ANTIGENIC DIFFERENCES BETWEEN A RESPIRATORY AND A NEUROVIRULENT STRAIN OF BOVINE HERPESVIRUS-1

BY

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CHAPTER I

INTRODUCTION

The Herpesviridae Family

The herpesviruses represent a large, clearly defined group of viruses. They are responsible for a variety of human and animal diseases, and nearly 100 herpesviruses have been characterized to date (Fields *et al.*, 1990). Herpesviruses have been isolated from man, most domestic animals, wild mammals, birds and fish (Stranberg *et al.*, 1965; Fabricant *et al.*, 1974; Wolf *et al.*, 1971). The herpesviridae family is divided into three subfamilies, α-herpesvirinae, β-herpesvirinae, and γ-herpesvirinae. This classification is based on thier biological properties and genomic structure (Roizman *et al.*, 1982).

The α-herpesviruses characteristically have a short replication cycle, are highly cytopathic, and establish latent infections in nerve cells, particularly neurons in the trigeminal ganglia (Roizman *et al.*, 1982; Baringer, 1976). Human herpes simplex virus (HSV) type 1 and type 2, varicella zoster virus, simian agent 8, B virus, pseudorabies virus and equine herpesvirus (EHV) types 1, 3 and 4 are all examples of α-herpesviruses. In the bovine species, bovine herpesvirus-1 (BHV-1), bovine

herpes mammillitis virus (BHV-2), BHV-5 and BHV-6 (Roizman, 1982; Plummer *et al.*, 1969 and 1973; Mortin *et al.*, 1966) are members of the α-herpesvirus subgroup.

The β-herpesviruses are the cytomegaloviruses (CMV). They are characterized by a relatively long replication cycle, their species-specificity, and establishment of latent infections in the salivary glands, kidneys and lympho-reticular tissues (Plummer *et al.*, 1969; Wittmann, 1989). The β-herpesviruses include human CMV, EHV-2 and BHV-4 (Roizman *et al.*, 1982; Wittmann, 1989).

The γ-herpesviruses include human Epstein-Barr virus, BHV-3, and gallid herpesvirus-2 (Churchill *et al.*, 1967). The γ-herpesviruses commonly cause oncogenic transformation of T- and B-lymphocytes and establish latent infections in the lymphoid tissues (Wittmann, 1989).

Bovine Herpesvirus Type 1 (BHV-1)

General Virion Structure

The bovine herpesviruses are largely comparable to other members of the herpesvirus family (Roizman, 1978). BHV-1 consists of an icosahedral nucleocapsid with a diameter 95-110 nm which is made up of 162 capsomeres. The capsid encloses the viral DNA genome which has a total length of approximately 137 kilo base pairs (kbp) (Mayfield *et al.*, 1983). The nucleocapsid is surrounded by an electron-dense material called the tegument which in turn is surrounded by a lipid bilayer envelope, forming a

pleomorphic virion 150-200 nm in diameter (Valicek *et al.*, 1976; Wittmann, 1989). The tegument is probably involved in maintaining the shape and structure of the virion (Fong *et al.*, 1973). The envelope, derived from the host cell membrane, has several viral glycoproteins anchored in it (Epstein, 1962). These surface glycoproteins appear as spikes by electron microscopy (Linda, 1986).

Classification of BHV-1 into Subtypes

BHV-1 isolates or strains have been subdivided into 3 subtypes: BHV-1.1, BHV-1.2, and BHV-1.3 (Metzler *et al.*, 1985; Metzler *et al.*, 1986; Friedli *et al.*, 1987; Wittmann, 1989). These subdivisions are based on restriction endonuclease (RE) cleavage pattern of the viral genome, viral protein profiles, and reactivity of the virus with monoclonal antibodies. To date, most BHV-1 isolates can be assigned to one of these three subtypes. Subtype 1, which the Cooper strain typifies, are commonly associated with infectious bovine rhinotracheitis (IBR). Subtype 2 of which strain K22 is the protype are associated with infectious pustular vulvovaginitis (IPV). Subtype 3 strains are few and are associated with encephalitis. Subtype 2 can be further subdivided into types 2a and 2b by differences in their viral polypeptides. Subtype 3 can also be further subdivided in types 3a and 3b, represented by the N-569 and A-663 strains respectively (Metzler *et al.*, 1987). Subtype 3, also known as bovine encephalitis virus (Studdert, 1989), is responsible for outbreaks of neurological disease in calves. These outbreaks have been recorded in Australia and Argentina (French, 1962.,

Carrillo *et al.*, 1983; Metzler *et al.*, 1986). Although outbreaks of fatal encephalitis caused by BHV-1 have been recorded in the United States (Barenfus, 1963., Eugester *et al.*, 1974) the virus isolated from such outbreaks were only identified by serology as BHV-1 virus.

Significant differences have been observed between the genomes of the different BHV-1 subtypes. The RE migration pattern of BHV-1.1 and BHV-1.2 strains are different, but only slightly (Engels *et al.*, 1981). The RE enzyme Hpa I produces two distinctly different cleavage patterns (Gregerson *et al.*, 1984). Whereas the BstE II RE migration pattern of BHV-1.1 and BHV-1.2 are very similar with only sight differences in the mobility of the G fragment, the BHV-1.3 BstE II migration pattern is very different. BstE II also distinguishes BHV-1.3a and BHV-1.3b (Engels *et al.*, 1986/87). The differences between BHV-1.1 and BHV-1.2 are restricted to the regions of the genome corresponding to map units 0.09-0.2 and 0.83-0.92, whereas differences in BHV-1.1 and BHV-1.3 are distributed throughout the genome (Engels *et al*, 1986/87). There is 95% homology between BHV-1.1 and BHV-1.2 strain genomes by DNA/DNA hybridization, whereas the similarity between the genomes of BHV-1.1 and BHV-1.3 is only 85% (Engels *et al.*, 1986/87).

Bagust (1972) compared four different BHV-1 strains serologically and reported that the antigenic nature of N-569 BHV-1.3 was significantly different from the three other isolates by kinetic neutralization test. Separation of BHV-1.1 and BHV-1.2 by virus neutralization (VN) tests using rabbit hyperimmune sera was not possible (Bowling *et al.*, 1969), although

slight antigenic variation could be observed by using neutralizing kinetic assays (Bagust, 1972; House *et al.*, 1972).

Viral Polypeptides and Glycoproteins

BHV-1 has at least 33 structural (virion) polypeptides, and of these, 11 are glycoproteins (Misra *et al.*, 1981). These viral polypeptides can be divided into alpha (immediate early), beta (early), and gamma (late) proteins on the basis of order of appearance in the infected cells. The glycoproteins gI, gII and gIII are the major BHV-1 glycoproteins involved in virus neutralization; gI and gII are beta proteins and gIII is a gamma protein. Glycoproteins gI, gII and gIV were found to induce high levels of antibodies in cattle, which could neutralize virus and participate in antibody dependant cellular cytotoxicity (ADCC) (Babiuk *et al.*, 1987).

The nomenclature for the BHV-1 viral glycoproteins was suggested by van Drunen Littel van den Hurk and Babiuk (1986). Four unique glycoproteins or glycoprotein complexes were recognized by a panel of monoclonal antibodies to BHV-1. Glycoprotein gI corresponds to the glycosylated viral polypeptides (GVP) 6/11a/16K (130,000 mol. wt. glycoprotein), and gII and gIII to GVP 7 and GVP 9, respectively. There is also a gIV which corresponds to the GVP 11b protein. Bolton *et al.*, (1983) found that the gp9 and gp11 described by Misra *et al.*, (1982) corresponded to their GVP 8 and GVP 13, and constituted the major surface antigen which comprised the envelope of BHV-1.

The beta proteins are more immunogenic than the gamma proteins

(Ludwig *et al.*, 1987). Glycoprotein gI is an external surface (beta) protein of infected cells and participates in virus neutralization and complement mediated immunocytolysis of infected cells (Misra *et al.*, 1982) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of BHV-1 infected cells revealed two major immunogenic glycoproteins, GVP 93 (gIII) and GVP 74 (gII) which induced neutralization antibodies (Gregerson *et al.*, 1985). gIII induced both neutralizing and hemagglutination inhibiting antibodies (Trudel *et al.*, 1987). Using monoclonal antibodies to different antigenic sites on BHV-1 glycoproteins, it was found that some antigenic glycoproteins mediate virus neutralization both with and without complement (Okazaki et al., 1986). Using monospecific sera and monoclonal antibodies, major BHV-1 glycoproteins involved in viral neutralization were 180/97K, (gIII) 150/77K (gIV) and to lesser extent 130/74/55K (gI) (Marshall *et al.*, 1986).

