rapid supplemental diagnostic assay for the screening and study of sheep-associated MCF in domestic cattle. Until the virus of sheep-associated MCF is isolated and the pathogenesis of MCF is fully investigated, the interpretation of the significance of seropositive healthy cattle or cattle clinically ill with sheep-associated MCF remains tenuous.

## References


1. Castro, A. E., G. G. Daley, M. A. Zimmer, D. L. Whitenack and J. Jensen:1982 Malignant catarrhal fever in an Indian gaur and greater kudu: Experimental transmission, isolation, and identification of a herpesvirus. Am. J. Vet. Res., 43:5.

2. Castro, A. E. and G. G. Daley:1982 Electron microscopic study of the African strain of malignant catarrhal fever virus in bovine cell cultures. Am. J. Vet. Res., 43:576.

3. Hamdy, F. M., A. H. Dardiri, C. Mebus, R. E. Pierson and D. Johnson: 1979 Etiology of malignant catarrhal fever outbreak in Minnesota. Proc. USAHA 82th Ann. Mtg., pp. 248-267.

4. Heuschele, W. P. and H. R. Fletcher:1984 Improved methods for the diagnosis of malignant catarrhal fever. Amer. Assn. Vet. Lab. Diag. 27th Ann. Proc., pp. 137-150.

5. Heuschele, W. P. and A. E. Castro. Malignant catarrhal fever, Chapter 7. in: Comparative Pathobiology of Viral Diseases. R. G. Olson, S. Krakowka and J. R. Blakeslee, Jr. eds, CRC Press, Boca Raton, Florida. PP115-125, 1985.

6. Lennette, E. H.: General principles underlying laboratory diagnosis of viral and rickettsial infections, in Diagnostic Procedures for Virus and Rickettsial Diseases. T. Francis, Jr. ed., American Public Health Association, N.Y., 1965.

7. Liggitt, H. D., J. C. DeMartini, A. E. McChesney, R. E. Pierson, and J. Storz:1978 Experimental transmission of malignant catarrhal fever in cattle and histopathologic changes. Am. J. Vet. Res., 39:1249.

8. Liggitt, H. D. and J. C. DeMartini:1980a The pathomorphology of malignant catarrhal fever. I. Generalized lymphoid vasculitis. Vet. Pathol., 17:58.

9. Liggitt, H. D. and J. C. DeMartini:1980b The pathomorphology of malignant catarrhal fever. I. Multisystemic epithelial lesions. Vet. Pathol., 17:73.

10. Mushi, E. Z. and F. R. Rurangirwa:1981 Epidemiology of bovine malignant catarrhal fever, a review. Vet. Res. Comm., 5:127.

11. Orsborn, J. S., C. J. Mare, J. L. Ayers and R. E. Reed:1977 Diagnostic features of malignant catarrhal fever outbreaks in the western United States. Amer. Assoc. Vet. Lab. Diag. 20th Ann. Proc., pp. 215-224.

12. Pierson, R. E., D. Thake, A. E. McChesney and J. Storz:1973 An epizootic of malignant catarrhal fever in feedlot cattle. JAVMA 163:349.

13. Pierson, R. E., F. M. Hamdy, A. H. Dardiri, D. H. Ferris and G. M. Schloer:1979 Comparison of African and American forms of malignant catarrhal fever: Transmission and clinical signs. Am. J. Vet. Res., 40:1091.

14. Plowright, W.:1968 Malignant catarrhal fever. JAVMA 152:795.

15. Plowright, W., K. A. J. Herniman, D. M. Jessett, M. Kalunda and C. S. Rampton:1975 Immunization of cattle against the herpesvirus of malignant catarrhal fever: Failure of inactivated culture vaccines with adjuvant. Res. Vet. Sci., 19:159.

16. Reid, H. W., D. Buxton, W. Corrigall, A. R. Hunter, D. A. McMartin and R. Rushton:1979 An outbreak of malignant catarrhal fever in red deer (Cervus elephus), Vet. Rec., 104:120.

17. Reid, H. W., D. Buxton, E. Berrie, I. Pow and J. Finlayson:1984 Malignant catarrhal fever. Vet. Rec., 114:581.

18. Rossiter, P. B., E. Z. Mushi and W. Plowright:1977 The development of antibodies in rabbits and cattle infected experimentally with an African strain of malignant catarrhal fever virus. Vet. Micro., 2:57.

19. Rossiter, P. B., E. Z. Mushi and W. Plowright:1978 Antibody response in cattle and rabbits to early antigens of malignant catarrhal fever virus in cultured cells. Res. Vet. Sci., 25:207.

20. Rossiter, P. B.:1983 Antibodies to malignant catarrhal fever virus in cattle with non-wildebeest-associated malignant catarrhal fever. J. Comp. Path., 93:93.

21. Rossiter, P. B., D. M. Jessett and E. Z. Mushi:1980 Antibodies to malignant catarrhal fever virus antigens in the sera of normal and naturally infected cattle in Kenya. Res. Vet. Sci., 29:235.

