# In vitro characteristics of bovine viral diarrhea virus infection in two bovine cell types: Effect of host cell

by

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# INTRODUCTION

The infectious agent bovine viral diarrhea virus (BVDV) is implicated in causing respiratory, gastrointestinal, and reproductive-teratogenic disease world-wide among domestic cattle. Descriptions of disease conditions ascribed to BVDV-infection range from mild, upper-respiratory tract involvement through more severe syndromes such as acute ulcerative enteritis, fetal abortion and congenital defects, and fatal mucosal disease. The virus has been incriminated in causing persistently infected animals and affected cattle are susceptible to superinfection with a heterologous strain of BVDV.

BVDV is one of three species of the genus pestivirus, family Togaviridae. Other members of the pestivirus genus are hog cholera virus and border disease virus which affects sheep. Collectively, therefore, the pestiviruses are recognized as significant pathogens by food-animal producers and veterinarians world-wide.

Previous in vitro research on BVDV, characterizing cytopathic strains of BVDV with respect to viral growth parameters and protein synthesis has used well-characterized bovine cell types, such as turbinate, testis, and kidney cells. While providing information about the basic features of viral infection, the early work has not adequately characterized and made comparisons with noncytopathic BVDV strains. In addition, no literature to date has been found that reports on BVDV infection of more specialized bovine cell types, such as the vascular endothelial cell.

The vascular endothelial cell is a highly specialized cell and a major cell type found in the respiratory tract, representing part of the

cellular interface between the blood-gas exchanging surface in the lung.

This research was conducted to: 1) provide information about BVDV behavior in the endothelial cell, 2) examine the role, if any, that cells play in the expression of virus-induced cytopathology, and 3) provide a foundation for future work on the direct role that BVDV may play in the pathogenesis of bovine respiratory disease.

# LITERATURE REVIEW

## Perspective

Bovine viral diarrhea virus (BVDV) infection in cattle is associated with a myriad of clinical disease patterns that ranges from a mild, subclinical or inapparent infection to a fatal condition called mucosal disease. Viral infection may result in an acute enteritis, immunosuppression, congenital deformities and abortions, chronic enteritis, and persistently infected (PI) animals. Bovine virus diarrhea virus has been implicated in the development of the bovine respiratory disease (BRD) complex or shipping fever pneumonia.

## History

Initial reports characterized two disease patterns associated with BVDV infection. Olafson, MacCallum, and Fox (1946) in the United States and Childs (1946) in Canada, described a previously unreported disease condition occurring in North American cattle. Affected cattle displayed clinical signs that included anorexia, depression, hypersalivation, a transient pyrexia and leukopenia, and a watery, sometimes hemorrhagic diarrhea with an accompanying tenesmus (Olafson et al. 1946). Further, Olafson et al. (1946) observed a mild, subclinical form of disease, as well as the potential for abortion to occur among pregnant cows, 10 to 90 days following clinical signs of disease. Olafson's group was able to experimentally transmit the disease to susceptible cattle by oral drenching with fecal suspensions from clinically ill cattle and by subcutaneous injections of blood or splenic emulsions collected from affected cattle during the pyrexic phase of the disease. Due to the

transmissible nature of the syndrome, and the lack of visible agent(s) causing disease, Olafson coined the term "virus-diarrhea" to describe this condition in cattle (Olafson and Rickard 1947)

Hedstrom and Isaksson (1951) provided evidence on the world-wide distribution of the disease in their report of a similar clinical syndrome occurring in Swedish cattle during the late 1940s. Their animal-to-animal transmission studies were equivocal, however, they reported that feces from affected cattle was the best source for transmitting the disease and they felt confident that, due to a lack of bacteriological evidence, disease was due to a filterable agent, or virus as proposed by Olafson in 1947 (Hedstrom and Isaksson 1951).

Childs (1946) gave a detailed description of lesions in cattle affected with virus-diarrhea, or in his terminology, "X-disease." The most remarkable observation was erosions and ulcerations of the mucosa lining the alimentary canal.

Shortly after the initial publications on virus-diarrhea, Ramsey and Chivers (1953) described a second major disease condition in cattle that is now recognized as a result of BVDV infection and termed mucosal disease (MD). In their report, cattle displayed clinical signs similar to virus-diarrhea affected cattle including depression, anorexia, pyrexia, oculonasal discharge, and a watery to hemorrhagic diarrhea. In contrast to virus-diarrhea, however, the morbidity rate in mucosal disease outbreaks was relatively low (2-50%) while the mortality rate was high, often above 90%. Olafson et al. (1946) and Pritchard et al. (1956) observed that morbidity far exceeded mortality in virus-diarrhea cases

with morbidity rates ranging from 33% to 100% and mortality rates often less than 20%.

Further differentiating mucosal disease from virus-diarrhea, histopathological examination of the intestinal mucosa of cattle succumbing to mucosal disease revealed marked alterations in this tissue. These included vascular congestion, hemorrhages, and severe erosions and ulcerations. Lesions were consistently observed in the submucosal lymphoid tissue of Peyer's patches ranging from lymphoid depletion to varying degrees of necrosis (Ramsey 1956).

Because of the more extensive erosion of the alimentary mucosa, as compared to cases of virus-diarrhea, Ramsey applied the term "mucosal disease" to affected cattle and claimed that differentiation between mucosal disease (MD) and virus-diarrhea could be based on symptomology and pathological examination (Ramsey 1956).

Subsequent research took advantage of advances in tissue culture technology to further study virus-diarrhea and MD. Using a splenic suspension from the original New York isolation of virus-diarrhea, Lee and Gillespie (1957) were able to infect and pass the virus through 20 passages of a bovine embryonic skin-muscle cell culture. Because this New York isolate did not induce cytological changes in vitro, proof of the ability of the virus to replicate and maintain full virulence was shown by serially diluting infected supernatant from the 20th cell culture passage of virus and injecting this material into susceptible cattle. In addition to reproducing clinical signs of virus-diarrhea in inoculated cattle, these animals were protected from clinical disease following subsequent

inoculations with virus-diarrhea-virus infected cell culture supernatant (Lee and Gillespie 1957)

As stated above, a major observation made by Lee and Gillespie (1957) was that this New York isolate of virus-diarrhea-virus remained noncytopathic in tissue culture. Infected cells showed no morphological changes following infection and the lack of cell damage following repeated passage of the New York isolate in tissue culture was a consistent finding. In contrast to the noncytopathic nature of the New York isolate, Gillespie et al. (1960) reported a virus isolate from a virus-diarrhea infected bovine in Oregon (strain C24V) that, when inoculated into tissue culture, resulted in cell vacuolation, nuclear pyknosis, and eventual cell lysis and death. To date, this display of virus-induced cell damage and death (cytopathic effect, CPE), or lack thereof, remains as an important classification tool in the study of BVDV isolates. Strains of BVDV that induce cell damage and death following inoculation into cell culture are called cytopathogenic (CP) strains. Conversely, those strains that do not induce cellular changes during replication are termed noncytopathogenic (NCP) strains. Gillespie et al. (1962) demonstrated that tissue culture cells, preinfected with NCP-BVDV for at least three days, were resistant to subsequent infection with a CP-BVDV strain. Neutralization of NCP-BVDV with convalescent antiserum prior to inoculation into tissue culture abolished this virus-induced resistance and cells remained susceptible to CP-BVDV infection (Gillespie et al. 1962).

By the mid-1950s virus-diarrhea and MD had been reported in 20 states in the United States (Ramsey 1956). Many of the isolates were identified

by the name of the state where the outbreak of disease had occurred, such as New York, Oregon, and Indiana. From these geographically distinct outbreaks, researchers, coupling in vitro virus isolation techniques with in vivo infectivity studies, began to establish patterns of antigenic similarity among virus isolates. Protection or cross-immunity, for example, against the New York virus-diarrhea-virus could be demonstrated in cattle previously infected with the Oregon strain C24V (Gillespie et al. 1960). Gillespie and Baker (1959) further demonstrated antigenic relationships between these two isolates and the Indiana-46 strain of virus-diarrhea-virus isolated by Pritchard et al. (1956) using cross-immunity tests.

Underdahl et al. (1957) reported the first in vitro isolation of a cytopathic virus from two clinical cases of MD, one case occurring in Nebraska and the second in Iowa. Virus from the Nebraska outbreak was isolated in fetal bovine kidney cells. Following isolation, virus-infected cell culture supernatant was inoculated into sheep in experimental transmission studies. A comparison of pre-inoculation and convalescent sera from the infected sheep demonstrated the development of virus neutralizing antibodies specific to the inoculated, Nebraska virus, as well as to the Iowa virus (Underdahl et al. 1957). Probably not recognized as a major observation at the time of their publication, yet providing evidence of the ubiquity of BVDV, Underdahl's group noted that in vivo studies using their MD-virus isolates were impossible due to the presence of neutralizing antibodies in test cattle obtained from six geographically separate herds (Underdahl et al. 1957).

Use of the CP-Oregon C24V strain of virus-diarrhea-virus in virus-neutralization assays enabled researchers to establish antigenic relationships across several virus-diarrhea and mucosal disease isolates. Kniazeff and Pritchard (1960) demonstrated neutralization of the C24V strain of virus-diarrhea-virus with sera collected from cattle following disease outbreaks attributed to the NY1 strain of virus in geographically isolated areas. They further showed neutralization of C24V with sera collected from several MD cases occurring in Indiana, Iowa, North Dakota, and England. Based on the apparent close relationship between C24V and NY1 displayed in virus-neutralization assays, they concluded that an antigenic relationship existed between the NY1 strain of virus-diarrhea-virus and the MD viruses (Kniazeff and Pritchard 1960). Lack of virus neutralizing capacity for a virus-diarrhea isolate was demonstrated by using convalescent sera from animals affected with other clinically similar disease conditions, such as sheep blutongue, hog cholera, and infectious bovine rhinotracheitis (IBR) (Kniazeff and Pritchard 1960). This data supported the classification of virus-diarrhea/MD viruses as a distinct group.

Gillespie et al. (1961) also observed virus-neutralization patterns suggesting antigenic similarities using Oregon-C24V, three New York virus-diarrhea-virus isolates, the Indiana-46 strain, and two Nebraska virus isolates.

In contrast to demonstrating cross-strain neutralization, Kniazeff and Pritchard (1960) also reported the failure to neutralize strain C24V with convalescent sera harvested from animals clinically diagnosed as

infected with virus-diarrhea-virus.

Darbyshire (1962) demonstrated a serological relationship between BVDV and the hog cholera virus (HCV) in an agar-gel immunodiffusion experiment. Substantiating Darbyshire's work, Mengeling et al. (1963) showed a positive antigenic relationship between HCV and BVDV using immunofluorescence assays. Currently, the two viruses are recognized as antigenically related members of the Pestivirus genus (Westaway et al. 1985a).

## Clinical Aspects

Bovine virus-diarrhea (BVD) in cattle is manifested by a variety of clinical presentations such as an acute ulcerative enteritis, subclinical infection, persistent infection (PI), fatal mucosal disease (MD), chronic BVD, congenital deformities, abortions, immunosuppression, and respiratory disease. Reviews and clinical-format presentations on the pathogenesis and immunological features of infection, as well as the different BVDV strains associated with these disease syndromes have recently been published (Ernst et al. 1983, Perdrizet et al. 1987, Baker 1987).

In addition to clinical disease, serious problems for research and industry arise from the potential of NCP-BVDV to infect animals and then remain undetectable in harvested sera and tissues. The two major sources of contamination are fetal bovine sera and bovine cells persistently harboring NCP-BVDV (Nuttall et al. 1977, Rossi et al. 1980). One survey found that about two-thirds of nonirradiated lots of bovine fetal sera were contaminated with NCP-BVDV, while gamma-irradiation of sera reduced contamination to around 10% of tested lots; further, heat-inactivation of

bovine fetal sera at 56 C for 30 minutes was not reliable in inactivating BVDV (Rossi et al. 1980). Contamination is of major importance in viral vaccine production in which extensive use of fetal bovine sera and bovine cells is practiced (Nuttall et al. 1977).

Direct and indirect virus transmission in cattle have been reported, with direct virus transmission being the more common route of infection. Infection typically occurs via inhalation or ingestion of infected saliva, oculonasal secretions, and/or infected feces and urine (Baker 1987). BVDV isolation from semen of a persistently infected bull has been reported (McClurkin et al. 1979). While normal calves resulted from pregnancies using the infected semen, a relatively high number of services were required for seronegative cows to conceive and these cows developed high BVDV-specific serum antibodies (McClurkin et al. 1979).

The myriad of clinical outcomes following BVDV-infection has been simplified by concomitantly considering the immunological status of the susceptible animal and its potential response to virus. Current disease classifications include 1) acute bovine virus-diarrhea (BVD), 2) chronic BVD, 3) mucosal disease (MD) and 4) persistent BVDV infection (PI). Another simplification scheme considers BVDV infection of the normal animal, the fetus, and the persistently infected (PI) animal (Perdrizet et al. 1987).

BVDV infection of the normal animal

Clinical disease Based on various serological sampling surveys, 50% to 90% of the adult cattle population carry antibodies to the BVDV (Ernst et al. 1983, Baker 1987). This widespread nature of immunological

response without reports of epizootic disease indicates that the majority of virus infections result in subclinical disease. Cattle typically develop a mild fever and leukopenia and a sufficient immune response to neutralize the virus (Baker 1987).

The neonatal calf passively acquires anti-BVDV-immunoglobulins through the ingestion of colostrum. As these antibodies normally decay within three to eight months, calves become seronegative to BVDV and are then susceptible to infection (Ernst et al. 1983). Acute, clinical disease generally occurs in cattle from 6-24 months of age and the clinical signs of infection include: a transient pyrexia and leukopenia, depression, anorexia, oculonasal discharge, occasionally a watery to hemorrhagic diarrhea and the infrequent appearance of oral ulcerations (Baker 1987). Adult, lactating cows may experience a decrease in milk production and infrequently, BVDV-infected cattle develop hyperemia and ulcerations of the interdigital area of the hoof, the teat epithelium and the mucocutaneous junctions of the vulva or prepuce (Ernst et al. 1983). Severe cases of diarrhea result in dehydration and acidemia with death as the final sequela. Clinical diagnosis of BVD is made by virus isolation from tissues or body secretions and the demonstration of a rising antibody titer between acute and convalescent sera.