Herpesvirus attachment and penetration are mediated by glycoproteins (Liang *et al.*, 1991). Penetration occurs by fusion of the viral envelope with that of the host cell plasma membrane (Wudunn and Spear, 1989). After penetration, the nucleocapsid is transported to the cytoplasm and viral DNA is released into the nucleus, where virus replication occurs. Newly assembled nucleocapsids acquire their envelope from the inner lamella of the nuclear membrane, from the cytoplasmic membrane, or from the plasma membrane (Fong *et al.*, 1973). Mature virions accumulate in the membrane systems of the host cell and may be released slowly by vacuolar membrane fusion and exocytosis, or by the lysis of the infected cells

(Wittmann, 1989; Gibbs *et al.*, 1977).

Cell to cell spread of BHV-1 may be facilitated by gIII (Okazaki *et al.*, 1987). Tunicamycin blocks transport of glycoproteins gI and gIII to the cell surface and inhibits production of infectious particles (van Drunen Littel van dan Hurk *et al.*, 1985). Liang *et al.*, (1991), using a BHV-1 gIII gene-deleted mutant, demonstrated that gIII function is not required for growth of virus in cell culture. However, some impaired functions were noted such as defective attachment, delay in replication and lower titer of extracellular viruses; gIII appeared to be the major glycoprotein involved in cell attachment, although gI and gIV also contributed to some extent.

Clinical Disease in Cattle

BHV-1 is a major viral pathogen of cattle. It can cause several clinical entities which include IBR, IPV and meningoencephalitis.

Infectious Bovine Rhinotracheitis. IBR was first described as a new respiratory tract disease of feedlot cattle in the US in 1955 (Miller, 1955). According to McKercher *et al.*, (1950) the disease was first observed in a feedlot in Colorado in 1950. The virus was isolated soon thereafter (Madin, 1956). It was recognized in dairy cattle in California in 1953 and Schroeder *et al.*, (1954) reproduced the disease in experimental cattle and suggested that the disease may be caused by some virus. IBR virus has also been isolated from cattle affected with conjunctivitis (Abinanti and Plummer, 1961) and from the uterine exudate of cows showing fever and metritis with

mucopurulent discharge (Lomba *et al.*, 1976). Metritis may also occur as a result of artificial insemination with semen contaminated with BHV-1 (Kendrick and McEntee, 1965; Karhs, 1977). BHV-1 has also been associated with alimentary tract infection and has been isolated from calf feces without any diarrhea (Baker *et al.*, 1960). More frequently, diarrhea is a clinical sign of the generalized and often fatal BHV-1.1 virus infection of young calves (Curtis *et al.*, 1966). BHV-1.1 has also been associated with and isolated from cases of mastitis (Gibbs *et al.*, 1977). Abortion is also characteristic of BHV-1.1 infection, and modified live IBR vaccine has caused abortion in cows (McKercher, 1964). Kendrick (1965) demonstrated experimentally that IBR virus causes abortion.

Infectious Pustular Vulvovaginitis. IPV was first described as a venereal disease by a Swiss veterinarian in the year 1841 (Wittmann, 1989). This disease was noticed in heifers, dairy cows and bulls (Kendrick, 1958). The infection arises invariably from the introduction of virus to the mucosa of the genital tract by coitus or via external agents rather than from a viremic phase associated with BHV-1 lesions elsewhere in the body (Gibbs *et al.*, 1977). The mucosal surface of the genital tract does not need to be abraded for infection to become established (Collings *et al.*, 1972). The BHV-1.2 virus does not interfere with fertility (Saxegaad, 1970) and does not cause abortion (Gibbs *et al.*, 1977).

Meningoencephalitis. Occasionally, BHV-1 causes neurological disease in young calves between the ages of 3-10 months. This is characterized by incoordination, muscular tremor, recumbency, aimless circling, ataxia, blindness and eventually death (Eugester *et al.*, 1974; French, 1962; Carrillo *et al.*, 1983). Outbreaks of the BHV-1 encephalitis seem to be more prevalent in Australia and Argentina than elsewhere in the world. Recently it was also observed in Brazil (Weiblen *et al.*, 1989). In the USA, an outbreak of BHV-1 encephalitis was first reported in dairy calves in Los Angeles, California (Barenfus *et al.*, 1963). Another outbreak was reported from Texas in 1974 in range calves (Eugester *et al.*, 1974).

The first case of BHV-1 encephalitis to be reported was in Australia in 1959-61 (Johnston *et al.*, 1962). The virus isolate from that outbreak was designated as N-569 (French, 1962). This encephalitic strain was shown to produce encephalitis in experimental calves inoculated intranasally or intravaginally (Bagust *et al.*, 1972). Hall *et al.*, (1966) reported that calves infected with N-569 developed encephalitis whereas standard respiratory and genital strains of BHV-1 inoculated in similar ways did not produce encephalitis. Bagust *et al.*, (1972) indicated that N-569 is antigenically different from BHV-1 IBR strain. Brake and Studdert (1985) studied the molecular epidemiology and pathogenesis of ruminant herpesviruses including buffalo, caprine and bovine encephalitis herpesviruses. They reported that three epidemiologically unrelated encephalopathic bovine herpesviruses had similar restriction enzyme fingerprints that were totally different from BHV-1.1 and BHV-1.2 and

other ruminant herpesviruses. They proposed bovine encephalitis herpesvirus as the prototype of a new bovine herpesvirus type.

Latent Infection in Cattle

All herpesviruses are capable of establishing latent infection in their host as a sequel to primary infection (Gibbs *et al.*, 1977). After multiplication at the local site of infection, BHV-1 enters the peripheral nervous system and is transported presumably by centripetal spread via neurons, mainly to the trigeminal and sacral ganglia (Narita *et al.*, 1981). After invading nerve fibers or ganglia, BHV-1 is no longer accessible to humoral antibodies (Narita *et al.*, 1980). Virus residing in trigeminal ganglia are responsible for recurrent infections (Baringer, 1976). After treatment with dexamethasone (DM), BHV-1 is reactivated and travels centrifugally through the nerve fiber to mucous membranes where they replicate. When cattle, that had been previously exposed experimentally to BHV-1 and recovered, were inoculated intravenously with DM daily over a period of several days, virus was excreted in nearly all animals examined (Davies and Carmichael, 1973). Reactivation of BHV-1 resulted in an anamnestic IgG response and a secondary IgM antibody response. Reactivation of latently infected BHV-1 stimulated the formation of antiviral antibody of the IgG1 and IgG2 classes, which can distinguish this response from a primary infection in which IgG2 was not produced (Guy and Potgieter, 1985).

Immune Response of Cattle to BHV-1 Infection

Following natural infection or vaccination with a modified live BHV-1 vaccine, cattle develop both a humoral and a cell-mediated immune (CMI) response (Kahrs, 1977; Babiuk *et al.*, 1974).

Humoral Immune Response

Systemic humoral immune responses depend on serum immunoglobulins (Rouse and Babiuk, 1978). The production of antibodies to BHV-1 in cattle begins at 8-12 days postinfection (PI), and may persist for 66 months (Chow, 1972). IgM antibodies appear first, followed by IgG. During the first month, both classes require complement for neutralization, but IgG becomes complement-independent and predominates in anamnestic responses (Rossi and Kiessal, 1976). Guy and Potgieter (1985) reported that maximum IgM activity was detected on day 14 PI, whereas maximum IgG antibody titers were reached by day 35 PI. IgG declined more slowly after infection than IgM which declined rapidly. The presence of IgM indicates recent exposure to the virus (Guy and Potgieter, 1985).

The role of antibodies in recovery from BHV-1 infection is questionable. Antibody appears very late in BHV-1 infection (Kahrs, 1977; Rouse and Babiuk, 1978). BHV-1, like other herpesviruses, escapes the immune system by spreading through intercellular bridges or through neural ganglia. However, antibodies are known to limit the spread of some other viruses, such as picornaviruses, which spread by the way of

extracellular route (Rouse and Babiuk, 1978). Antibodies cooperate, *in vivo*, with other components of the immune system; complement (antibody mediate complement lysis) (Rouse *et al.*, 1975), leukocytes (ADCC) (Shore *et al.*, 1974) or both (ADCC-C). Complement mediated destruction of antibody sensitized virus infected cells *in vitro* has been reported (Babiuk *et al.*, 1975). ADCC occurs when an effector cell, equipped with a receptor for the Fc portion of immunoglobulin (IgG), binds with the Fc portion of an antibody which in turn binds to viral antigens on the surface of a virus infected cells (Shore *et al.*, 1974). The ADCC-C is effective in early recovery when IgM is predominant, levels of antibodies are low, and number of effector cells are low.