22. Rossiter, P. B.:1981 Antibodies to malignant catarrhal fever virus in sheep sera. J. Comp. Path., 91:303.

23. Rossiter, P. B.:1982 Immunoglobulin response of rabbits infected with malignant catarrhal fever virus. Res. Vet. Sci., 33:120.

24. Rossiter, P. B.:1983 Proliferation of T-lymphoblasts in rabbits fatally infected with the herpesvirus of malignant catarrhal fever. Clin. Exp. Immunol., 54:547.

25. Rurangirwa, F. R. and E. Z. Mushi:1982 Effect of malignant catarrhal fever virus infection on the immune response of rabbits to sheep red blood cells. Comp. Immun. Microbiol. Infect. Dis., 5:423.

26. Russell, P. H.:1980 The in vitro response to phytohaemagglutinin during malignant catarrhal fever of rabbits and cattle. Res. Vet. Sci., 28:39.

27. Snyder, M. L. and G. A. Erickson:1982 Pseudorabies serodiagnosis using the enzyme-linked immunosorbent assay (ELISA). A procedural guide. USDA, APHIS, NVSL, P. O. Box 844, Ames, Iowa 50010.

28. Tizard, I., An Introduction to Veterinary Immunology, W. B. Saunders Co., Philadelphia, 1982, pp. 38-53.

29. Wan, S. K. and A. E. Castro:1984 Development of an enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to MCF virus (Abstract). The 65th Ann. Mtg. Conf. Res. Workers in Anim. Dis., p. 13.

30. Wilks, C. R. and P. B. Rossiter:1978 An immunosuppressive factor in serum of rabbits lethally infected with the herpesvirus of bovine malignant catarrhal fever. J. Inf. Dis., 137:403.

31. Zimmer, M. A., C. P. McCoy and J. M. Jensen:1981 Comparative pathology of the African form of malignant catarrhal fever in captive Indian gaur and domestic cattle. JAVMA 179:1130.

Table 1. Antibody to alcelaphine herpesvirus-1 in sera from domestic cattle with sheep-associated MCF and other respiratory diseases

| Group No. | Origin of Cattle | Clinical History of Cattle | ELISA No. of Positive Sera / No. Tested | ELISA % Positive | SVN Test No. of Positive Sera / No. Tested | SVN Test % Positive |
|---|---|---|---|---|---|---|
| 1 | California and Colorado | Clinical Outbreaks of MCF | 30/128 | 23 | 0/69* | 0 |
| 2 | Colorado | Clinical and Experimental MCF Cases** | | | | |
| | | Preinoculation | 1/24 | 4 | 0/10 | 0 |
| | | Post-inoculation | 4/21 | 19 | 1/14 | 7 |
| | | Clinical MCF | 6/24 | 25 | 3/15 | 20 |
| 3 | Indiana | Respiratory Disease | 11/35 | 31 | N.D. | |
| 4 | Oklahoma | Respiratory Disease | 0/88 | 0 | N.D. | |

*Sera were tested at a starting dilution of 1:8 and 33 sera were toxic to cell cultures.
**Total 69 sera were collected from 47 cattle naturally or experimentally infected with sheep-associated MCF.
N.D.: Not Done.

# VITA

Siao-Kun Wan

Candidate for the Degree of

Doctor of Philosophy

Thesis: A STUDY OF THE BIOLOGICAL PROPERTIES OF MALIGNANT CATARRHAL FEVER VIRUS (ALCELAPHINE HERPESVIRUS-1) AND DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DETECTION OF ANTIBODIES TO ALCELAPHINE HERPESVIRUS-1 IN DOMESTIC AND EXOTIC RUMINANTS

Major Field: Veterinary Parasitology

Biographical:

Personal Data: Born in Taipei, Taiwan, Republic of China, October 6, 1952, the daughter of Mr. and Mrs. F. P. Wan.

Education: Graduated from Shing-Chuo Girl's High School, Shing-Chuo, Taiwan, in May, 1971; received Diploma, Taiwan Provincial Pingtung Institute of Agriculture in 1975; received Taiwan National License of Doctor of Veterinary Medicine in December, 1975; received the Master of Science degree at Oklahoma State University in July, 1982; enrolled in Ph.D. program at Veterinary College, Oklahoma State University in August, 1982; completed requirements for the Doctor of Philosophy degree at Oklahoma State University in December, 1986.

Professional Experience: D.V.M., Chong-May Animal's Hospital, Taipei, Taiwan, 1975; Research Assistant, in charge of Tissue Culture Section, Taiwan Provincial Research Institution for Animal Health, 1975-1979; Student Assistant, Department of Microbiology, Oklahoma State University, 1979-1981; Research Assistant, Department of Microbiology, Oklahoma State University, Summers, 1980, 1981; Graduate Teaching Assistant, Department of Microbiology, Oklahoma State University, 1980-1982; Research Assistant, Medical Entomology Laboratory, Department of Entomology, Oklahoma State University, Summer, 1982; Graduate Teaching Associate, Department of Parasitology, Microbiology and Public Health, College of Veterinary Medicine, Oklahoma State University,

August 1982-June 1985; Research Investigator, Oklahoma Animal Disease Diagnostic Laboratory, Oklahoma State University and Oklahoma City Zoo, July 1983-September 1985.