Lesions Gross lesions in acute BVD cases are variable with mucosal ulcerations of the alimentary canal an undependable feature (Jubb et al. 1985). If present, oral lesions are generally found on the palate, tips of the buccal papillae, and the gingiva. Further into the alimentary canal, linear erosions or ulcerations of the esophagus, ulceration of the

nonvillous portions of the rumenoreticulum, and punctate ulcers in the abomasum are characteristic findings. Peyer's patches are grossly eroded or ulcerated and are usually covered with coagulated blood and fibrin. Histologically, there is lysis of the follicular lymphoid tissues. Important to those involved in regulatory veterinary medicine, Jubb et al. stress that the lesions in acute, fulminant BVD resemble those associated with rinderpest infection and thus, this disease should remain high on the list of differential diagnoses.

Immune system effects The leukopenia and lesions in lymphoid tissues in BVDV-infected animals point out the predilection of the virus for the cells and tissues of the immune system. Affinity of the virus for leukocytes, even in subclinical infections, has been demonstrated by isolating BVDV from washed buffy coat leukocytes (Malmquist 1968). Following experimental BVDV-infection of adult, seronegative steers, Reggiardo and Kaeberle (1981) detected an endogenous bacteremia in 85% of the animals during the first five days post-infection.

Using several in vitro experimental protocols, Truitt and Shechmeister (1973) showed that 1) bovine peripheral blood macrophages and lymphocytes, from either immune or nonimmune animals, were permissive to BVDV-infection and supported viral replication, 2) adding BVDV-neutralizing antibody to preinfected macrophages masked the appearance of progeny virus, but virus reappeared following the removal of antibody, whereas the addition of antibody at the time of infection prevented viral adsorption to susceptible macrophages, and 3) the addition of a mitogen, phytohemagglutinin (PHA), to BVDV-infected lymphocytes

enhanced viral replication compared to nonmitogenized lymphocytes.

Rossi and Kiesel (1977) speculated that because the macrophage is essential in the elicitation of the immune response, and because this cell is permissive to BVDV infection, malfunction of the infected macrophage is no less important than lymphocyte defects during clinical viral infections.

Other in vitro research with bovine peripheral blood lymphocytes (PBL) has shown that normal lymphocytes infected with BVDV display a depressed blastogenic response to phytohemagglutinin (PHA) (Muscoplat et al. 1973a). BVDV-infected lymphocytes have significantly depressed chemotactic responses (Ketelsen et al. 1979). Peripheral blood lymphocytes isolated from BVDV-infected cattle were recovered in lower numbers and had a reduced capacity to secrete immunoglobulin-G (IgG) (Muscoplat et al. 1973b) and likewise, showed a diminished blastogenic response to PHA (Johnson and Muscoplat 1973).

Atluru et al. (1979) also observed that bovine splenic lymphoid cells coincubated with mitogen and BVDV for five days showed a significant depression in plasma cell development and ability to synthesize and secrete IgG or IgM. The depression was not observed in cell cultures coincubated with mitogen and heat-inactivated BVDV.

Bolin et al. (1985a) observed that B- and T-lymphocyte populations declined in normal cattle inoculated with BVDV, and while the decline lasted seven days post-inoculation, all cattle recovered and seroconverted to the virus. Research done with peripheral blood mononuclear leukocytes (Bolin et al. 1987, Ohmann et al. 1987) collected from persistently

infected (PI) cattle has shown NCP-BVDV association with B- and T-lymphocyte populations and monocytes. Virus association with monoculear cells (4.4%) and T-lymphocytes (5.4%) was higher than association with B-lymphocytes (2.1%) (Bolin et al. 1987).

Roth et al. (1981) demonstrated in normal cattle challenged with BVDV that a reduction in circulating polymorphonuclear leukocyte (PMN) numbers was accompanied by functional defects in PMNs. Functional defects were observed in PMN degranulation and the mediation of antibody-dependent cell-mediated cytoxicity (ADCC). Healthy, BVDV-naive cattle vaccinated with a modified-live BVDV-vaccine (MLV-BVDV) showed comparable changes in PMN numbers and function (Roth and Kaeberle 1983).

In contrast, PMN populations isolated from cattle persistently infected with BVDV showed impaired particle ingestion capability while PMN degranulation and ability to mediate ADCC remained unimpaired (Roth et al. 1986).

An indirect immunosuppressive effect of BVDV has been observed in vitro. Bovine fetal lung cells inoculated with BVDV released substances into the cell culture supernatant that suppressed the blastogenic response of PBL stimulated with a mitogen. The addition of indomethacin to infected cells abolished this release and therefore, it was speculated that the immunosuppressive substances were related to prostaglandins (Markham and Ramnaraine 1985).

Respiratory disease Toth and Hesse (1983) found that bovine alveolar macrophages also would support BVDV infection and replication. Studying several pneumotropic viruses and BVDV, Rossi and Kiesel (1977)

observed that parainfluenza-3 virus (PI-3), infectious bovine rhinotracheitis (IBR) virus, and BVDV readily destroyed ciliary function in tracheal ring organ cultures.

Interaction of BVDV with peripheral blood leukocytes and phagocytic cells of the respiratory system illustrates the potential synergism that could result from BVDV and other bovine pathogens. The precise role BVDV plays in the pathogenesis of bovine respiratory disease remains unanswered due to the difficulties encountered in experimentally inducing clinial pneumonia following virus infection (Baker 1987).

In a one year study of bovine pneumonia in Texas feedlots, 21% of virus isolates from pnuemonic lungs were BVDV compared to a 16% isolation rate for the IBR-virus (Reggiardo 1979). Evidence for a synergism between BVDV and Pasteurella hemolytica (P. hemolytica) was the finding that seroconversion rates to these two agents occurred in 72.4% of the animals clinically diagnosed as pneumonic.

Barber et al. (1985) reported that during a two year clinical follow-up of a BVDV outbreak in a dairy herd, the greatest cause of calf loss was pneumonia. The association of BVDV with bacterial bronchopneumonia was suggested by histological examination of pneumonic lungs and the high numbers of pneumonic calves that were viremic at the time of death. Further, calves born to cows infected with BVDV during gestation had a significantly higher death rate (Barber et al. 1985). BVDV isolated from tissues of pneumonic calves, as well as from aborted fetuses, was NCP-BVDV, whereas CP-BVDV was isolated from cases of clinical mucosal disease (MD) and from a hydrocephalic calf (Barber et al. 1985).

Despite the clinical association of BVDV-infection and bovine respiratory disease, experimental reproduction of respiratory disease with BVDV has remained equivocal. Lopez et al. (1982) failed to reject their working null hypothesis that preinfecting calves with BVDV or Mycoplasma bovis (M. bovis) from three to seven days prior to aerosol challenge with P. hemolytica significantly impaired the pulmonary clearance of bacteria. Clearance rates were determined on bacterial recovery following culture of sections of lung tissue after challenged calves were euthanized (Lopez et al. 1982).

Also from this study, Lopez et al. (1986) reported that following aerosol exposure with BVDV, virus was recovered from lungs of calves at three, five, and seven days post-challenge. Virus inoculation, however, did not induce gross or microscopic changes in pulmonary tissue and inflammatory lesions suggestive of pneumonia were not apparent.

Potgeiter et al. (1984a) demonstrated the pneumo-pathogenicity of BVDV in calves following endobronchial inoculation of virus, and sequential inoculation of P. hemolytica. Compared to aerosol challenge, endobronchial inoculation of infectious agents provided a more severe and consistent challenge of the lower respiratory tract (Potgeiter et al. 1984a). After inoculating BVDV alone or P. hemolytica alone, calves developed pneumonic lesions involving 2-7% of total lung volume and 15% of total lung volume, respectively. In marked contrast, calves sequentially inoculated with P. hemolytica 5-days after BVDV challenge developed severe respiratory tract disease with pneumonic lesions involving 40-75% of total lung volume (Potgeiter et al. 1984a).

The five-day sequence used in Potgeiter's study was based on data from Reggiardo and Kaeberle (1981) showing the detrimental effects of BVDV infection in cattle and the subsequent development of clinical bacteremia, 3 to 5 days after virus infection (Potgeiter et al. 1984a). Further observations from Potgeiter's study included the recovery of large numbers of P. hemolytica from lungs and tracheas of dual-challenged calves compared to lower recovery rates in calves inoculated with P. hemolytica alone. In addition, mixed populations of bacteria were recovered from the lower respiratory tracts of calves inoculated with BVDV alone, providing further evidence of viral impairment of normal defense mechanisms (Potgeiter et al. 1984a).

Potgeiter et al. (1984b) also examined the effects of BVDV on the distribution of infectious bovine rhinotracheitis (IBR) virus. Inoculating IBR/BVD-free-calves by aerosol exposure with IBR-virus alone, or seven days after exposure to BVDV, comparisons were made on the distribution of recoverable IBR-virus three to eight days post-aerosolization. In calves receiving IBR-virus alone, virus was recovered from the cranial and caudal portions of the respiratory tract in lower concentrations compared to BVDV and IBR-virus challenged cattle that displayed a wider IBR-virus distribution pattern (Potgeiter et al. 1984b). Calves receiving both viruses were shown to have recoverable IBR-virus in high concentrations throughout the upper and lower respiratory tract, as well as in bronchial lymph nodes, thymus, liver, spleen, mesenteric lymph nodes, brain, gastrointestinal tract, and joint fluid. Conclusions drawn from this work were that co-infection with BVDV appeared to influence the

wide dissemination of the IBR-virus, and that the role of BVDV may be critical as a synergist for other bovine pathogens.

Potgeiter et al. (1985) expanded their research on the role of BVDV in respiratory disease, comparing CP- and NCP- isolates of the virus. Similar to earlier observations, calves sequentially challenged endobronchially with P. hemolytica after BVDV inoculation showed impaired pulmonary clearance of bacteria and developed severe respiratory tract disease. The CP-BVDV was associated with more severe respiratory effects (more pneumopathogenic) compared to the NCP-BVD-virus isolate used in the study (Potgeiter et al. 1985).

BVDV infection of the fetus

Effects on the developing fetus following BVDV-infection are influenced by the cytopathogenicity of the infecting strain of virus (CP or NCP) and the stage of fetal development at the time of viral infection (Perdrizet et al. 1987). Transplacental transmission of the BVDV in the seronegative dam is the major route of infection of the fetus (Van Oirschot 1983).

Considering virus strains and time of infection, there are four outcomes of viral infection: reproductive failure, congenital defects, persistent infection (PI), and a normal, seropositive calf.

First trimester Infection shortly after conception or during the first trimester (50-100 days of gestation) may result in fetal resorption, mummification, or abortion (Baker 1987). Grahn et al. (1984) showed that in seronegative cows, viral infection caused a failure of fertilization or embryo development. McClurkin et al. (1979) demonstrated that

seronegative cows required a higher number of services to conceive using BVDV-infected semen, and the cows developed a high antibody-titer to the virus. While BVDV- infection is not associated with a high incidence of abortion, or abortion "storms", the scenario of early reproductive failure and repeat breeding is observed (Ernst et al. 1983).

Second trimester Two outcomes are possible following BVDV-infection of the fetus during the second trimester of gestation, congenital defects and persistently infected (PI) calves. BVDV-infections of the fetus from 100-150 days of gestation are associated with congenital defects such as microencephaly, cerebellar hypoplasia, spinal cord dysmyelination, ocular defects, mandibular brachygnathism, in utero growth retardation, thymic aplasia, pulmonary hypoplasia, and hypotrichosis and alopecia (Ernst et al. 1983, Baker 1987).

Ross et al. (1986) isolated a NCP-BVDV from a blind and brachygnathic calf and reported similarly affected live-born calves and abortions in a dairy herd. This herd had previously experienced a respiratory infection accompanied by a febrile response. An infectious agent(s) was not identified during the clinical respiratory syndrome, however, a presumptive diagnosis of BVDV-infection was made following virus isolation from the malformed calf. At the time of the clinical syndrome, pregnant cows that aborted or delivered malformed calves were from 40-140 days into gestation (Ross et al. 1986).

Done et al. (1980) experimentally infected 15 pregnant, BVDV-seronegative heifers with a "cocktail" of CP-BVD-viruses at 100 days of gestation, and showed the pathogenicity of the virus for developing

fetuses. While none of the cows showed clinical disease, six fetuses died in utero (5 aborted, 1 mummified), and 10 fetuses survived to term with all 10 showing growth retardation with or without evidence of dysmyelination of the central nervous system (Done et al. 1980). Only two of the 10 live-borns demonstrated serum-antibodies to BVDV. NCP-BVDV was isolated from the remaining eight calves (Done et al. 1980). The recovery of NCP-BVDV from calves suggests contamination of the CP-BVDV cocktail, or dams persistently infected with virus at the time of experimental challenge.

Typically, fetal infection during the second trimester with NCP-BVDV is associated with BVDV persistence; affected cattle demonstrating a BVD-viremia and developing no or low levels of serum antibodies to the strain of virus throughout the remainder of their lives (McClurkin et al. 1984, Perdrizet et al. 1987, Baker 1987).

McClurkin et al. (1984) experimentally showed that the time-frame for fetal infection resulting in PI-calves was 42-125 days of gestation and that seronegative, pregnant cows exposed to NCP-BVDV during this time were at risk in delivering PI-calves. Calves born PI with BVDV were often weak and debilitated at birth, and did not survive to weaning, however, two normal appearing, PI heifers in the study did survive to breeding age and produced normal appearing, PI calves (McClurkin et al. 1984). Thus, the PI cow represents the major reservoir of BVDV for the bovine population (Dubovi 1986, Baker 1987).

Lack of demonstrable serum immunoglobulins in PI calves to the infecting strain of BVDV is indicative of an immunotolerance or paralysis.

This tolerance or paralysis is not complete as calves born PI with BVDV were shown to be immunocompetent to IBR, PI-3 and P. hemolytica antigens (McClurkin et al. 1984). The development of immunocompetence in the bovine fetus has been postulated to occur from 150-200 days of gestation and immunotolerance to BVDV is uncommon after 100-125 days of gestation (Baker 1987).

Due to high death rates in PI calves during the first year of life, prevalence rates of PI cattle from slaughter surveys may be inaccurate, however, a Danish slaughter check found a prevalence rate of PI cattle of about 1% (Baker 1987). Bolin et al. (1985b) studied select cattle herds with previous experience of BVDV infections and found 6 of 66 herds with PI members (9%), with 54 of 3157 individuals showing a BVD-viremia (1.7%).