Cell Mediated Immune Response

Cell mediated immunity is thought to play a crucial role in resistance and recovery from BHV-1 infection (Davies and Carmichael, 1973; Rouse and Babiuk, 1974). Rouse and Babiuk (1974) observed a lymphocyte blast cell response soon after infection and the disappearance of virus from nasal secretions before the appearance of significant amounts of circulating antibodies. They used a blast cell assay to confirm their finding that peripheral blood lymphoctytes (PBL) were able to prevent viral plaque formation in the cell monolayer infected with BHV-1. The inhibition was shown to be immunologically specific and involved suppression of viral replication rather than destruction of virus infected cells. Two main factors essential for CMI are antigen recognition and effector or mediator function.

These functions are implemented by T-lymphocytes and phagocytes (macrophages) (Tizard, 1987). T-lymphocytes produce mediator factors (lymphokines) which require reciprocal interaction with phagocytes (Rouse and Babiuk, 1978). The T-cells can be directly cytotoxic, activate macrophages and produce interferon (Rouse and Babiuk , 1978).

Non-Specific Immunity

Interferon (IFN) plays an important role in non-specific immunity and in protecting animals from BHV-1 infection. INF can be induced in cattle by infecting them intranasally or via the genital tract (Babiuk *et al.*, 1985). Viral infection induced high levels of IFN locally, which is considered important in recovery from BHV-1 infection (Rouse and Babiuk, 1978). d'Offay and Rosenquist (1988) reported that although BHV-1 virus induce IFN production in nasal secretions of calves, the amount of viral replication was not directly correlated with amount of IFN produced, nor did greater interferon production in animals result in less virus excretion.

Natural killer (NK) cells exhibit spontaneous cytotoxicity against neoplastic cells and viral infected cells (Tizard, 1987). Following infection with BHV-1, a transient increase in NK-cell activity was observed (Babiuk *et al.*, 1985).

Quantitating Antibody Response in Cattle

Several procedures have been used to quantitate antibodies in serum of cattle. These include the indirect fluorescence antibody test, indirect

hemagglutination test, reverse passive hemagglutination, complement fixation test, enzyme linked immunosorbant assay (ELISA) and virus neutralization (VN) test (Engvall *et al.*, 1972; Edward and Gitoa, 1987; Wardly and Crowther, 1982).

Virus Neutralization Test

The detection of neutralizing antibodies in the serum of cattle is indicative of infection. The VN test is the only serological test that measures virus neutralizing antibodies (Carbery *et al.*, 1972). Standards for virus growth rate, dose response curve, variation with and between tests, and relationship of amount of virus to serum titer were established by Mohanty and Lillie (1965). A standard serum neutralization test for BHV-1 has been established by Carbary *et al.*, (1972). The constant virus and varying serum dilution neutralization test was compared to the constant serum and varying virus. It was found that the constant serum and varying virus procedure was more sensitive, but with constant virus and varying serum dilution, accurate results were obtained when 50 to 100 $TCID_{50}$ of virus were added to 2-fold serum dilutions (House and Baker, 1970). Although the VN test is accepted as the standard test for the serological diagnosis of the BHV-1 (Collins *et al* 1984; House and Baker, 1970), it is slow, expensive and takes days before the results can be read.

Enzyme linked Immunosorbant Assay (ELISA)

ELISA was first introduced by Engvall and Perlmann in 1972. They

used it for the quantification of specific antibodies by enzyme labelled antiglobulin. The technique is as sensitive as the radioimmunoassay. The ELISA has wide applications and has been used with protozoa, helminth, bacteria, mycoplasma, and to diagnose viral diseases both by detecting viral antigen or antibody immune response (Wardley and Crowther, 1982). Payment *et al.*, (1979) used the ELISA technique to detect BHV-1 antibodies in sera. The detection of BHV-1 antigen was increased 50-fold by using biotin and avidin interaction to amplify the reaction (Edward and Gitao, 1987).

Experimental Inoculation of Rabbits with BHV-1

Rabbits were found susceptible to BHV-1 and have been used as an experimental model for BHV-1 (Lupton *et al.*, 1980). Neonatal rabbits may be useful to study the pathogenesis of BHV-1 infection (Kelly, 1977). In adult rabbits treated with DM, infection was exacerbated and resulted in systemic infection similar to neonatal infection (Lupton *et al.*, 1974). Armstrong *et al.*, (1961) produced a mild erythematous lesion in rabbits inoculated intradermally with BHV-1. Rabbits have also been used to study the neuropathogenicity of BHV-1 in our laboratory (d'Offay *et al.*, 1990). When the Cooper and an encephalitic (EC) strains of BHV-1 were inoculated on the scarified cornea of weanling rabbits, at least half of the rabbits inoculated with the EC strain developed fatal encephalitis between 12-14 days PI, whereas rabbits inoculated ocularly with same dose of Cooper strain did not develop encephalitis (d'Offay *et al.*, 1990).

Specific Objectives of this Study

The first objective of the study was to determine the immune response of rabbits to infection with two stains of BHV-1, a respiratory (Cooper) strain and an encephalitic (EC) strain. These viruses were inoculated on the scarified cornea and sera were collected from infected rabbits sacrificed on various days PI. Rabbits were also inoculated with the viruses intranasally and sera collected on day 21 PI. Serum neutralizing antibodies were determined by the VN test, and the ELISA was used to determine serum antibodies that bound to viral antigen.

The second objective of the study was to determine to what extent serum antibodies of infected rabbits recognized the two virus strains. VN tests were performed using each virus strains as test virus, and ELISA was carried out using both the EC and Cooper viruses as antigens. Also, viral proteins separated by SDS-PAGE, were transferred to nitrocellulose paper and sera were reacted to these by western blot to determine the viral polypeptides recognized by the serum antibodies. Furthermore, viral polypeptides and glycosylated viral polypeptides were radio-labelled metabolically, separated by SDS-PAGE and identified by autoradiography.

The third objective was to identify determinants responsible for the antigenic differences observed between the two strains. Hyperimmune sera and sera made to certain specific viral glycoproteins (gI and gIII) were reacted to viral polypeptides by western blotting.

CHAPTER II

MATERIALS AND METHODS

Viruses and Cells

Two strains of BHV-1 were used in these studies: the Cooper strain (ATCC VR-864 strain; Colorado-1, Cooper-1) (York, 1957) and the EC strain (Eugester *et al.*, 1974). The Madin Darby bovine kidney (MDBK) cell line was used for the propagation of viruses. MDBK cells were grown in Eagle minimal essential medium (MEM) supplemented with non-essential amino acids, L-glutamine (200 mM), antibiotics (penicillin,100 units/ml; streptomycin, 100 ug/ml; fungizone, 0.25 ug), 25 mM HEPES buffer, 7.5% sodium bicarbonate and 5% fetal calf serum (FCS).

Both strains were plaque purified three times before making the virus stock for inoculation purposes. For virus stock preparation, MDBK cells were infected at a multiplicity of infection of approximately 1. When cells exhibited 4+ cytopathic effect (CPE), monolayers were scraped and infected cells centrifuged at 1800 x g at 4°C for 10 minutes. Pellets were resuspended in MEM containing 5% FCS, freeze/thawed 3 times, centrifuged briefly, and the supernatant (virus stock) stored in individual vials at -70°C. Virus titration was done by plaque assay on MDBK cells.

The EC strain virus stock contained $2.0x10^8$ PFU/ml and the Cooper virus stock $2.2x10^8$ PFU/ml.

Animal Inoculation

Weanling New Zealand White rabbits weighing 1-2 lbs were used for all studies.

In the first study, two groups of rabbits (18/group) were inoculated ocularly on the scarified cornea with 10^7 PFU of the Cooper strain (Group 1) or with 10^7 PFU of the EC strain (Group 2). Blood was collected by cardiac puncture (2 rabbits/group) on days 1, 3, 5, 8, 11, and 14 PI. Sera were collected by centrifugation and stored at -70°C.

In the second study, anaesthetized rabbits were inoculated with virus intranasally. Four rabbits were inoculated with $2x10^7$ PFU of the EC strain virus and three with the same dose of the Cooper strain. One control rabbit was not inoculated. Blood was collected on day 21 PI from all surviving animals.

Production of Hyperimmune Sera

Two groups of rabbits were used for the production of hyperimmune sera using the EC and Cooper strains. The first group (n=2) was injected at 10 day intervals intramuscularly with the EC strain mixed with incomplete Freund's adjuvant. The second group (n=4) were inoculated with Cooper (2) or EC (2) intradermally in the foot pad and after one month were inoculated every 2 weeks with virus mixed with incomplete Freund's adjuvant. One of

the rabbits inoculated with the Cooper strain died during the experiment. Blood were collected prior to each virus exposure and serum neutralizing antibodies were measured in the sera on each occasion. For the rabbit anti-Cooper gI and anti-Cooper gIII sera, the glycoproteins were purified using preparative SDS-PAGE gels and SDS-hydroxylapatite column chromatography. After purification, glycoprotein was inoculated into rabbits as described (Eberle and Courtney, 1980).

Serum Neutralization Test

Neutralizing antibody titers in sera were determined by a microtiter serum neutralization test, using 96-well tissue culture plates (Falcon Labwear) as described by Carbery *et al.*, (1972) with some modifications. All sera were heat inactivated at 56°C for 30 minutes before being tested. Sera were diluted 2-fold and four wells were used per dilution. Approximately 100 $TCID_{50}$ of either the EC strain or Cooper strain were added to each well and the virus-serum mixture incubated at 37°C for one hour after which trypsinized MDBK cells (approximately 75000 cells/ well) were then added to each well. The plates were incubated at 37°C in 5% CO_2 atmosphere for day 4 after which time the results were read. Titers were recorded as the reciprocal of the highest final serum dilution which completely protected at least 3 of the 4 wells from CPE.

Preparation of Antigen for ELISA, Gel Electrophoresis and Western Blot.

MDBK cells grown in 150 cm^2 flasks were infected with the EC and Cooper strains at a multiplicity of 1 as described earlier. One flask was not infected and served as uninfected control. When cells in the infected flasks showed 4+ CPE, cells in both infected and uninfected flasks were scraped and centrifuged at 1800 x g. Cell pellets were washed with PBS (phosphate buffer saline) and re-centrifuged. The pellets were then suspended in 20ml of PBS containing 0.5% Triton X-100 (Bio Rad) and 0.1% SDS. This mixture was sonicated and then stored at -70°C. These cells lysates were tested for protein concentrations by the Lowry method (Lowry *et at.*, 1951). Protein concentrations of the lysates were equalized before they were used in ELISAs.

For gel electrophoresis, MDBK cells in 15 cm^2 wells were infected with 10^8 PFU of EC or Cooper strain in 1 ml of medium. After incubation at 37°C for 1 hour, the medium was replaced with MEM with 2% FCS and incubated for a further 4 hours. ^{35}S-methionine and ^{14}C-glucosamine were then added to final concentration of 2 uCi/ml and 1 uCi/ml, respectively, and the plates incubated overnight (Eberle and Courtney, 1980). At 24 hours PI when the cells showed 4+ CPE, they were scraped, centrifuged at 1800 x g and resuspended in water.

Immunoblot (Western Blot) antigen was prepared by infecting 150 cm^2 flasks of MDBK cells with the Cooper or EC strain as described above. When there was 4+ CPE, cells were scraped and centrifuged at 1500 x g for 5 minutes. Pellets were washed with PBS, centrifuged and resuspended in 600ul of water containing 0.5% TX-100 and 0.1% of SDS.

ELISA Procedure

Infected and uninfected MDBK cells lysates were used as antigens for the ELISA. The assay was performed according to instructions provided by Vector Laboratories for its Vecta Stain ABC kit (Vector Laboratories, CA). Round bottom 96-well polyvinyl plates were coated overnight at 4°C with cell lysates diluted in 1 X Hanks' balanced salt solution containing O.375% sodium bicarbonate. Wells in one column of each plate were not coated with antigen and were used as blanks. After overnight incubation, wells were blocked with 1% bovine serum albumin (BSA) in 10% gelatin for one hour. Test sera plus standard positive and negative sera were diluted 2-fold in 10% gelatin with 1% BSA before transfer to the wells. 50ul of different serum dilutions were added to wells containing the antigens. The plates were rocked for 1 hour at room temperature after which they were washed with PBST (0.5% Tween 20 in PBS). Then 50ul of 1:4000 biotinylated rabbit anti-IgG were added to all wells except those used for blanks, plates rocked for an additional hour, washed again with PBST, after which 50ul of ABC reagent (made according to kit instructions) were added. After further incubation, wells were washed once more, and 100ul of

substrate solution (diaminobenzidine tetrahydrochloride in citrate buffer with 5ul of 30% H_2O_2 added as catalyst) was added to each well. After 30 minutes incubation in dark, the reaction was stopped by adding 50ul of 2M H_2SO_4. The absorbance of the solutions in each well were determined in a micro-ELISA plate reader using a wavelength mode of 490 nm. The final ELISA titer was read as the reciprocal of the highest serum dilution with an absorbance value on virus antigen which was at least 3 times higher than that on uninfected cell antigen.

Gel Electrophoresis

^{35}S-methionine and ^{14}C-glucosamine labelled cell lysates were diluted 1:2 in electrophoresis sample buffer (1 M urea, 2% SDS, 2% mercaptoethanol and 8% glycerol with tracking dye), heated at 100°C for 2 minutes, and electrophoresed on 7% SDS-polyacrylamide gels as described by Laemmli (1970). Gels were run at constant current of 15-20 mAmp/gel in running buffer (0.1% SDS, 25 mM Tris and 192 mM glycine). When the dye band was about 1 cm from the bottom of the plate, electrophoresis was stopped. The gels were fixed in destain (25% MEOH, 7% acetic acid) for 15 minutes, and then transferred to Whatman paper. The gels were dried under vacuum with heat for 1-2 hours. The dried gels were put on Fuji X-ray film (24.3cm x 25.4cm) and kept at -70°C. Films were developed after 1-3 days.

Western Blotting

Antigens for the western blot were diluted in sample buffer and electrophoresis conducted in 7% SDS-PAGE as described above. After electrophoresis, the rest of procedures were adopted as described by Eberle and Mou, (1983). After electrophoresis, the gel was placed against a sheet of nitrocellulose and the proteins were electrophortically transferred from the gel onto the nitrocellulose paper (Burnette, 1981). The electroelution procedure was carried out in transfer buffer (20% methanol, 25 mM Tris and 192 mM glycine) at 1.50-2.00 Amps for 1-2 hours.

Prior to addition of sera, nitrocellulose membranes with bound antigens were blocked with 2% BSA in 10X NT (NaCl 0.9%, Tris HCl 10 mM) for 1 hour at room temperature. Following this, test sera were allowed to react with the nitrocellulose bound antigens for 3 hours. After washing 5 times with PBST, bound antibodies were detected by using I^{125}-labelled Staphylococcus protein A and autoradiography. Nitrocellulose membranes were dried and exposed to Fuji medical X-ray film (24.3cm x 25.4cm) for 12-20 hours at room temperature.

CHAPTER III

RESULTS

Clinical Signs Associated with Infection

Rabbits Inoculated Ocularly

In the first study, rabbits were inoculated with 10^7 PFU of either the Cooper or the EC strain on the scarified cornea. Cooper strain infected rabbits developed conjunctivitis on the second day following infection which lasted for at least a week. Mucopurulent conjunctivitis developed in a few animals. In rabbits infected ocularly with the EC-strain, conjunctivitis was much milder and was not observed beyond day 5 PI. Overall, rabbits inoculated with the Cooper strain developed a more severe infection than those inoculated with the EC strain. A rabbit inoculated with EC strain developed convulsions on day 10 PI and was euthanized. None of the rabbits inoculated with the Cooper strain developed clinical encephalitis.

Rabbits Inoculated Intranasally

In the second study, rabbits were inoculated intranasally with $2x10^7$ PFU of either the Cooper or EC strains. Most rabbits became depressed (hunched and hardly moved) on the second day after inoculation.