Third trimester Infection by either strain of BVDV from 150 days to the end of gestation has little effect on the bovine fetus. Although the potential exists for the calf to undergo in utero growth retardation at any stage of gestation (Baker 1987), fetuses have developed a competent immune system during the third trimester and typically mount an immune response to the virus and are born normal, testing seropositive to BVDV prior to ingesting colostrum (Perdrizet et al. 1987, Baker 1987).

BVDV infection of the persistently infected (PI) bovine

Not only can the virus infect susceptible, normal cattle and developing fetuses, but the PI bovine, immunotolerant to BVDV, is at risk to "superinfection" by heterologous BVDV strains. Syndromes associated with BVDV superinfection are acute and chronic mucosal disease (MD), both ultimately ending with the death of the affected animal (Ohmann and Babiuk

1986, Baker 1987). Virus isolations from cattle with acute and chronic forms of MD revealed the presence of both NCP- and CP-BVD-viruses in over 70% of spleens from MD cases (McClurkin et al. 1985).

Acute MD, as initially described by Ramsey and Chivers (1953), may occur as a rapid-onset clinical condition of low morbidity and high mortality with affected animals presenting with pyrexia, leukopenia, profuse watery diarrhea containing blood and fibrin, and developing extensive and severe mucosal ulcerations. There is a marked depletion of lymphoid cells and frequently, lymphoid tissues and follicles are necrotic (Ohmann and Babiuk 1986, Baker 1987).

The acute form of MD is characterized by rapid onset of clinical signs and death of the animal, frequently occurring 3-10 days later. A chronic form of MD has been described with a prolonged clinical course of anorexia, marked weight loss and emaciation, and chronic bloating accompanied by a continual or intermittent diarrhea (Baker 1987). In addition, erosive lesions may be found in the oral cavity, the interdigital areas and heels of the hooves, and around the perineum; poor wound healing, chronic lameness and hoof lesions, and secondary bacterial infections may be indicators of chronic MD (Baker 1987).

Experimental reproduction of clinical MD was reported by Brownlie et al. (1984) and Bolin et al. (1985c) infecting cattle PI with NCP-BVDV with a heterologous, CP-strain of BVDV. Thus, the pathogenesis of MD involves four critical elements 1) the immunologically incompetent fetus, 2) NCP-BVDV infection of the fetus in utero, 3) the birth of the PI calf, and 4) exposure of the PI calf to CP-BVDV later in life (Dubovi 1986).

An important consideration in the pathogenesis of MD is the mode of exposure of the PI bovine to CP-BVD-viruses, as virus isolation studies have shown that both NCP- and CP-BVDV were recovered from spleens of cattle, following vaccination with MLV-BVDV vaccines (McClurkin et al. 1985). A follow-up study vaccinating PI cattle with both MLV- and killed-CP-BVDV vaccines, however, failed to experimentally substantiate these findings, in that none of the cattle developed clinical MD following vaccination (Bolin et al. 1985d). Demonstrating the inability of the immune system of the PI animal to mount a protective response to the tolerated virus, the vaccinated, PI cattle were challenged with CP-BVDV six weeks post-vaccination and all developed clinical MD (Bolin et al. 1985d).

This group speculated that the failure to experimentally produce clinical disease following CP-BVDV vaccination, in spite of reported post-vaccinal MD outbreaks, might indicate that the pathogenesis of MD may be more complex than the superinfection of PI cattle with "random" strains of CP-BVDV. Further, the expression of fatal MD may depend on the animal's response to antigenic differences or similarities of the co-infecting BVDV (Bolin et al. 1985d). Some supporting evidence for this hypothesis has recently been proposed. Corapi et al. (1987) used a panel of monoclonal antibodies to study NCP- and CP-BVDV isolated as pairs from clinical cases of MD. BVDV pairs showed very similar (and sometimes undistinguishable) reactivity patterns.

## Virus Classification and Cultural Characteristics

Currently, definitive placement of BVDV into a virus family is in a state of flux; the virus is classified as a member of the family Togaviridae, genus pestivirus. The pestiviruses are not transmitted by insects and are therefore called nonarthropod-borne, or nonarbo-Togaviruses. Recent studies of the genomic organization of BVDV suggests that the virus' replication and protein transcription strategies more closely resemble members of the family Flaviviridae (Collett et al. 1987).

### Physical characteristics

As a pestivirus, BVDV is antigenically related to the other two viruses in the genus, hog cholera virus (HCV) and border disease virus affecting sheep. As reviewed by Westaway et al. (1985a), the pestiviruses are small, enveloped viruses 50-60 nm in diameter. The envelope is a lipid bilayer with two glycoprotein projections, one with a relative molecular mass (Mr) of 55-57 kilodaltons (kD) and the second with Mr of 44-46 kD (Westaway et al. 1985a). The nucleocapsid is presumed to be spherical and composed of the core protein with Mr of 34-36 kD and the viral genome, a positive-sense, single stranded RNA with a sedimentation coefficient of 38-40 S (Westaway et al. 1985a). Estimates of the molecular weight of the genome range from 3.0 x 10(3) to 4.0 x 10(3) kD based on sedimentation and gel electrophoresis analyses (Horzinek 1981).

Other characteristics of pestiviruses include stability at pH 6-9, heat inactivation of virus at 56 C., sensitivity to lipid solvents, ionic and nonionic detergents, and trypsin, and rapid viral inactivation by ultraviolet light (Westaway et al. 1985a).

Virus genome, replication, and morphogenesis

Common to members of the Togaviridae, the strategy of viral replication involves one or more polyadenylated (poly A) subgenomic-lengths of RNA for protein translation. Nonstructural proteins are translated directly from the 5'- end of the RNA and structural proteins genes are located at the 3'-end of the genome, typically on a subgenomic-length of RNA (Westaway et al. 1985a).

In contrast to the aforementioned characteristics, and thus controversial for the classification of pestiviruses, Purchio et al. (1983) found several features of the BVDV genome more similar to the Flaviviruses. They observed that the resolved 8.2 kb viral genome failed to bind to an oligo(dT) column which indicated the absence of a polyadenylated 3'-end. Similar to the Uganda-S and West Nile flaviviruses, Purchio et al. found no apparent subgenomic RNA for the BVDV, one viral-RNA species serving as message for structural and nonstructural proteins (Purchio et al. 1983a).

Renard et al. (1985) and Collett et al. (1987) confirmed Purchio's observation on the lack of the 3'-poly A tail, and further, molecular cloning techniques used in the analysis of viral genomic sequences demonstrated a viral genome representing 12.5 kb. Characterizing the BVDV genome by purifying the viral RNA from infected cells and using this RNA as a template for complementary DNA (cDNA) synthesis, these molecularly cloned fragments of cDNA hybridized to viral RNA in infected cells and showed that the nucleic acid was a single RNA-species of 12.5 kb (Renard et al. 1985).

Viral morphogenesis differs between the Togaviridae and the Flaviviridae (Westaway et al. 1985a, 1985b). Viral morphogenesis for members of the Flaviviridae involves undefined processes in maturation, virion accumulation within endoplasmic reticulum (ER) cisternae, and no definitive proof exists for the budding of virus progeny through cellular membranes (Westaway et al. 1985b). Except for the pestiviruses, members of the Togaviridae mature progeny virus via budding of nucleocapsids through cellular plasma or intracytoplasmic membranes (Westaway et al. 1985a).

For BVDV, replication occurs in the cell cytoplasm and viral particles, within reported BVDV diameter limits, have been associated with modified tubular structures of the ER (Gray and Nettleton 1987). In ultrastructural studies of animal tissues and cell cultures infected with a cytopathic Danish strain of BVDV (UG-59), Ohmann and Bloch (1982) observed virus-like particles mostly associated with smooth membrane proliferations. They observed no evidence of a budding process by virions or virion-like particles through cytoplasmic or surface membranes (Ohmann and Bloch 1982).

Chasey and Roeder (1981) observed budding tubules and the formation of tubular networks from distended regions of the ER in an ultrastructural study of BVDV-infected bovine turbinate cells. While observing virus-like particles within unmodified ER cisternae and vacuoles, and being unable to substantiate viral budding through internal cellular membranes, they speculated that virion association with ER-membranes and absence of budding seen in BVDV-infected cell cultures were consistent with

flavivirus morphogenesis (Chasey and Roeder 1981).

Gray and Nettleton (1987) reported similar findings, yet reached another conclusion regarding the classification of the BVDV. From their ultrastructural study of BVDV-infected cell cultures, infected cells displayed modified ER-tubules, virus replication appeared to be associated with the ER, and virions were not observed budding through cellular membranes or being released from infected cells. Gray and Nettleton (1987) concluded that their ultrastructural observations substantiate the continued classification of BVDV as a member of the pestiviruses, family Togaviridae.

Virus-specified polypeptides

Like virus classification, determination of the products of BVDV-directed protein synthesis has been in a developmental phase during recent years. Pestiviruses are characterized by three major, structural proteins, two envelope glycoproteins and a nucleocapsid, or core protein (Westaway et al. 1985a). More recent reports credit the BVDV with more than three, virus-specified polypeptides.

Using two radioactive labels coupled with co-electrophoresis techniques, Pritchett and Zee (1975) were able to differentiate (14C)-labelled host cell proteins from (3H)-labelled virus proteins. They reported four major viral polypeptides synthesized by the NADL strain of BVDV with Mr of 93-110 kilodaltons (kD), 70 kD, 50-59 kD, and 25 kD.

Matthaeus (1979) and Coria et al. (1983) each purified preparations of CP-BVDV-infected cell cultures prior to characterizing virus-specified proteins. Matthaeus (1979) radiolabelled, sucrose-gradient purified, and

immunoprecipitated three, BVDV-specified viral proteins. The polypeptides migrated with Mr of 57 kD, 44 kD, and 34 kD. Further analysis labelling the sucrose moieties with (3H)-fucose showed that the 57 kD and 44 kD polypeptides were glycoproteins. Coria et al. (1983) resolved four major viral proteins synthesized by the cytopathic Singer strain of BVDV. Using a series of techniques to concentrate and purify virus, thus keeping viral morphology intact, isolated proteins had Mr of 75, 66, 54, and 26 kD, and further, the 75 and 54 kD proteins were determined to be glycoproteins by dansyl hydrazine staining (Coria et al. 1983).

Akkina (1982) and Purchio et al. (1984) radioimmunoprecipitated (RIP) BVDV-infected cell lysates and resolved five virus-specific polypeptides by sodium-dodecyl-sulfate polyacrylamide-gel-electrophoresis (SDS-PAGE). Polypeptides migrated with Mr of 115, 80, 55, 45, and 38 kD. Both groups used two-dimensional electrophoretic analysis of polypeptide digests to show that the 115 kD and 80 kD peptides were structurally related and observed in cell lysates, and thus, were likely to be nonstructural polypeptides. Pulse-chase experiments failed to establish any precursor-product relationship between these two polypeptides (Purchio et al. 1984).

Akkina (1982) observed differences between BVDV strains in protein profiles. For a NCP-BVDV, NY1 isolate, he noted a marked reduction in the quantity of the 80 kD protein (vp 2) compared to protein profiles from CP-BVDV strains. Speculation on failure to isolate nonstructural viral proteins in earlier research was based on purification of viral preparations, rather than examining BVDV-infected cell lysates (Akkina

1982).

Pocock et al. (1987) studied viral protein synthesis in calf testis cells using RIP analysis and five BVDV isolates, three CP-BVDV strains (NADL and two field isolates) and two NCP-BVDV strains. RIPs from NADL-infected cell lysates resolved eight proteins with Mr of 120, 87, 69, 57, 49, 37, 33, and 23 kD, while the two field isolates of CP-BVDV had the same number of proteins with minor variations in polypeptide migration patterns (Pocock et al. 1987).

Examination of the published values for the Mr of vp 2 shows a discrepancy between research groups utilizing RIP with CP-BVDV-infected cell lysates. Akkina (1982) and Donis and Dubovi (1987a, 1987b) have reported a vp 2 with Mr of 80 kD, while Pocock et al. (1987) observed a vp 2 of 87 kD. This controversy is probably not minor, as vp 2 is widely held to be the differentiating feature between CP- and NCP-isolates of the BVDV. Consequently, an accurate and consistent Mr value for vp 2 requires further definition.

Among the NCP-BVDV isolates included in the study by Pocock's group and consistent with published work, the 87 kD protein (vp 2) was absent. Further analysis with (3H)-glucosamine radiolabelling showed that the 69, 57, 49, and 23 kD proteins were glycosylated (Pocock et al. 1987).

Pocock et al. (1987) further showed that bovine antiserum specific for a NCP-BVDV isolate could immunoprecipitate the 87 kD vp 2 from CP-BVDV-infected cell lysates. Antiserum specific for a CP-BVDV isolate failed to detect and precipitate a vp 2, or "vp 2-like" protein from NCP-BVDV-infected cell lysates. Thus it appears that NCP-BVDV displays

antigenic components or epitopes that are similar to the CP-BVDV vp 2 to the immune system. Synthesis of the vp 2 by NCP-BVDV has not been observed in vitro, however, the structural relatedness of the 115 and 80 kD polypeptides suggests potential antigenic relatedness. Pocock et al. speculated that NCP-BVDV isolates possess vp 2, as shown by their RIP data, but do not induce cytolysis because of their slower in vitro replication rates (Pocock et al. 1987).

An interesting feature of Pocock's research, a pair of BVDV, one CP, and the second NCP, was isolated from a clinical case of mucosal disease. Comparison of these viral polypeptide profiles by RIP/SDS-PAGE showed marked similarities in polypeptide migration except for the absence of the 87 kD vp 2 from NCP-BVDV-infected cell lysates (Pocock et al. 1987). This lends support to the observations of similar reactivity patterns with monoclonal antibodies for BVDV pairs isolated from one animal (Corapi et al. 1987).

Donis and Dubovi (1987a) studied BVDV-specific polypeptides in infected cells using a hypertonic initiation block and SDS-PAGE, complemented with RIP procedures using anti-BVDV hyperimmune serum. The hypertonic initiation block is a technique that enables the direct visualization of virus-synthesized proteins without relying on the specificity of immune antiserum. Thus, under hypertonic conditions the cell's protein synthesis is selectively shut down while viral command of the cell's translation machinery remains undisturbed (Nuss et al. 1975, as cited in Donis and Dubovi 1987a). Twelve proteins were identified from Singer-strain, BVDV-infected fetal bovine testicle cells with Mr of 165,

135, 118, 80, 75, 62, 56-58, 48, 37, 32, 25, and 19 kD (Donis and Dubovi 1987a).