Depression was more severe in the rabbits inoculated with the Cooper strain and lasted for at least one week. Respiration rates increased in many rabbits, but particularly in those inoculated with the Cooper strain. Anorexia were also observed in some of the animals after inoculation. Only one rabbit inoculated with the EC strain developed fatal encephalitis on day 7 PI. None of the rabbits inoculated with Cooper strain developed encephalitis. Again it was noted that rabbits inoculated with the Cooper strain were more severely affected clinically following intranasal inoculation.

Immune Response to Infection

First Study

In the first study, 36 rabbits were infected (18 with each virus strain) and 4 animals sacrified on days 1, 3, 5, 8, 11 and 14 PI. Sera were obtained at the time of sacrifice. These sera were tested for neutralizing antibodies and for the presence of IgG antibodies to BHV-1 by ELISA.

Serum neutralizing antibodies were first detected on day 14 PI, and only in rabbits inoculated with the Cooper strain virus. None of the rabbits inoculated with the EC strain seroconverted by day 14 PI in first study. The seropositive sera were obtained from both rabbits sacrificed on day 14 PI, and titers of 1:8 and 1:16 were recorded (Table I).

When sera were tested for the presence of antiviral IgG by ELISA, antibodies were detected as early as 8 days PI in both groups of rabbits

TABLE I

VIRUS NEUTRALIZING AND ELISA ANTIBODY TITERS IN SERA OF RABBITS INOCULATED OCULARLY WITH THE COOPER STRAIN VIRUS

Animal Number	Postinoculation days	Neutralizing Ab titers	ELISA Ab titers against	
			Cooper	EC-strain
1	1	0[a]	0[b]	0
2	1	0	0	0
4	3	0	0	0
6	3	0	0	0
7	5	0	0	0
8	5	0	0	0
10	8	0	800	100
11	8	0	400	50
13	11	0	1600	100
14	11	0	1600	50
16	14	8	6400	800
17	14	16	12800	1600

[a] For neutralizing antibody titers, 0 = <4
[b] For ELISA antibody titers, 0 = <25

(Tables I and II). In rabbits inoculated with the Cooper strain, an average titer of 600 was recorded on day 8 PI and this increased to 9600 by day 14 PI. In rabbits inoculated with the EC strain, antibodies were detected in only one of the two serum samples tested on both day 8 and day 11 PI. Rabbit #34, which was to be sacrificed on day 14 PI, died of encephalitis on day 10. It had, at this time, detectable amounts of antibodies in its serum.

When the sera from rabbits inoculated with the Cooper strain were tested against the EC strain and the Cooper strain antigen by ELISA, antibody titers in individual rabbits were at least 8-fold higher on the homologous Cooper than with the heterologous EC strain antigens (Table I). However, when sera from the rabbits inoculated with the EC strain were similarly tested, titers were almost the same irrespective of the antigens used (Table II).

Second Study

In the second study, sera from rabbits were obtained on day 21 PI, except for the one sample collected on day 7 PI from the rabbit that developed fatal encephalitis. Serum neutralizing antibody titers and IgG antibody titers were determined for all these sera, and results are given in the Table III. Of the 4 rabbits inoculated with EC strain, only two seroconverted by day 21 PI. One of the rabbits that did not seroconvert died on day 7 PI. All 3 rabbits inoculated with the Cooper strain seroconverted by 21 day PI with NA titers ranging from 1:16 - 1:32.

TABLE II

VIRUS NEUTRALIZING AND ELISA ANTIBODY TITERS IN SERA OF RABBITS INOCULATED OCULARLY WITH THE EC-STRAIN VIRUS

Animal Number	Postinoculation days	Neutralizing Ab titers	ELISA Ab titers against	
			Cooper	EC-strain
19	1	0[a]	0[b]	0
20	1	0	0	0
22	3	0	0	0
24	3	0	0	0
25	5	0	0	0
26	5	0	0	0
28	8	0	0	0
29	8	0	200	200
34[c]	10	0	400	800
31	11	0	0	0
32	11	0	400	400
36	14	0	400	400

[a] For neutralizing antibody titers, 0 = <4
[b] For ELISA antibody titers, 0 = <25
[c] Rabbit died of encephalitis on day 10 PI

Neutralizing titers in two of those rabbits were 4-fold lower when the heterologous EC strain was used as the test virus (Table III). Similarly, NA titers in sera from rabbits inoculated with the EC strain tended to be higher when the homologous virus was used as the test virus. This indicated that virus neutralizing antibodies in recovered rabbits differed in their ability to neutralize the two virus strains.

When the sera from rabbits in the second study were tested for presence of IgG antibodies by ELISA, the rabbits inoculated with the Cooper strain had, on average, higher serum antibody titers then rabbits inoculated with EC strain (Table III). However, all these sera were tested only against the Cooper antigen.

Hyperimmune sera obtained in the third study were also tested by VN test. The BHV-1 Cooper strain hyperimmune serum had a neutralizing antibody titer of 1:256 when tested with the Cooper strain, but the titer was 4-fold lower against the EC strain. The EC strain hyperimmune serum had a titer of 1:64 when tested against the EC strain but had a titer of 1:16 when tested with the Cooper strain. These results indicate once more, that the neutralizing capacity of sera from hyperimmunized rabbits were different for the two strains.

Analysis of Viral Proteins

Since the VN tests and ELISA assays suggested that the two viruses differed antigenically, a comparison of their protein profiles was performed.

TABLE III

VIRUS NEUTRALIZING AND ELISA ANTIBODY TITERS IN SERA OF RABBITS INOCULATED INTRANASALLY WITH THE EC- OR COOPER STRAINS

Animal Number	Inoculated with:	ELISA Ab titers[b]	NA titers[a] when tested against	
			Cooper	EC-strain
7	EC	51200	4	32
8	EC	0[c]	0[d]	0
9	EC	25600	8	16
10[e]	EC	0	0	0
11	Cooper	51200	16	16
12	Cooper	51200	32	8
13	Cooper	102400	32	8
14	Control	0	0	0

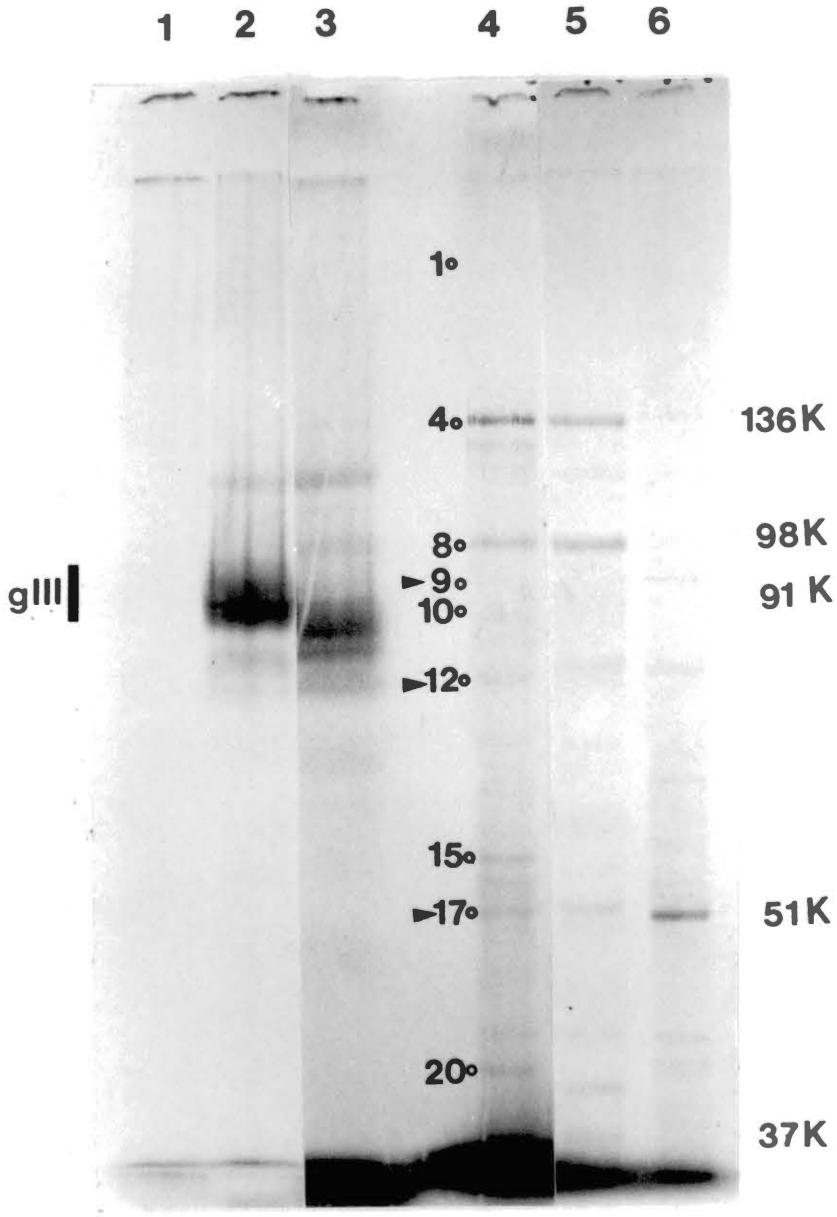
[a] All sera (except for animal #10) were obtained 21 days PI
[b] ELISA performed using Cooper strain viral antigen
[c] For ELISA antibody titers, 0 = <25
[d] For neutralizing antibody titers, 0 = <4
[e] Rabbit died of encephalitis on day 7 PI

Cells infected with the Cooper strain, cells infected with the EC strain and uninfected MDBK cells were labelled metabolically with ^{35}S-methionine. When the radio-labelled proteins were separated by SDS-PAGE, autoradiography revealed very similar polypeptide patterns for both viruses as can be seen in Figure 1. Thirty MDBK cell proteins were identified. Approximately 21 virus-specific protein bands were detected for the Cooper strain and 20 virus-specific bands for the EC strain. Although there is a lot of similarity between the two strains, several differences between the EC strain and the Cooper strain can be seen. These differences are in proteins # p9, p12 and p17.

Since it has been established that immune response to BHV-1 is mediated predominantly against the viral glycoproteins, MDBK cells infected with the EC strain and the Cooper strain were metabolically labelled at 4 hours PI with ^{14}C-glucosamine. Proteins were separated by the SDS-PAGE and autoradiography revealed several different glycoprotein bands in the infected cells. The glycoprotein bands corresponded to specific viral polypeptides. In addition to minor differences in the glycoprotein bands (Figure 1), a major difference appeared to exist in the region of gp 10. This gp 10 corresponds to the gIII glycoprotein in the case of the Cooper strain.

These results indicate that the EC strain protein profile, especially its glycoproteins, are different from the Cooper strain and that one of these differences is in the gIII region.

Figure 1: SDS-PAGE analysis of BHV-1 Cooper and EC glycoproteins and polypeptides. Uninfected MDBK cells (Lanes 1, 6) and MDBK cells infected with BHV-1 Cooper (Lanes 2, 5) or EC (Lanes 3, 4) were labelled with ^{14}C-glucosamine (Lanes 1-3) or ^{35}S-methionine (Lanes 4-6). Polypeptides were separated by SDS- PAGE on 7% gels. Viral proteins are numbered in lane 4. The polypeptides differing between the Cooper and EC are indicated with an arrowhead. Molecular weight of certain major polypeptides are shown at right.


1
2
3
4
5
6
gIII
1°
4°
8°
9°
10°
12°
15°
17°
20°
136K
98K
91 K
51K
37K

Western Blotting

In order to determine which viral polypeptides were recognized by antibodies in the sera of infected rabbits, viral proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane and reacted with sera by western blotting. Sera from rabbits inoculated with the EC strain did not bind to any viral antigens. Only one serum sample from a rabbit inoculated with the Cooper strain (collected day 14 PI) reacted with the Cooper virus antigens. This serum recognized two lower molecular weight proteins of 50 K and 39 K daltons.

Since most of the above mentioned sera did not react by western blotting, hyperimmune sera which contained higher titers of antiviral antibodies were used for the next stage of the study. The hyperimmune sera were reacted to viral proteins separated by SDS-PAGE and results showed different reactivity patterns for the two viruses (Figure 2). In Lane 4, it can be observed that the anti-EC hyperimmune serum recognized several differents EC strain protein bands with very intense reactivity with the 91 K protein. Anti-EC strain hyperimmune serum also reacted with the Cooper antigen but recognized fewer different protein (lane 5). In lane 6 containing uninfected MDBK cells protein, no reactivity with anti-EC hyperimmune sera was observed.

Figure 2 also shows anti-Cooper hyperimmune sera reacted with uninfected MDBK cell, Cooper strain and EC strain antigens. As can be seen, the sera reacted with a few polypeptides of the Cooper strain only and

there is intensive reactivity in the gIII glycoprotein region. When EC strain, Cooper strian and uninfected cells were treated with normal rabbits serum (Figure 3, Lanes 4-6), no reactivity was observed.

Because the anti-Cooper serum did not recognize the glycoproteins of the EC strain virus, and because there appeared to be differences in the glycoproteins of the two viruses when ^{14}C-glucosamine labelled infected cells proteins were examined, polyclonal hyperimmune antisera raised to the purified gI and gIII glycoproteins of BHV-1 (Cooper) were tested against the Cooper strain, the EC strain antigens and uninfected MDBK cells by western blot. The gI and gIII sera recognized only Cooper strain antigens . The gI anti serum reacted with gI glycoprotein and similarly gIII anti serum recognized only gIII glycoprotein (Figure 3).

These results indicated that anti-Cooper serum, and anti gI and gIII sera recognized only Cooper strain antigens; they did not react with EC strain antigens. Looking at these western blots, it appears that the Cooper strain is antigenically different from the EC strain. This difference is particularly evident in the gIII glycoprotein.

Figure 2: Immunogenicity of BHV-1 (Cooper) and BHV-1 (EC) polypeptides. Proteins of uninfected MDBK cells (Lanes 1, 6), MDBK cells infected with BHV-1 Cooper (Lanes 2, 5), or EC (Lanes 3, 4) were separated by SDS-PAGE and transferred to a nitrocellulose membrane for western blot analysis. The membranes were reacted with hyperimmune rabbit anti-BHV1 Cooper serum (Lanes 1-3) or hyperimmune rabbit anti-BHV1 EC serum (lanes 4-6). The position of the Cooper gIII glycoprotein is indicated at left.