Further characterizing their initial observations, Donis and Dubovi (1987b) studied BVDV-specified polypeptides using several CP- and NCP-strains of the virus. Confirming earlier observations, the most abundant protein in CP-BVDV-infected cells (and absent in NCP-BVDV-infected cells) was the 80 kD vp 2 (Donis and Dubovi 1987b).

Among the NCP-BVDV isolates, the 118 kD protein was the most abundant protein recovered from infected cell lysates. In addition, all NCP-BVDV strains in the study synthesized 75 and 90 kD polypeptides (Donis and Dubovi 1987b). The 75 kD protein was also observed in CP-BVDV strains, but required longer autoradiogram exposure times for visualization of the band. The 90 kD band migrated as a doublet in NY1-infected embryonic bovine testicle cells (Donis and Dubovi 1987b).

As noted by Pocock et al. (1987), Donis and Dubovi (1987b) observed that antisera specific for NCP-BVDV would immunoprecipitate vp 2 from CP-BVDV-infected cell lysates, suggesting that epitopes displayed by NCP-BVDV strains are immunologically cross-reactive with vp 2 of CP-BVDV strains.

A summary of BVDV polypeptide profiles, including investigator(s), year of publication, and strain of BVDV used, is presented in Table 1.

Virus growth characteristics

The types and susceptibility of primary and low-passage cell cultures, and cell lines for BVDV have been reviewed (Horzinek 1981). The preference for a specific cell type used in BVDV research typically

Table 1. Summary of published observations on number and molecular mass (Mr) of BVDV-specified polypeptides

| Range of Mr in kD | Investigators[a] | | | | | |
|---|---|---|---|---|---|---|
| | A | B | C | D,E | F | G |
| 200 | | | | | | |
| | | | | | | 165 |
| | | | | | | 135 |
| | | | | | 120 | |
| | | | | 115 | | 118 |
| | 93-110 | | | | | |
| | | | | | 87 | |
| | | | | 80 | | 80 |
| | | | 75 | | | 75 |
| | 70 | | | | 69 | |
| | | | 66 | | | 62 |
| | 50-59 | 57 | | | 57 | 56-58 |
| | | | 54 | 55 | | |
| | | | | | 49 | 48 |
| | | 44 | | 45 | | |
| | | | | 38 | 37 | 37 |
| | | 34 | | | 33 | 32 |
| | 25 | | 26 | | | 25 |
| | | | | | 23 | |
| | | | | | | 19 |
| 0 | | | | | | |

[a]Investigators and strain of BVDV:
A - Pritchett and Zee, 1975: NADL
B - Matthaeus, 1979: NADL and C24V
C - Coria et al., 1983: Singer
D - Akkina, 1982: NADL and C24V
E - Purchio et al., 1984: NADL
F - Pocock et al., 1987: NADL
G - Donis and Dubovi, 1987a: NADL.

depends on the collective experience(s) in the laboratory, as well as knowledge of the behavior of the BVDV in a particular cell type.

Studies using CP- and NCP-strains of BVDV indicate that the expression of cytopathology, or lack thereof, serves as a genetically stable marker for a particular strain (Horzinek 1981). To characterize strain differences, comparisons of viral growth parameters between CP- and NCP-strains of BVDV have been made.

Hafez and Liess (1972) studied four CP-BVDV isolates, three from Germany and one from the U.S.A. (strain Oregon C24V). Several observations from their work included: 1) the influence of cell culture supernatant pH on the expression of viral cytopathology, 2) virus neutralization (plaque reduction) by antiserum demonstrated that viral adsorption and penetration occurred 60-75 minutes post-infection, and 3) with minor variations, times of maximum release of virus and the expression of cytopathology were dependent on infective dose, while length of eclipse phase was independent of dose (Hafez and Liess 1972).

Nuttall (1980) contrasted replication characteristics of CP- and NCP-BVDV isolates and reported that replication cycles of both strains were similar in kinetics. Extracellular virus quantities exceeded titers of cell-associated virus. At low input multiplicities (m.o.i.), the lag or eclipse phase extended to 6-8 hours post-infection (HPI) with the exponential phase lasting to 12 HPI; the exponential phase time was longer or shorter by manipulating the m.o.i. with lower or higher infective doses of virus, respectively (Nuttall 1980). Differences were reported on the yield of virus between the two strains, with the CP-BVDV yielding

approximately 58X more virions than the NCP-isolate (Nuttall 1980).

Differences were observed between CP- and NCP-BVDV yields using actinomycin-D in infected cell cultures. At concentrations of 0.1-1.5 ug/ml of actinomycin-D, yields of CP-BVDV were reduced by 30-100% compared to increases of 20-100% in yields of NCP-BVDV (relative to virus yields obtained in the absence of the drug) (Nuttall 1980).

## Bovine Endothelial Cells

Essential to the integrity of the cardiovascular system of blood-transporting vessels, this network is lined by the endothelium: a single layer of highly specialized epitheloid-type cells of mesodermal origin, called endothelial cells. Endothelial cells have been under intense investigation in the field of human medicine to better understand their role(s) in hemostasis, hypertension and atherosclerosis, and more recently, as active participants in immunomodulation. Much of the research has been conducted, for convenience and ethical reasons, by extrapolating observations obtained from experiments using endothelial cells from various animal species. Thus, the literature contains many reports on the culture, characterization, and properties of mammalian endothelial cells.

Macarak et al. (1977) observed that following harvest and culture, bovine endothelial cells (BEC) obtained from calf aortas display a characteristic epitheloid, polygonal morphology described as "cobblestone," and the cells react specifically in an immunofluorescence (FA) assay for Factor VIII:von Willebrandt-factor-related-antigen (VIII:vWF-RAg).

Factor VIII is a glycoprotein complex. The major component is a 200-225 kD disulfide-linked structure that can be detected by indirect immunofluorescence (FA) assay (Jaffe 1982). In general, the complex, Factor VIII:vWF, plays a vital role in hemostasis and platelet adhesion (Jaffe 1982).

Most normal cultured mammalian endothelial cells, including those of bovine origin, stain by FA assay for VIII:vWF-RAg and synthesize the complex in vitro (Jaffe 1982). Immunofluorescence assay is an accurate and convenient method of detecting the presence of these cells in culture.

Qualitative, quantitative, and functional differences exist between endothelial cells from various organs (Ryan et al. 1978). Collectively, however, the endothelium plays vital roles in hemostasis, inflammation, and leukocyte adherence to the vascular lining (margination) with subsequent infiltration into inflamed and/or diseased tissues. These cells are actively involved in immunoregulation (Cotran 1987, Kasukawa et al. 1980, Roska et al. 1984, Wagner et al. 1984, Harlan 1985).

Studies of the various roles played by the endothelium in the regulation of coagulation pathways led to observations on the responses of endothelial cells to cytokines such as interleukin-1 and tumor necrosis factor (Cotran 1987). Monoclonal antibodies have been used to demonstrate a surface antigen on cytokine-stimulated endothelium and further, immunoprecipitation studies indicate that this antigen is an adhesion molecule that mediates leukocyte adhesion. Leukocyte adhesion can be reduced by blocking or complexing surface adhesion antigens of stimulated endothelial cells by pretreatment with the monoclonal antibody (Cotran

1987). This antigen has been designated endothelial-leukocyte adhesion molecule 1, and has not been observed on unstimulated endothelial cells (Cotran 1987).

Recent studies have shown that lymphocyte migration into peripheral lymphoid organs and mucosa-associated lymphoid tissue is regulated and mediated primarily by specialized postcapillary high endothelial venules (HEV) (Duijvestijn et al. 1988). A monoclonal antibody has demonstrated a surface determinant specific to endothelial cells lining HEV. In immunoperoxidase assays using this monoclonal antibody, HEV stained intensely in lymphoid organs while vascular endothelium remained unstained; and lymphocytes were observed intraluminally and within endothelial cells of HEV presumably in various stages of extravasation (Duijvestijn et al. 1988). Inflammation is a stimulus for the differentiation of vascular endothelial cells into specialized HEV, and the development of HEV may occur in areas of extensive lymphoid infiltration (Duijvestijn et al. 1988).

Receptors for the Fc portions of immunoglobulin G (IgG) molecules and for the C3b component of the complement system have been demonstrated in sections of aortas from man, the ox and the pig (Kasukawa et al. 1980).

The activation of T-lymphocytes in an immune response requires that the antigen must be presented in the context of class II antigens (determinants) of the major histocompatibility complex (MHC). Class II MHC-antigens are present on mononuclear phagocytes (macrophages), thus these cells have been termed "antigen-presenting-cells (APC)." Endothelial cells have been shown in vitro to potentially fulfill

functions attributed to APC. In some vascular beds the endothelium has been shown to display class II antigens, influence the proliferation of T-lymphocytes, and secrete immunoregulatory molecules, such as interleukin-1 and prostaglandin E-2 (Roska et al. 1984, Wagner et al. 1984, Harlan 1985).

Friedman et al. (1981) speculated that endothelial cell injury caused by an infectious agent could have ramifications in the pathogenesis of disease; and the functional/architectural damage to the endothelium may be as severe as other experimental methods of inducing endothelium injury, such as by mechanical trauma, hypercholesterolemia, hypertension, and immune-mediated vasculitis. This group compared endothelial cells from human umbilical veins and bovine aortas in their permissiveness to infection by 11 human viruses (Friedman et al. 1981).

Three parameters were used to document successful viral infection of endothelial cells; these were cytopathology, viral growth curves, and the detection of viral antigens via immunofluorescence (Friedman et al. 1981). The origin of the endothelial cells, human versus bovine, appeared to influence the ability of the viruses to adsorb and penetrate the cells, however, the effects of infection on the cells could generally be classified as 1) severe and lytic, 2) chronic and less destructive, and 3) no apparent change in cellular morphology. In addition, the results suggested that endothelial cell changes following viral infection did not differ from documented changes observed in "known" permissive cells (Friedman et al. 1981). This group acknowledged that their in vitro observations may not transcend the laboratory and parallel in vivo events,

however, they speculated that endothelial sites of viral replication may conveniently aid the spread of virus to other tissues (Friedman et al. 1981).

In a separate study, this group observed that viral infection of endothelial cell monolayers enhanced polymorphonuclear cell (PMN) adherence and they speculated that, in vivo, this augmentation in PMN adherence may promote cellular damage and vasculitis via PMN degranulation (MacGregor et al. 1980, Friedman et al. 1981).

Incubating PMNs with supernate from virus-infected endothelial cells also enhanced PMN adherence compared to preincubation with noninfected endothelial supernate and thus, virus-infected endothelium appear to synthesize and release substances that promote early migratory events in PMN redistribution into infected tissues (MacGregor et al. 1980). This augmentation of PMN adherence was independent of complement and antibody, as washed PMNs suspended in Hank's buffered saline solution also showed enhanced adherence. In addition, while normal, uninfected endothelium treated with trypsin inhibited PMN adherence to cell monolayers, treatment of virus-infected endothelium with trypsin had no effect on adherence, and it appears that the mechanism of PMN adherence to infected endothelium is a function of a unique viral-induced signal (MacGregor et al. 1980).

Andrews et al. (1978) demonstrated that the human dengue virus, a flavivirus, could replicate in vitro in rabbit and human endothelial cells. The dengue virus, a member of the Flaviviridae and therefore possibly closely related to the BVDV based on virus replication strategies, causes hemorrhagic shock syndrome (HSS) in man. Disease

following dengue infection results in an interruption in vascular integrity or intravascular coagulation and has been speculated to involve the formation of immune complexes.

Parameters of cellular permissiveness to the dengue virus were growth curves and the demonstration of intracellular viral antigens via indirect immunofluorescence assays (Andrews et al. 1978). While the research did not demonstrate a direct cytopathic effect on dengue-infected endothelial cells, the authors did observe previously unreported evidence of a direct, virus-cell interaction between endothelial cells and a virus associated with clinical HSS. Further, they proposed that permissiveness to virus infection probably translates into functional alterations in infected cells; and these changes, if occurring in vivo, could provide a functional-defect-pathway for HSS, in addition to the proposed immune-mediated mechanisms of HSS (Andrews et al. 1978).

In an immunohistological study of BVDV-infected bovine tissues, Meyling (1970) observed that viral antigens could be demonstrated in endothelial cells, thus establishing virus-infection of the endothelium. However, in addition to demonstrating the endothelial cell component of BVDV-infection, viral antigens could be demonstrated in the tunica media and adventitia of blood vessels from the BVDV-infected tissues (Meyling 1970).

Evaluating lesions in tissues harvested from clinically healthy, persistently infected cattle, Cutlip et al. (1980) observed a widespread distribution of BVDV antigen by immunofluorescence assays; viral antigen was observed in vascular endothelium of the lung, liver, adrenal gland,

heart, and aorta. Histologically, renal tissue was characterized by focal thickening of the glomerular basement membrane, suggestive of glomerulonephritis (Cutlip et al. 1980). The microscopic lesions and positive immunofluorescence in the glomeruli led the authors to conclude that immunotolerance to BVDV (in these cattle) was incomplete and the antibody complexed with viral antigen and was deposited along the glomerular basement membrane (Cutlip et al. 1980). In the histologic evaluation of sections of brain from the same group of cattle, blood vessels were surrounded by a cuff of mononuclear cells and the endothelium was hyperplastic in appearance (Cutlip et al. 1980).

Zakarian et al. (1976), using morphological and histochemical observations, reported a periarteritis, with a predilection for the central nervous system, occurring in fetal ovine tissues following experimental, in utero border disease virus infection. Also classified as a pestivirus, the border disease virus of sheep is closely related antigenically to the BVDV and the hog cholera virus. Based on the morphology of the lesion, this research team postulated that the periarteritis was associated with a complex, cell-mediated immunological response; no direct involvement with the endothelium was proposed (Zakarian et al. 1976).

Based on these observations, along with the demonstration of Fc receptors on the surface of endothelial cells, Ohmann (1983) examined the distribution of BVDV-antigens in the tissues of virus-infected calves. She observed that viral antigen was only occasionally associated with endothelial cells lining the blood vessels; the predominant

antigen-positive cells were mononuclear cells diapedesing through blood vessels. The distribution of BVDV-antigen in lymphoid tissues corresponded with the localization of "antigen-presenting-cells " (APC) and a hypothesis on the pathogenesis of BVDV infection includes extensive interaction of the virus with APC (Ohmann 1983).

# MATERIALS AND METHODS

## Cells and Media

Bovine turbinate cells (BTU) were obtained from the National Veterinary Services Laboratory, Ames, Iowa at passage level eight and grown in Eagle's minimal essential media (MEM), supplemented with 10% fetal bovine serum (McClurkin et al. 1974). The cells were adapted to horse serum (HS; Hyclone, Logan, Utah) via a series of sequential cell passages using declining levels of fetal calf serum (FCS; KC Biological, Inc., Lenexa, KS) and increasing levels of HS. Cells were used after stabilization in 8% HS and 2% FCS, from passage 11-19. Cells were screened for BVDV by direct anti-BVD-virus fluorescent antibody (FA) assay.

The growth media for BTU consisted of F-15 Eagle's MEM (GIBCO), 1% PKS (penicillin 1000 U/ml, streptomycin 10 ul/ml, kanamycin 10 ul/ml), and 10% v/v total serum. Both FCS and HS were heat-inactivated for 30 minutes at 56 C. The FCS was screened for adventitious virus and 0.22 um filtered by the supplier. Further, the serum was sampled pre-purchase and if purchased, was custom-treated with beta-propriolactone by the supplier. The HS was also screened for adventitious virus and 0.1 um filtered by the supplier.

Bovine endothelial cells (BEC) were obtained by enzymatic digestion of isolated segments of calf thoracic aortas as described by Macarak et al. (1977), with the following procedural modifications. Healthy, neonatal calves from a BVDV-negative herd were euthanized by intravenous (i.v.) barbiturate overdose following i.v. administration of 10,000 Units

of heparin. One, ten centimeter segment of thoracic aorta was aseptically removed and placed in a transport media of Hank's buffered saline solution (HBSS) containing 1% PKS, 1% gentamycin (82 ug/ml), and 1% amphotercin B (10 ug/ml). Aortas were processed within one hour following harvest.

Following a rinse in transport media plus 0.2% hypochlorite (Clorox), the aorta segment was trimmed of excess fat and connective tissue and the narrower (caudal) end of the segment was ligated with a short length of sterile, nonabsorbable suture material. A 10 ml sterile, glass pipette was inserted into the lumen of the aorta and this entire assembly was placed into a slightly larger diameter, sterile vessel, so that the patent end of the aorta was parallel with the vessel opening. A volume of sterile 2.5% agar (56 C.) was carefully poured around the aorta and allowed to harden. The 10 ml pipette was removed when the agar was firm. This technique produced a "tube-within-a-tube" and effectively sealed small vessels coming off the aorta.

The enzyme solution for endothelial cell removal was HBSS-transport media containing 0.1% collagenase type IV (Sigma) and 0.4% dispase (protease neutrale, Boehringer Mannheim). The aorta was filled with a volume of the enzyme solution and four, 15 minute digests were sufficient to obtain cells. Digests were centrifuged at 1,000 rpm for 5 minutes, the enzyme solution was discarded, and cell pellets were resuspended in growth media of 10% FCS, F-15 MEM containing 1% PKS, 1% gentamycin, and 1% amphotercin B supplemented with 0.5% lactalbumin hydrolysate (LAH) and 50 ug/ml of endothelial cell growth supplement (ECGS; Collaborative Research, Inc.). Resuspended cells were pooled and seeded in 1-2 ml volumes into

precoated wells of a 12-well tissue culture plate cluster.

Prior to BEC seeding, two to three wells of a 12-well tissue culture plate were precoated with bovine fibronectin (Sigma) 45-60 minutes prior to the initiation of the digest. Supplier's recommendations were followed for proper product reconstitution and storage at -20 C. After thawing, stock fibronectin was added to serum-free MEM (SF-MEM) at 10 ul/ml and a final volume of 2 ml was used to precoat each of the 2-3 wells on a 12-well plate. Wells were precoated for 45 minutes at room temperature (RT). Fibronectin-MEM was discarded and precoated wells were rinsed with SF-MEM prior to seeding with resuspended cell digests.

When BEC approached near confluency in 12-well plates, cells were briefly treated with a solution of 0.05% trypsin and 0.02% sodium-ethylenediaminetetracetate (EDTA) in a calcium and magnesium-free saline. Cells were then dispersed with a disposable cell scraper. Cell suspensions were pooled and centrifuged at 1,000 rpm for 5 minutes and cell pellets were resuspended in growth media and seeded into tissue culture flasks (25 sq. cm.(cm2), Costar). Following the second to third passage, BEC were adequately grown in growth media without added LAH or ECGS. Harvested BEC were determined to be endothelial cells by their characteristic "cobblestone" morphology in tissue culture flasks and by positive staining in an indirect-FA assay for Factor VIII:von Willebrandt factor-related-antigen (VIII:vWF-RAg). Cells were screened for the presence of BVDV by direct FA assay.

## Virus Strains

The CP-BVDV, strain Oregon C24V (C24V), was obtained from the National Animal Disease Center (NADC), Ames, Iowa (McClurkin and Coria, 1978). The virus was plaque-purified three times and viral stocks were grown in BTU cells in maintenance media (MM) of 3% HS, Eagle's F-15 MEM with 1% PKS. Virus was quantified in microtiter plates and viral titers were calculated according to Reed and Muench (1938), by observing the highest virus dilution that caused cytopathic effect (CPE) in 50% of target cells (BTU) plated in wells of a 96-well microtiter plate (Truitt and Shechmeister, 1973). Titers of virus were calculated as 50% tissue-culture infective dose per ml (TCID-50/ml), expressed in base 10 logarithms. The C24V strain grew very well and viral titers of 10(7.0)-10(7.6) TCID-50/ml were common.

The NCP-BVDV, strain New York 1 (NY1), was also obtained from the NADC, Ames, Iowa (McClurkin and Coria, 1978). This strain was purified by limiting dilutions and virus was also grown in MM in BTU cells. Virus was quantified according to Nuttall (1980), as follows. Ten-fold dilutions of the NY1 virus (in 50 ul MM) were placed into microtiter wells and BTU cells (in 100 ul MM) were added. Plates were incubated at 37 C. in a humidified, 5% CO2 atmosphere for 4 days, at which time NY1-infected cells were challenged with 50 TCID-50/ml of stock C24V virus. Uninfected control cells were also challenged with the C24V virus at 50 TCID-50/ml, and at 1:10, 1:100, and 1:1,000 dilutions of the challenge dose. Cells were observed for C24V-typical CPE at 3, 4, and 5 days post-challenge. Titers were calculated by the 50%-endpoint method of Reed and Muench

(1938) by observing the highest NY1 dilution that prevented CPE in 50% of the microtiter wells (Nuttall, 1980). Titers of NY1 were usually one log lower than for C24V, with values ranging from 10(5.9)-10(6.5) TCID-50/ml.

Two other strains of CP-BVDV, NADL and Singer, were studied in radioimmunoprecipitation/SDS-PAGE analysis of viral polypeptides. These strains were obtained from the NADC, however, passage levels and titers were unknown. These two strains were used for internal comparison with C24V-infected BEC cells.

## Viral Growth Curves

Growth characteristics of C24V and NY1 strains of BVDV were determined using modifications of procedures outlined by Nuttall (1980). Growth of each virus strain was done in each cell type, BTU and BEC.

Both cell types were dispersed from tissue culture flasks and resuspended in a volume of growth media sufficient to plate cell suspensions in a 1 ml:1 well ratio in 24-well plates (Falcon). Four wells, with adjacent empty wells, were used for each virus so that 8 wells on a plate were filled with 1 ml of the appropriate cell suspension. Seven post-infection observation times were specified at 4, 6, 8, 10, 12, 14, and 16 hours. For this protocol, fourteen 24-well plates were used in each single-step growth experiment. Two single-step curves were conducted, 4 replicates of the 4 possible virus-cell combinations performed in each growth curve.

Cells were allowed to reach near-confluency (approximately 33,000cells/well) in appropriate growth media. At the time of infection growth media was discarded from each well, cells were rinsed once with

warm, SF-MEM, and infected with 0.5 ml volume per well of the appropriate virus in MM. The multiplicity of infection (m.o.i.) for C24V and NY1 was 6.2 and 5.2, respectively. Infection was carried out for 1 hour on a rocker platform at 37 C. Infective media was then removed from each well and cells were rinsed twice with a 1 ml volume of warm MM and reincubated for 2 hours with a fresh 1 ml volume of MM. At the end of 2 hours, media was removed from each well and cells were twice rinsed with a 1 ml volume of warm MM and reincubated with a fresh 1 ml volume of MM.

At the specified times, plates were centrifuged for 1 minute at 1,000 rpm and the 1 ml of media from each monolayer was pipetted into an immediately adjacent well. This volume represented extracellular virus. Cell monolayers were then rinsed with a 1 ml volume of 4 C. MM and plates were centrifuged for 1 minute at 1,000 rpm. The cold media was discarded from each monolayer and a fresh 1 ml volume of 4 C. MM was added to each monolayer. This volume represented cell-associated virus. Plates were sealed and frozen in a 95% ethanol/dry-ice slurry and stored at -70 C. for viral titration.

Viral titrations for each time interval were conducted as outlined. Briefly, the four samples from each of the two-virus by two-cell combinations (32 volumes) were diluted ten-fold. Four, 50 ul samples per dilution were delivered into microtiter wells and overlayed with 100 ul of MM containing BTU cells. Microtiter plates were incubated at 37 C. Cells infected with C24V were observed for CPE on days 3, 4, and 5. Cells infected with NY1 were challenged with 50 TCID-50/ml of stock C24V on day 4 post-NY1-infection and observed for interference of C24V-induced CPE on

days 3, 4, and 5 post-challenge.

## Cytotoxicity Assays

### Tetrazolium ring (MTT) cleavage assay

Modified from a procedure developed by Mosmann (1983), 50 ul volumes of MM alone, C24V in MM, and NY1 in MM were aliquoted into wells of a 96-well microtiter plate. Five sample wells of each treatment were made for both BTU and BEC cell types. This was done in 5 microtiter plates, one plate for days 1, 2, 3, 4, and 7 post-infection. Cells were added (10,000 cells/100 ul) in MM and plates were incubated at 37 C.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma, M2128) was dissolved in PBS at 5 mg/ml and filtered through a 0.1 um syringe-tip filter. At specified times post-infection, media was aspirated from each well, cells were rinsed once with 100 ul of SF-MEM, and 100 ul of fresh MM was added back to each well. MTT was then added to each well at 10ul/100 ul and the plate was reincubated for 3.5-4 hours. At the end of the incubation period, a 100 ul volume of 0.04N HCl-isopropanol was added to each well to dissolve MTT-formazan crystals. After 15 minutes, the volume in each well was mixed with a multi-well micropipetter and transferred to a fresh microtiter plate to eliminate interference by any remaining cells. Care was taken to re-pipet well contents in the same configuration on the new microtiter plate to insure accurate absorbance readings.

Plates were read within 30 minutes of processing on a microelisa plate reader (Dynatech, MR 580) at a test wavelength of 570 nm and a reference wavelength of 630 nm. The threshold and calibration readings

were set at 1.99. The average absorbance (O.D. 570-630) was calculated for the 5 samples, and OD-values were plotted as a function of day post-infection for each cell type.

(51-Cr)-chromium-uptake assay

Based on a procedure outlined by Neville (1987), BTU and BEC cells (10,000/100 ul) were seeded into wells of 96-well microtiter plates in MM. Twelve wells of each cell type were seeded on each of four plates so that observations could be made on four samples of mock-infected controls, NY1-infected, and C24V-infected cells on days 1, 2, 4, and 6 post-infection. Appropriate virus or media alone was added to the designated wells in a 50 ul volume of MM and plates were incubated. The m.o.i. for each virus was 1.0.

At specified times post-infection, medium was aspirated from each well, 10ul sodium (51-Cr)-chromate (1mCi/ml, specific activity 250-500 mCi/mg Cr, Amersham) was added to each well at a concentration of 1.0 uCi per well and the plate was reincubated for 30 minutes. Following incubation, this volume containing (51-Cr)-chromate was aspirated from each well and discarded. Wells were rinsed three times with 200 ul of SF-MEM and cells were lysed with 100 ul of 1.5% acetic acid for 15 minutes at RT. Fifty microliters of each cell lysate were pipetted into disposable, 5 ml plastic tubes and counted on a gamma counter (Beckman). The mean counts-per-minute (CPM) was calculated for the four samples and CPM-values were plotted as a function of day post-infection.

Direct immunofluorescence (FA)-assay

Both cell types were trypsinized from confluent cultures, resuspended and seeded into four-chambered slides (Labtek, Miles Laboratories) in appropriate growth media. Enough slides were used so that, following viral inoculation, one slide per infected cell type could be fixed and stained at 2, 4, and 6 days post-infection.

Prior to virus infection, growth media was discarded and cells were rinsed with a 0.5 ml volume of warm SF-MEM. Cells adsorbed C24V- and NY1-BVDV in a 0.5 ml volume of MM for 1 hour on a rocking platform at 37 C. Multiplicity of infection for both BVDV strains was calculated to be approximately 1.3.

One chamber of each slide served as a mock-infected control and the "infective" media was the same MM, except for lack of virus. Following a one-hour adsorption, media was removed and a fresh volume of MM was added to each chamber and the slides were reincubated.

At specified times post-infection, slides were fixed in acetone for 7 minutes at RT. Infected and control cells were overlayed with anti-BVDV immune serum conjugated to fluorescein isothiocyanate (FITC) (anti-BVDV-reagent 8802, 1:10 dilution, NADC, Ames, Iowa) and incubated at 37 C. for 30 minutes in a humidified atmosphere. Slides were then treated with two, 5 minute rinses in phosphate buffered saline (PBS) and then quick dipped in distilled water and air-dried. Slides were mounted with 90:10 v/v, glycerol:phosphate-buffered-saline solution (PBS, pH 7.4) and viewed under a coverslip using a Leitz fluorescence microscope.