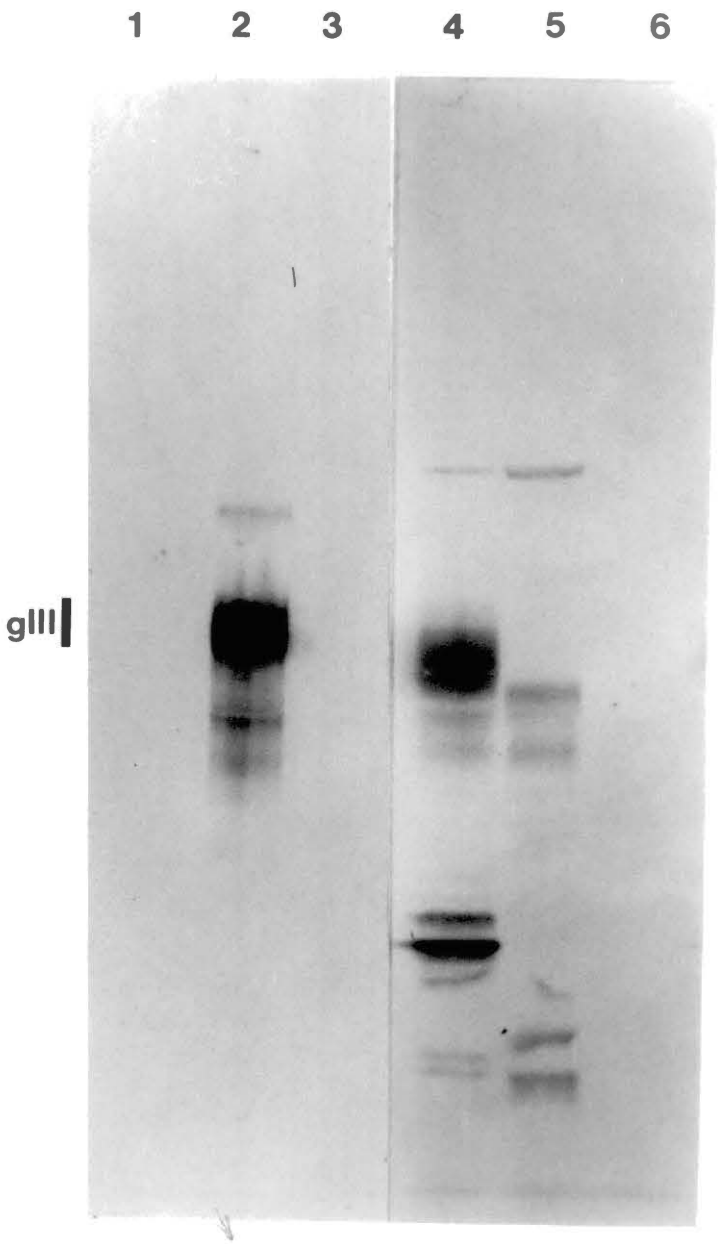
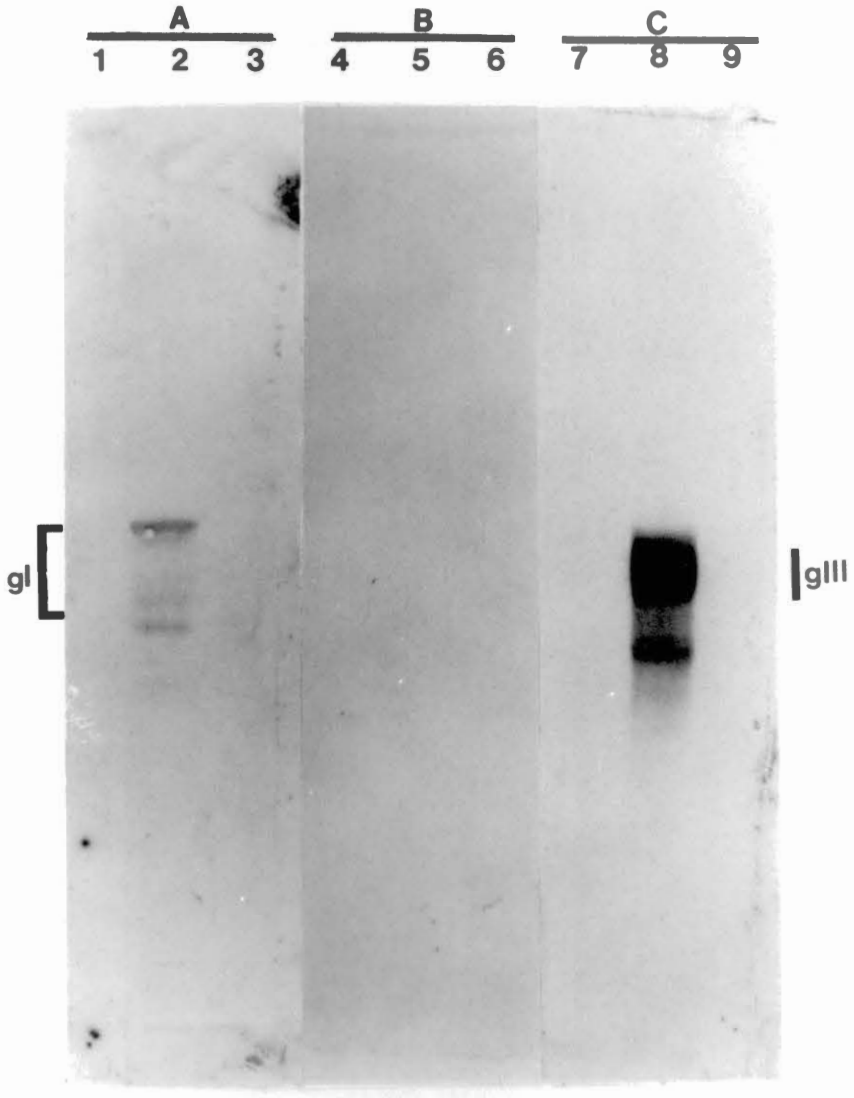

1
2
3
4
5
6
gIII

Figure. 3: Reactivity of antisera to BHV1 Cooper gI and gIII glycoproteins with the Cooper and EC glycoproteins. Proteins of uninfected MDBK cells (Lanes 1, 4, 7), MDBK cells infected with BHV-1 Cooper (Lanes 2, 5, 8) or EC (Lanes 3, 6, 9) were separated by SDS-PAGE and transferred to a nitrocellulose membrane for western bolt analysis. The membranes were reacted with rabbit anti-BHV1 Cooper gI serum (Lanes 1-3) nonimmune rabbit serum (lanes 4-6), or rabbit anti-BHV1 Cooper gIII serum. The position of the gI glycoprotein is indicated at left and of gIII at right.


A
B
C
1 2 3
4 5 6
7 8 9
gI
gIII

CHAPTER IV

DISCUSSION

Two strains of BHV-1 were used in this study: the respiratory Cooper strain (York, 1957) and the neurovirulent EC strain of BHV-1 originally isolated by Eugster *et al.*, (1974) in Texas. Preliminary work in our laboratory indicated that the RE migration pattern of the EC strain was very similar to the RE patterns reported for two other neurovirulent strains, the N-569 strain isolated in Australia by French (1962) and the A-663 Argentine isolate reported by Carrillo *et al.*, (1983). The RE migation patterns of these isolates have been published (Brake and Studdert, 1985; Engels *et al.*, 1986/87). The neurovirulent N-569 strain is antigenically different from the respiratory IBR virus (Bagust, 1972) and has been shown to cause fatal encephalitis in calves when inoculated intranasally. The purpose of the present study was to compare the pathogenesis of our neurovirulent EC strain to the Cooper strain in rabbits, and to determine if the two strains were antigenically different.

Only the neurovirulent EC strain caused encephalitis in rabbits in the present study. Of the rabbits inoculated ocularly on the scarified cornea with 10^7 PFU of the EC strain virus, one rabbit had to be euthanized on day 10 PI when it suddenly developed convulsions. In those inoculated

intranasally with 10^7 PFU of the same virus, one (25%) developed clinical encephalitis on day 7 PI. Rabbits inoculated similarly with the same dose of the respiratory Cooper virus did not develop clinical encephalitis. This confirms previous observations made in our laboratory regarding the neuropathogenicity of the EC strain for rabbits. In these studies 25%-75% of rabbits inoculated ocularly with the virus developed clinical encephalitis 10-14 days PI; in the present study, only 1 out of 18 rabbits inoculated ocularly with the EC virus developed fatal encephalitis. This was partly because rabbits were sacrificed on various days PI and so did not survive to develop fatal encephalitis between 10-14 days PI.

Meningoencephalitis caused by BHV-1 occurs sporadically in cattle. An outbreak of encephalitis in calves caused by BHV-1 was first reported in the USA by Barenfus *et al.*, in (1963). Another ourbreak, reported by Eugster *et al* (1974) occured in Texas range calves. However, the virus isolated from the central nervous system of calves during these outbreaks were not compared by RE migratin pattern and were identified as IBR virus. As mentioned, our laboratoy confirmed that the virus isolated by Eugster *et al* (1974) was indeed a BHV-1 virus with a RE migration pattern resembling those of the two other well characterized neurovirulent strains N-569 and A-663, referred to as BHV-1.3 (Metzler *et al.*, 1986) or bovine encephalitis herpesvirus (Studdert, 1989). When the Austalian N-569 isolate was inoculated intranasally or intravaginally in cattle, calves developed clinical meningoencephalitis between days 10 and 14 PI (Hall *et al.*, 1966; Bagust, 1972). It is interesting to note that both rabbits and

calves tend to develop clinical encephalitis within the same time frame after virus inoculation.

Kelly (1977) infected rabbits with IBR virus and reported that they developed conjunctivitis post inoculation. Similarly, Lupton *et al.*, (1979) observed conjunctivitis in rabbits on the second day after inoculation with IBR virus. In our studies we noticed moderately severe conjunctivitis in rabbits inoculated with the Cooper strain starting on the second day PI. On the other hand, although rabbits inoculated with the EC strain also developed conjunctivitis, it was noticeably much milder. We also noticed clinical disease in some rabbits inoculated with the viruses intranasally, including depression, anorexia and some accelerated respiratory rates. However, none of the animals died as a result of respiratory complications, and rabbits inoculated with the Cooper strain virus were again more severely affected clinically.

The level of immune response to IBR virus varies with the route of infection. Bagust (1972) noted that serum NA antibody titers were highest when calves were inoculated by the conjunctival route and was intermediate after intranasal inoculation. Maximum IgG antibody titers occurred on day 35 PI when cattle were inoculated by the intranasal route (Guy and Potgieter, 1985). In our rabbits inoculated intranasally, serum NA titers were observed in all rabbits (n=3) inoculated with the Cooper virus, but in only 2 out of 3 rabbits inoculated with the EC strain. In rabbits inoculated ocularly, only those inoculated with the Cooper strain seroconverted by day 14 PI; antibodies were not measured after this time. Rabbit IgG antibodies

specific for BHV-1 were detected by ELISA as early as 8 days PI in rabbits inoculated ocularly and increased until day 14 PI at which time titers were 8-fold higher in sera of rabbits inoculated with the Cooper strain. The ELISA is a highly sensitive method for detecting presence of antibodies in serum of calves (Edward and Goita, 1987) and Collins *et al.*, (1984/85) detected antibody as early as day 8 PI in sera of BHV-1 infected calves. It appears, therefore, that rabbits inoculated with BHV-1 will develop a good humoral immune response postinoculation, and that rabbits inoculated with the Cooper strain tended to respond earlier with slightly higher serum NA titers and significanly higher ELISA titers than did rabbits inoculated with the EC strain.

The virus neutralization test is a sensitive test for measuring neutralizing antibody titers in sera (Carbey *et al.*, 1972). Using the virus neutralization test, various researchers have failed to detect antigenic differences between the respiratory (IBR) and the genital (IPV) strains of BHV-1 (Bowling *et al.*, 1969; House *et al.*, 1971). In fact Gregersene *et al.*, (1985) indicated that the antigenic separation of IBR from IPV by neutralization with rabbit hyperimmune sera was not possible. Bagust (1972) detected no antigenic differences between IBR and IPV by neutralization kinetic assays, but he mentioned that the antigenic nature of the neurovirulent N-569 virus was significantly different from IBR virus by neutralizing kinetics. Also, Freidli *et al.*, (1987) reported that monoclonal antibodies directed against BHV-1.1 only partially reacted with BHV-1.3 isolates, indicating that antigenic differences exist between these strains.

In the present study, sera from hyperimmunized rabbits and sera from rabbits inoculated intranasally with either the Cooper or EC strains neutralized both virus strains. In most instances, however, higher levels of rabbit anti-Cooper sera were required to neutralize the same quantity of EC as Cooper virus in the neutralization test. The same was true of serum from one EC strain innoculated rabbit, where 8-fold higher concentration of sera was needed to neutralize the same quantity of Cooper as EC strain. This would indicate that although the viruses are related antigenically, strain-specific antiserum could differentiate between the two BHV-1 strains by the virus neutralization test in this study. The antigenic difference between the two strains was underscored by the fact that antibodies in sera of rabbits inoculated ocularly with the Cooper strain had significantly higher titers when mearured against its homologous antigen by ELISA than against the heterologous the EC strain antigen.

The findings that virus-specific antibodies bound differently to the two viruses prompted us to analyse and compare the viral polypeptides of the two strains and to determine antigenic differences. Autoradiography of SDS-PAGE separated viral proteins revealed that the Cooper strain had 21 proteins whereas the EC strains had 20 proteins. It is impossible to compare exactly our results to those published by others, since detection procedures and percentage gels used varied. However, the number and molecular weight range of proteins in our study are in general agreement with those reported by others (Misra *et al.*, 1982; Trepanier *et al.*, 1986; Metzler *et al.*, 1985, 1986). The migrational pattern are obviously similar

but display certain differences which distinquish BHV-1.1 Cooper from the BHV-1.3 3 EC strain (Meztler *et al.*, 1986). The ^{14}C-glucosamine labelled proteins showed a big difference between strains in the region of gp10. The Cooper gIII glycoprotein is located in this region (van Drunen littel-van den Hurk *et al.*, 1986) and appears to be more glycosylated in the Cooper than in the EC strain.

Carbohydrate moieties of viral glycoproteins can have a strong influence on functional as well as antigenic and immunogenic activities (Alexandar *et al.*, 1984). Deglycosylated glycoproteins induce significantly lower antibody responses in rabbits than do native glycoproteins, and antibodies raised against the deglycosylated glycoproteins have much lower neutralizing titers (van Drunen Littel van den Hurk *et al.*, 1990). This suggests that the immunogenicity of several epitopes on gIII may be carbohydrate depended. If the Cooper strain glycoprotein is more glycosylated (Figure 1), it may explaine why the Cooper strain was more immunogenic in the rabbit.

In our first Western blot experiments, in which we tested sera from ocularly inoculated rabbits, only the serum of one Cooper inoculated rabbit collected on day 14 PI bound to some lower molecular weight Cooper virus protiens. These proteins appeared to be in the region of internal capsid proteins. Eberle *et al.*, (1985) using western blots noticed that early in primary infections, antibodies were directed against an internal capsid protein of HSV.

Studies with monoclonal antibodies have identified and recognized

two neutralizing epitopes on glycoproteins gIII (91 K mol wt) and gI (74 K mol wt) (van Drunen Littel-vanden Hurk *et al.*, 1985). Trudel *et al.*, (1987) also identified the 90 K mol wt glycoprotein (gIII) as a major immunogenic protein. Gregerson *et al.*, (1985) identified two major glycoproteins [93 K (gIII) and 74 K (gI) mol wt] that induced neutralizing antibodies and Tropanier *et al.*, (1986) also identified the 90 K mol wt glycoprotein (gIII) as an important neutralizing epitope.

By reacting hyperimmune sera to viral proteins in western blots, we found that a glycoprotein in the range of mol wt 90 K was a major antigenic protein. Rabbit anti-EC hyperimmune serum recognized this glycoprotein in the EC strain virus by western blotting, but did not recognize the related Cooper virus glycoprotein as intensely. When rabbit anti-Cooper gIII antiserum was tested, it recognized a protein band only in the Cooper virus; this protein band was located in the region of gIII glycoprotein (Figures 2 and 3). It appears, therefore, that the gIII glycoprotein of the Cooper and neurovirulent EC strain are antigenically different.

In conclusion, it appears that the neurovirulent BHV-1 EC strain is different from the respiratory Cooper strain both antigenically and in ability to cause clinical disease and fatal encephalitis in rabbits. It would be interesting to find out what role, if any, the antigenically different viral glycoprotein gIII plays in determining BHV-1 neurovirulence.

CHAPTER V

SUMMARY AND CONCLUSIONS

The purpose of these studies was to examine the humoral immune response of rabbits to infection with two different BHV-1 strains - a respiratory (Cooper) strain and a neurovirulent (EC) strain - and to determine if the two strains were antigenically different.

For this purpose, rabbits were inoculated with the two virus strains intranasally and ocularly. Ocularly infected rabbits developed conjuctivitis, whereas some intranasally infected animals became depressed, anorexic and developed increased respiratory rates. Rabbits inoculated with the Cooper strain tended to be more severely affected clinically. Two rabbits inoculated with the EC strain developed fatal encephalitis, one on day 7 and the other on day 10 PI. None of the rabbits inoculated with the Cooper strain died as a result of the infection.

Only rabbits inoculated ocularly with the Cooper strain developed detectable amounts of serum neutralizing antibodies by day 14 PI. In rabbits inoculated intranasally, 100% of those inoculated with the Cooper strain seroconverted by day 21 PI, whereas only 66% of those inoculated with the EC strain seroconverted by that time. Serum ELISA antibody titers were noted on day 8 PI in both groups of rabbits, and these titers

increased to day 14 PI. Titers in the Cooper virus inoculated rabbits tended to be higher at all times PI.

Serum virus neutralization tests indicated that antibodies in sera of rabbits inoculated with a particular strain of virus were better able to neutralize the homologous virus than the heterologous virus. Also, antibodies in sera of rabbits inoculated with the Cooper virus tended to bind to Cooper virus antigen to a significantly higher degree than the EC. This indicated that the different strains are antigenically distinct.

Viral protein profiles could distinguish between the two strains, although the differences were slight. The Cooper strain glycoproteins tended to be more glycosylated, especially in the region of the gIII glycoprotein. A big difference could be detected in this gIII region between the Cooper and EC strain glycoproteins.

Anti-EC hyperimmune serum recognized several proteins in both Cooper and EC strains by Western immunoblots. The anti-Cooper strain serum, however, recognized only Cooper strain proteins and showed intense activity in the gIII region. Anti-Cooper gIII sera, and to a much lesser extent anti-gI sera, reacted strongly to the Cooper virus glycoproteins, but not to any of the EC strain virus proteins by immunoblot.

It appears, therefore, that these two BHV-1 strains are antigenically distinct and differ in their ability to cause clinical disease in rabbits. Only the EC strain virus causes fatal encephalitis in rabbits, but the Cooper strain appears to be more immunogenic following inoculation. The gIII glycoprotein at least, can clearly differentiate the two strains antigenically.

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