## Radioimmunoprecipitation (RIP)/Sodium-dodecyl-sulfate polyacrylamide-gel-electrophoresis (SDS-PAGE) Analysis

### Isotopic labelling

Following protocols outlined by Akkina (1982), and Donis and Dubovi (1987a), BEC and BTU cells were seeded into 25 cm2 tissue culture flasks in their respective growth media and allowed to reach confluency (48 hours) prior to infection. Three flasks were seeded for each cell type, one flask served as mock-infected control, one flask was infected with C24V, and the third flask was infected with NY1. At confluency and just prior to infection, flasks were rinsed three times with warm, SF-MEM.

Cells were infected with C24V and NY1 in MM at input multiplicities of approximately 10.0. Virus was allowed to adsorb to cells for 1 hour on a rocking platform at 37 C. At the end of 1 hour, infective media was discarded from each flask, fresh volumes of MM were added, and flasks were reincubated for 20-24 hours, or until C24V-infected flasks showed 70% CPE.

Prior to labelling, media was removed from each flask and cells were rinsed three times with a warm volume of methionine-free MEM (met-free-MEM) and reincubated for 30 minutes in a fresh volume of met-free-MEM. L-(35-S)-methionine (15.0 mCi/ml, specific activity 1489 Ci/mmol; Amersham) was added to each flask in a 5 ml volume of met-free-MEM at a final concentration of 50-60 uCi/ml. Cells were reincubated for 8 hours. At the end of labelling, cells were dispersed into the media with a disposable cell scraper and transferred into disposable, 5 ml plastic tubes. Cells were pelleted by centrifugation at 3,000 rpm for 5 minutes. Radioactive supernatant was discarded, cell

pellets were washed with 1 ml of SF-MEM and transferred to 1 ml Eppendorf microcentrifuge tubes. Washed cells were then repelleted in a microcentrifuge at 14,000 x g for 2 minutes. The SF-MEM was discarded and pellets were resuspended in 0.25 ml of PBS and 0.5 ml of TNE-lysis-buffer (50mM-tris-HCl, pH 8.0, 500 mM-NaCl, 5 mM-EDTA) containing 1% Triton X-100, 20 ul phenylmethylsulphonyl fluoride (PMSF, 100mM) per 10 ml of TNE buffer, and 10 ul leupeptin (1 mM) per 10 ml of TNE-buffer. This volume of TNE-lysis-buffer containing cells was aspirated and expelled through a disposable 25 ga needle and incubated for 3 minutes on ice. Resulting lysates were repelleted for 30 minutes, 4 C., at 90,000 x g via ultracentrifugation to remove large molecular weight material. Supernatants were harvested and were either stored at -20 C. or were used immediately for immunoprecipitation.

Immunoprecipitation

For immunoprecipitation of radiolabelled, viral polypeptides, immunosorbent was prepared by mixing equal volumes of cell-lysate-purified-anti-BVDV-globulins and Protein A-Sepharose CL-4B beads (Sigma).

Sepharose beads were rehydrated and washed twice by low speed centrifugation (1,000 rpm, 5 minutes) in 25 ml of 0.25 M tris-tricine, 0.02% sodium azide ($NaN_3$) at pH 8.6 and reconstituted to 0.1 g/ml in a fresh volume of the tris-tricine-azide solution.

The anti-cell reactivity was removed from the bovine anti-BVDV-globulins by elution through BTU and BEC lysates bound to Affigel-10 beads (BioRad). Separate volumes of immunosorbent were required for each cell

type under study, therefore, each volume of cell lysate-purified globulins was mixed with Protein A-Sepharose in a 1:1 ratio (v/v).

The volume of immunosorbent was incubated for 90 minutes at 4 C. on a shaker (Labquake, Labindustries, Berkeley, CA). At the end of 90 minutes, immunosorbent was pelleted in a microcentrifuge for 30 s at 14,000 x g and rinsed three times with a 200 ul volume of TNE-lysis-buffer with 1% Triton X-100, PMSF and leupeptin. Immunosorbent was resuspended in a final volume of TNE-lysis-buffer to yield 50 ul of beads per treated lysate.

A 50 ul volume of immunosorbent was then added to 150 ul of labelled lysates in microcentrifuge tubes. This mixture was incubated for 90-120 minutes at 4 C. on the shaker. Immunosorbent-bound immune complexes were pelleted by microcentrifugation at 14,000 x g for 2 minutes and extensively washed 6 times with 200 ul of TNE-lysis-buffer with 1% Triton X-100, PMSF, and leupeptin. After the final wash, pellets were resuspended with 50 ul of sodium-dodecyl-sulfate (SDS) treatment buffer (0.125 M tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% mercaptoethanol) and boiled for 6 minutes. Tracking dye (2 ul of 1% bromphenol blue) was added to each sample prior to loading in separate wells of the polymerized resolving gel.

SDS-PAGE

For polypeptide resolution by sodium-dodecyl-sulfate polyacrylamide-gel-electrophoresis (SDS-PAGE), a discontinuous SDS-PAGE buffer system was used as described by Laemmli (1970). A 4% stacking gel and a 10% resolving gel were prepared with 20 ml of 30% acrylamide, 0.6 ml of 10% SDS and polymerized with 0.3 ml of 10% ammonium persulfate and 20

ul of N,N,N',N'-tetramethylethylenediamine (TEMED). Molecular mass standards (Mr in kilodaltons, kD) were electrophoresed simultaneously for orientation and included myosin (Mr=205 kD), beta-galactosidase (Mr=116 kD), phosphorylase B (Mr=97.4 kD), bovine serum albumin (Mr=66 kD), egg albumin (Mr=45 kD) and carbonic anhydrase (Mr=29 kD) (SDS6-H; Sigma).

Following electrophoresis at 30 mA for about 3 hours, resulting gels were stained and fixed in 1% Coomasie blue, 50% methanol, and 10% acetic acid for visualization of molecular weight standards. Gels were destained in a two-step destaining protocol; destain I with 50% methanol, 10% acetic acid, and destain II with 30% methanol, 0.3% acetic acid, and 0.3% glycerine. For fluorography, gels were rinsed for 30 minutes in 1 M Na-salicylate (Chamberlain 1979) and dried for 90 minutes under heat and vacuum. Dried gels were wrapped in plastic film and placed in direct contact with radiographic film (X-OMAT AR 5, Eastman Kodak) in an X-ray casette. Casettes were made light-tight and kept at -70 C. with typical exposure times of 1-3 days for detection of polypeptide bands. Radiographic film was developed according to manufacturer's specifications.

Antiserum preparation

A 5 month old bovine calf, BVDV-free and originating from a BVDV-negative herd, was inoculated intravenously with 10(6.9) TCID-50/ml of BVDV, strain C24V. The calf was inoculated again with two doses of C24V at 7 months of age by intramuscular inoculations of virus administered one week apart. Convalescent serum was collected 14 days after the last virus inoculation.

The globulin fraction was harvested from a 50 ml volume of convalescent serum using a saturated ammonium-sulfate solution in a three-step precipitation protocol. The precipitated globulin was resuspended to one-half of the original serum volume in a 1% tris-tricine solution, pH 8.6, containing 0.85% NaCl and 0.02% NaN3. Globulins were dialyzed against the 1% tris-tricine-saline at 4 C. with three changes of dialysate every 24 hours. Globulins were harvested from dialysis tubing and stored at 4 C.

Immunosorbent preparation

Cell lysates from BTU and BEC were prepared by growing cells in large, 75-cm2 flasks in their appropriate growth media. At confluency, growth media was removed from the flasks and a fresh volume of MM was added to each flask and cells were reincubated overnight. The next day cells were lysed with 25 ml of HNE-lysis-buffer (100 mM Hepes, pH 7.5, 500 mM NaCl, 5 mM EDTA) containing 20 ul PMSF/10 ml HNE-buffer and 10 ul leupeptin/10ml HNE-buffer.

Two 25 ml volumes of Affigel-10 agarose beads (BioRad) were prepared as per supplier's recommendations and then each volume was handled separately for each cell lysate. The BTU and BEC lysates (ligands) were bound to the beads by mixing cell lysate in a 1:1 v/v with the Affigel slurry and incubating overnight at 4 C. on a rocker platform. Following binding, each gel-ligand was washed extensively with 0.1M Hepes, pH 7.5 until wash-aliquots from each gel-ligand showed zero absorbance at 280 nm on an ultraviolet spectrophotometer. Each gel-ligand preparation was activated with a fresh 20 ml volume of 5 M ethanolamine, pH 8.0, for 2

hours at 25 C. and then extensively washed with 0.1 M Hepes, pH 7.5, until collected wash-aliquots from each preparation showed zero absorbance at 280 nm.

Each gel-ligand slurry was poured as a column and equilibrated with two washes of a tris-buffered-saline (TBS, 0.01 tris, pH 7.9, with 0.15 M NaCl and 0.02% NaN3). The previously harvested anti-BVDV-globulin was then divided into two volumes for elution through each gel-ligand column. The two volumes were diluted in a 1:1 v/v with TBS, and the TBS-globulin mixture was added to each column and allowed to react for 30 minutes at RT. Globulins were purified through each cell lysate by eluting with TBS and the elutant was recovered in 0.5 ml volumes for absorbance readings at 280 nm. Volumes of elutant that gave positive absorbance readings were pooled and concentrated 5X with a disposable multiple ultrafilter (Minicon B-15, Amicon, W.R. Grace & Co.). Purified globulin aliquots were stored at 4 C. until used in the RIP procedure.

# RESULTS AND DISCUSSION

## Endothelial Cell - Harvest and Culture

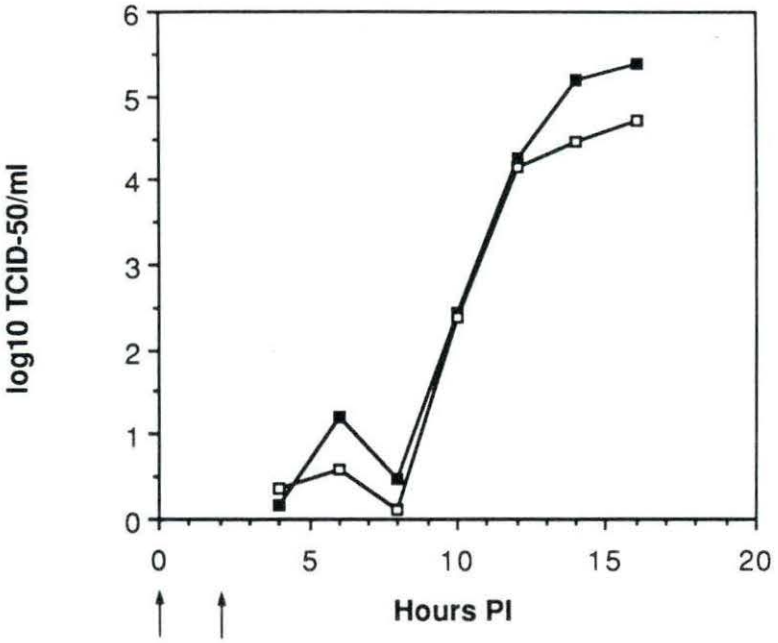
Following enzymatic digestion of bovine aortas, the BEC were seeded into precoated wells of a 12-well tissue culture plate. Cell attachment and subsequent proliferation was dependent on the precoating agent and for this research, bovine fibronectin (Sigma) was superior to CR-LAMININ (Collaborative Research, Inc., Lexington, MA) in promoting BEC adhesion to a plastic surface (data not shown). Following several cell passages, BEC behavior in vitro was monitored by morphological and functional criteria. The cobblestone appearance of confluent BEC served as the morphological determinant, while synthesis and incorporation of Factor VIII:vWF-RAg into cell membranes served as a convenient means to screen cell cultures for the presence of functionally differentiated BEC. By indirect FA-assay, Factor VIII:vWF-RAg was detected in 85-95% of BEC from passages 3-11. This percentage of positive staining cells declined to 50-60% from passages 12-19 and decreased even further to 30% and less from passage 20 and above (data not shown). Because of these results, BEC were not used in any experimental protocol beyond passage level 11.

## Viral Growth Curves

In comparisons made across both BVDV strains, C24V and NY1, regardless of cell type infected, the time of maximum virus release occurred between 8 and 12 HPI and no significant differences were observed in the rate of virus release per hour (Figures 1-4). The kinetics of viral replication and the time-frame observed for maximum virus release were consistent with published findings (Nuttall 1980). In all virus-cell

Figure 1. Single-step growth curve of CP-BVDV, strain C24V following infection of bovine BTU. Extracellular virus ( ■ ) and cell-associated virus ( □ ). Arrows denote times of 2X rinse of infected cell monlayers with warm MM

Figure 2. Single-step growth curve of NCP-BVDV, strain NY1 following infection of bovine BTU. Extracellular virus ( ■ ) and cell-associated virus ( □ ). Arrows denote times of 2X rinse of infected cell monolayers with warm MM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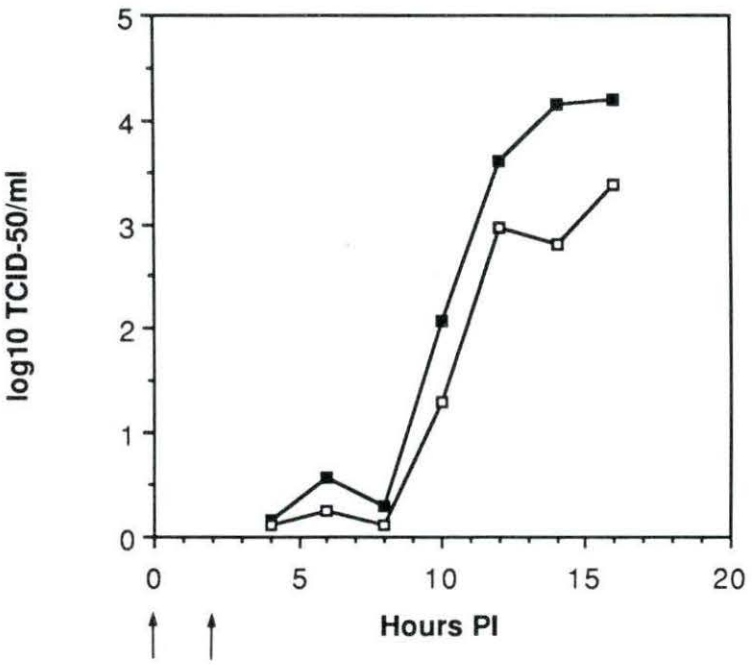
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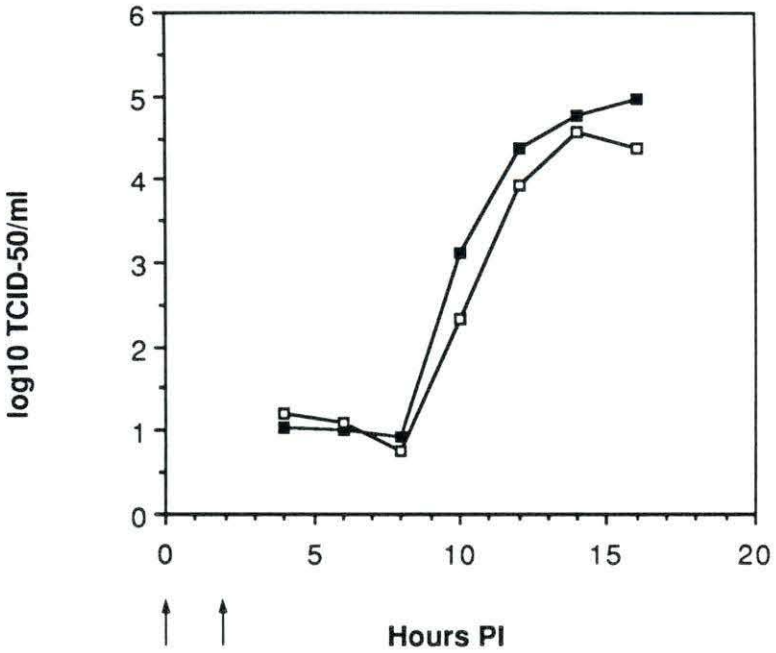
Figure 3. Single-step growth curve of NCP-BVDV, strain NY1 following infection of bovine BEC. Extracellular virus ( ■ ) and cell-associated virus ( □ ). Arrows denote times of 2X rinse of infected cell monolayers with warm MM

Figure 4. Single-step growth curve of CP-BVDV, strain C24V following infection of bovine BEC. Extracellular virus ( ■ ) and cell-associated virus ( □ ). Arrows denote times of 2X rinse of infected cell monolayers with warm MM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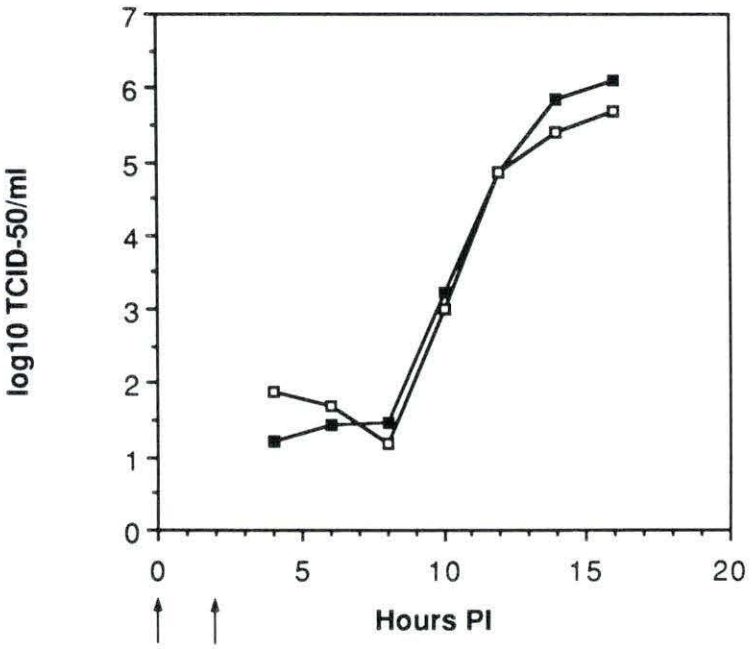
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combinations, mean extracellular titers of virus exceeded mean cell-associated titers during the time of maximum release and this difference was significant (P .05).

Within a virus strain, extracellular titers of virus replicated within BEC surpassed virus titers obtained in BTU. This held for both C24V and NY1 (Figures 5 and 6). Two explanations are offered for this observation: 1) the total number of infected BEC was greater than infected BTU, or 2) BEC enhanced replication of BVDV compared to BTU. In addition to titer differences observed between infected cell types, the four post-infection rinses of BVDV-infected BEC with MM did not reduce amounts of residual virus to comparable levels observed in infected BTU following the same protocol of post-infection rinsing (Figures 1-6). Again, this may be a result of greater numbers of infected BEC reaching equilibrium in adsorbing/releasing virions, or virus adsorption was enhanced in BEC.

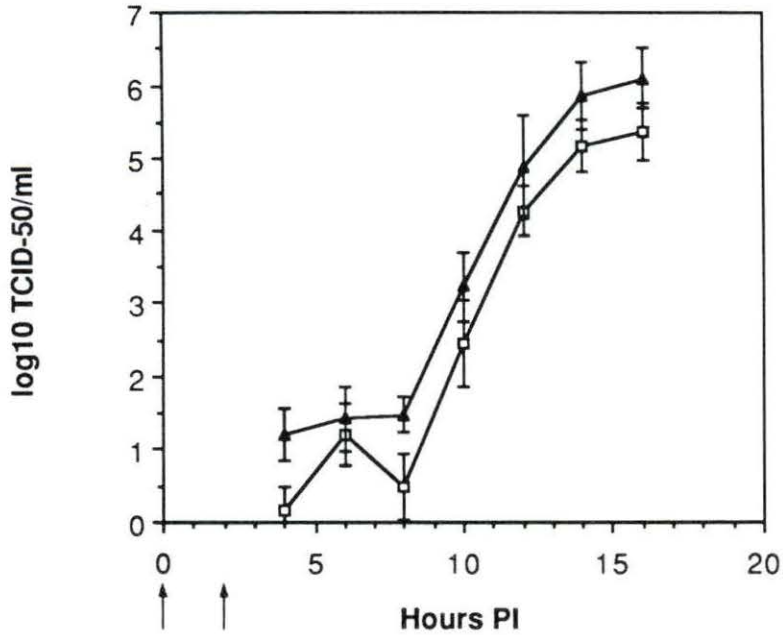
Comparisons were made between CP- and NCP-BVDV extracellular virus titers within infected cell types. In BTU, C24V titers surpassed NY1 from 12-16 HPI by approximately 1.0-1.5 base 10 log. This difference was significant (Figure 7). Similar results were found in BEC, however, the difference in titers between strains did not occur until 14 HPI and the difference was generally less than 1.0 base 10 log (data not shown).

The similarities in growth curves (up to 14 HPI) for NY1 and C24V in BEC indicated that NCP-BVDV behavior was, within reasonable limits, mimicking CP-BVDV growth patterns and titers. A curve was plotted comparing NY1 grown in BEC with C24V grown in BTU (Figure 8). The

Figure 5. Comparison of extracellular virus titers for CP-BVDV, strain C24V, grown in two bovine cell types, BTU ( □ ) and BEC ( ▲ ), +/- 2X S.E.M. Arrows denote times of 2X rinse of infected cell monolayers with warm MM



Figure 6. Comparison of extracellular virus titers for NCP-BVDV, strain NY1, grown in two bovine cell types, BTU ( ■ ) and BEC ( □ ), +/- 2X S.E.M. Arrows denote times of 2X rinse of infected cell monolayers with warm MM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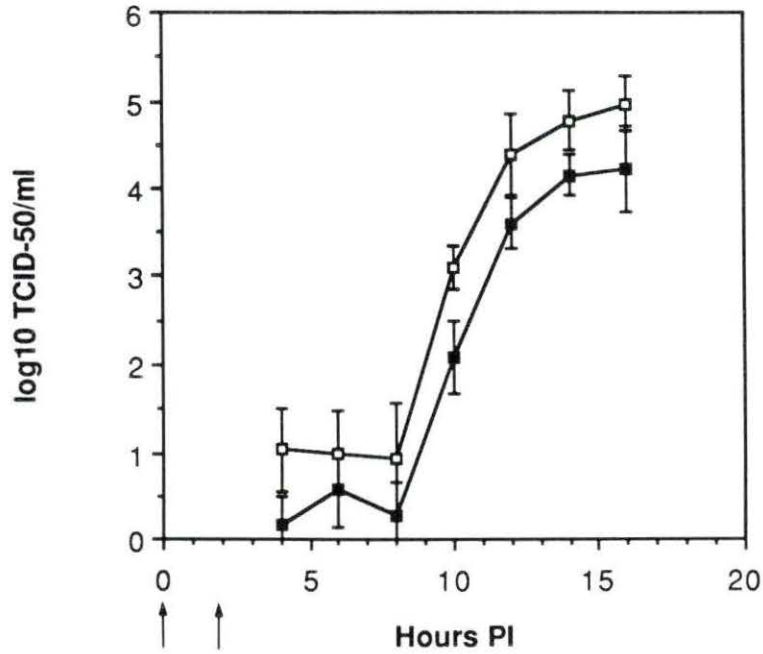
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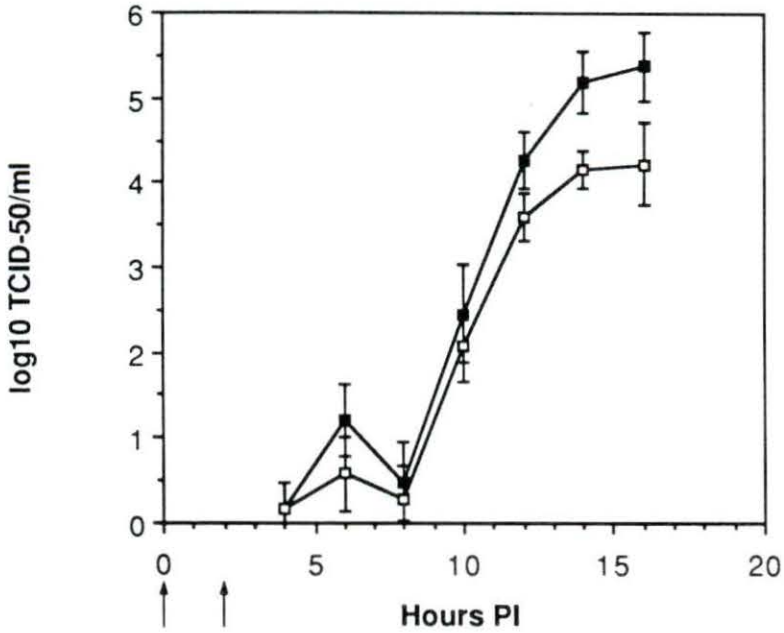
Figure 7. Comparison of extracellular virus titers for two strains of BVDV, grown in bovine BTU. CP-BVDV, strain C24V ( ■ ) and NCP-BVDV, strain NY1 ( □ ), +/- 2X S.E.M. Arrows denote times of 2X rinse of infected cell monolayers with warm MM

Figure 8. Comparison of extracellular virus titers for two strains of BVDV, following infection of two bovine cell types. CP-BVDV, strain C24V, grown in bovine BTU ( □ ) and NCP-BVDV, strain NY1, grown in bovine BEC ( ▲ ), +/- 2X S.E.M. Arrows denote time of 2X rinse of infected cell monolayers with warm MM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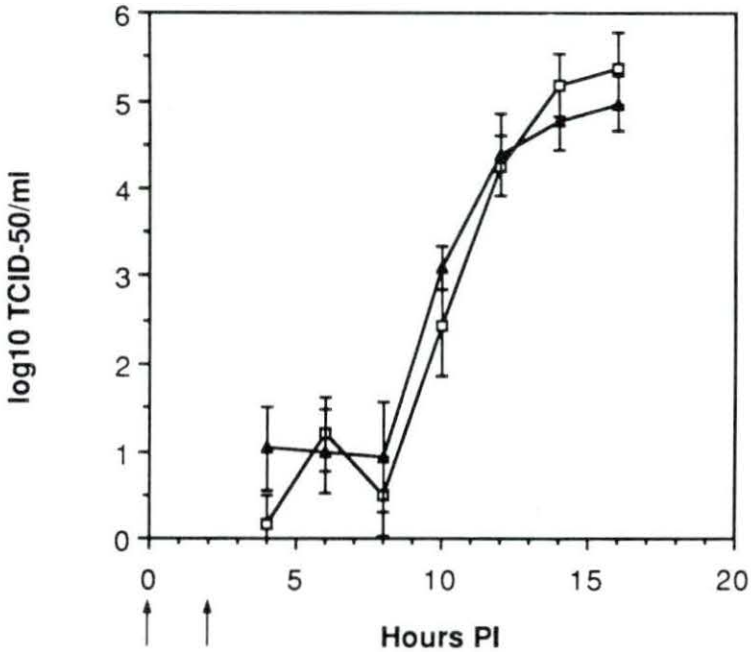
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replication kinetics and extracellular titers were parallel and showed no significant differences. From 8-12 HPI, titers of NY1 in BEC were greater than C24V titers in BTU, and again, these observations are suggestive of a larger total yield of virus from infected BEC.

## Cytotoxicity Assays

### Tetrazolium ring (MTT) cleavage assay

This quantitative assay was developed to objectively evaluate mammalian cell viability and proliferation. Primarily used for populations of mammalian cells of the leukocytic series, the assay has been used to measure the reduction of MTT to MTT-formazan in mitochondria of mitogen-stimulated T- and B-lymphocytes, myeloma, T-lymphoma, and macrophage-like tumor cell lines (Mosmann 1983).

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) forms a pale yellow solution upon dissolution into PBS. The cleavage of the tetrazolium ring by dehydrogenase enzymes results in the formation of dark blue MTT-formazan granules. Absorbance readings (O.D. 570-630nm) on an ultraviolet-light spectrophotometer quantitate enzymatic activity in cell mitochondria and thus, cell viability/activation can be indirectly determined. Quantities of MTT-formazan are directly proportional to cell numbers in a homogeneous cell population and the assay also provides an indication of cell activation (in the absence of proliferation) through the generation of MTT-formazan (Mosmann 1983).

Visible CPE in C24V-infected BTU was not correlated with the "expected" impairment and loss of cell function, as measured by the MTT-assay (Figure 9). In C24V-infected BTU from 3-7 DPI the increase observed

in O.D. readings suggests cellular activation and/or proliferation that was not evident morphologically, as cells displayed characteristic C24V-induced CPE. Throughout the time-frame of the study, media in C24V-infected BTU remained clear and there was no obvious contamination, however, a conceivable explanation for the cleavage of MTT would be the presence of a microorganism population containing the enzymatic machinery capable of tetrazolium ring cleavage (Neville 1987).

In C24V-infected BEC, cellular dysfunction, detected by reduced O.D. readings in the MTT-assay, correlated well with visible CPE characteristic of C24V virus infection (Figure 10).

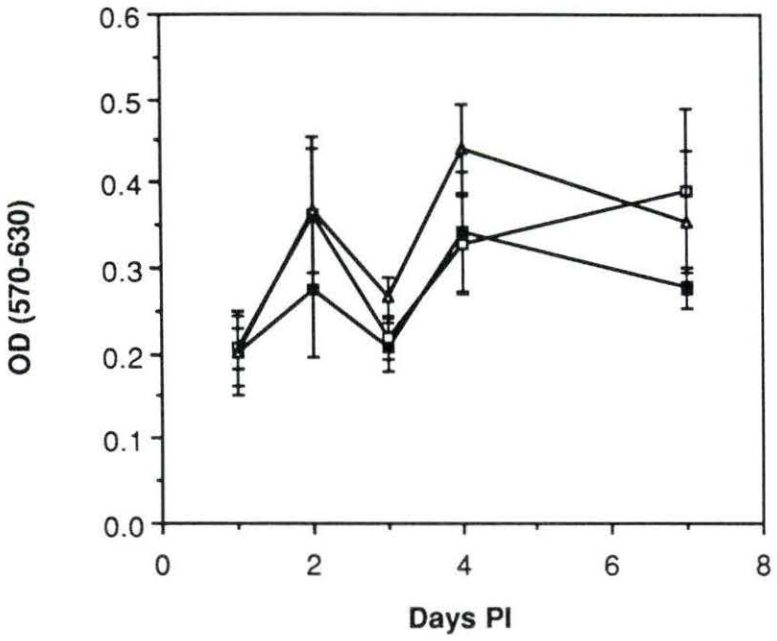
In either cell type, mock- and NY1-infected cells demonstrated a large day-to-day component of variability in O.D. readings (Figures 9 and 10). These readings differed between BTU and BEC, however, results were consistent between mock- and NY1-infected cells within a cell type.

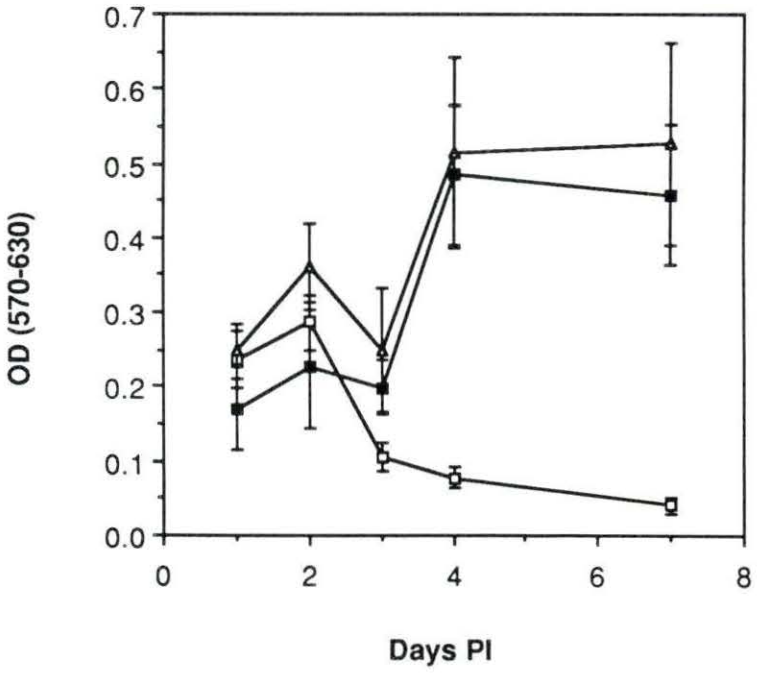
Mock- and NY1-infected BTU demonstrated functional cytotoxicity in the absence of visible CPE from 4-7 DPI, however, NY1-infected cells did not differ from control cells in their capability of MTT-cleavage (Figure 9). The reduction in metabolic activity may have been due to the sensitivity of BTU to accumulating cytotoxic cellular metabolites.

In contrast to BTU, mock- and NY1-infected BEC demonstrated an increase in metabolic activity as the assay progressed from 2-6 DPI (Figure 10). The MTT-assay, in evaluating cytotoxic events in a cell population, does not distinguish between cell proliferation/activation (Mosmann 1983). The consistency observed in absorbance readings between mock- and NY1-infected BEC precludes the conclusion that virus infection

Figure 9. Measurements of optical density for the MTT-assay following mock- and BVDV-infection of bovine BTU. Mock-infected control (△), CP-BVDV strain C24V (□), and NCP-BVDV strain NY1 (■)

Figure 10. Measurements of optical density for the MTT-assay following mock- and BVDV-infection of bovine BEC. Mock-infected control (△), CP-BVDV strain C24V (□), and NCP-BVDV strain NY1 (■)


0.6
0.5
0.4
0.3
0.2
0.1
0.0
OD (570-630)
0
2
4
6
8
Days PI


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
OD (570-630)
0
2
4
6
8
Days PI

alone stimulated cell metabolism and/or proliferation. The lack of demonstrable cellular impairment in BEC, compared to similarly treated BTU, addresses the hardiness of the cell type and the apparent higher metabolic activity of endothelial cells.

The results of the MTT-assay in mock- and BVDV-infected BTU did not parallel similarly treated BEC. Within the first 4 days of the assay, however, similar patterns of metabolic activity were observed in mock-infected cells, as well as NY1-infected cells. Despite this pattern, the large day-to-day variation in similarly treated cell types, the inability to differentiate between cellular proliferation or activation, and false-positive results (C24V-infected BTU) suggest that the value of the MTT-assay in evaluating virus-induced cytotoxicity is minimal.

(51-Cr)-chromium-uptake assay

To confirm and supplement results of the MTT-assay, a (51-Cr)-chromium-uptake assay was evaluated in mock- and BVDV-infected cell types. Major advantages of the chromium-uptake assay include: 1) the ability to conduct long-term cytotoxicity studies, 2) the measurement of viable cells with signal-strength being proportional to cell numbers, cell volume, and temperature, and 3) chromium-uptake is independent of DNA, RNA, and protein synthesis. Isotope uptake occurs by facilitated diffusion across cell membranes (Neville 1987).

The (51-Cr)-chromium-uptake assay was developed to overcome difficulties reported with the chromium-release and tetrazolium ring (MTT) cleavage assays in evaluating cytotoxicity (Neville 1987). Chromium-uptake is more reliable than chromium-release in evaluating long-term

cytotoxicity events (greater than 8 hours). The measurement of chromium-release in long-term assays tends to be distorted by excessive diffusion of isotope across cell membranes (Neville 1987). Theorized problems with the MTT-assay include false-positive results from bacterial cleavage of the tetrazolium ring and differences between cell populations in their ability to cleave MTT (Neville 1987).

The disparity in CPM between BTU and BEC, either mock- or BVDV-infected, was probably due to differences between the cell populations in cell volume and cell numbers (contrast Figures 11 and 12). Similar to the MTT-assay, a large component of variability was observed in the chromium-uptake assay for mock- and NY1-infected cell types (Figures 11 and 12). Overall, however, mock-infected control cells demonstrated less day-to-day variation in chromium-uptake compared to cleavage of MTT.

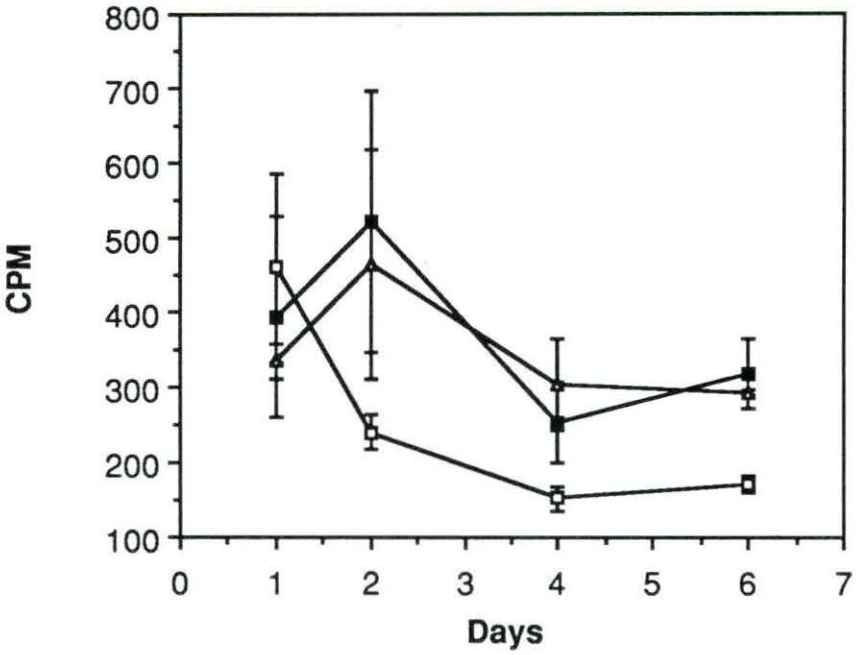
Visible CPE and destruction of the cell monolayer in C24V-infected BTU and BEC was adequately confirmed by the chromium-uptake assay (Figures 11 and 12).

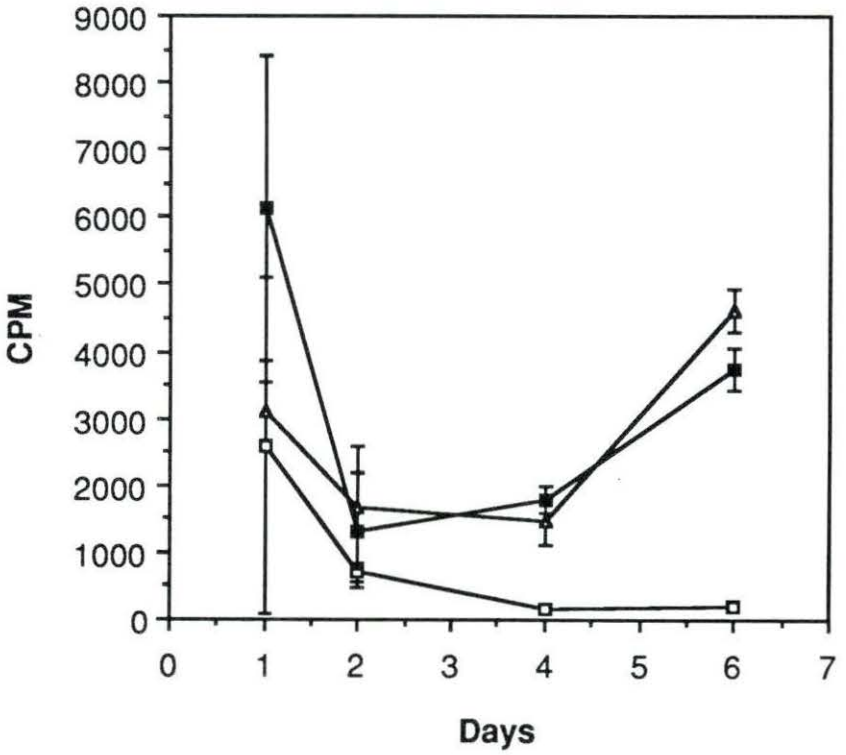
In mock- and NY1-infected BTU, uptake of (51-Cr)-chromium increased and then declined from 1-4 DPI (Figure 11). Based on the assay, this might suggest a peak of metabolic activity occurring 1-2 days following the change of media at the initiation of the assay or, a cytotoxic event affecting cell membranes from 2-4 days into the assay that was not visible morphologically.

Similar to BTU, mock- and NY1-infected BEC peaked in chromium internalization on day 1 of the assay and then declined in uptake through day 4 (Figure 12). Neither treatment group displayed morphological

Figure 11. Measurements of counts-per-minute (CPM) for the (51-Cr)-chromium uptake assay following mock- and BVDV-infection of bovine BTU. Mock-infected control cells (△), CP-BVDV strain C24V (□), and NCP-BVDV strain NY1 (■)

Figure 12. Measurements of counts-per-minute (CPM) for the (51-Cr)-chromium uptake assay following mock- and BVDV-infection of bovine BEC. Mock-infected control cells (△), CP-BVDV strain C24V (□), and NCP-BVDV strain NY1 (■)


800
700
600
500
400
300
200
100
CPM
0
1
2
3
4
5
6
7
Days


9000
8000
7000
6000
5000
4000
3000
2000
1000
0
CPM
0
1
2
3
4
5
6
7
Days

evidence of CPE during the time-frame of the assay and from 4-6 DPI, both groups showed a rebound in internalization of isotope. Virus effects appear to be precluded by the consistency observed in chromium-uptake between mock- and NY1-infected BEC.

Chromium-uptake is independent of cellular metabolism and dependent on viable cell membranes (Neville 1987). This suggests that cell proliferation or changes in BEC volume occurred during the assay. Typically, BEC form a stable monolayer following harvest that is contact-inhibited at confluence. When cultures are maintained at high densities for periods of 1-2 weeks, however, focal areas of a second growth pattern have been reported (Schwartz 1978). This pattern has been termed "sprouting" and is not due to overgrowth of a heterologous, contaminating cell type, but rather represents a second pattern of BEC growth (Schwartz 1978).

Direct immunofluorescence (FA)-assay

Examination of C24V-infected cells, both BTU and BEC, demonstrated that each cell type was permissive to the virus. Following infection both BTU and BEC displayed CPE characteristic of CP-BVDV, strain C24V (Gillespie et al. 1960). Direct FA-assay of C24V-infected cells after 1 DPI detected BVDV-antigens distributed throughout the cytoplasm. The fluorescence offset dark, nonstaining cytoplasmic vacuoles and cell nuclei (Figures 13 and 14).

As the C24V-infection progressed through BTU and BEC monolayers (2-3 DPI), vacuoles increased in size and number in the cytoplasm, cells stretched out and took on a "stringy" appearance, nuclei became pyknotic

and eccentric, and cells were lysed and shed from the monolayer. Typically, by 4 DPI, no significant cellular mass remained for direct FA-assay.

Monolayers of NY1-infected BEC were examined by direct FA-assay at 2, 4, and 6 DPI (Figures 15-17, respectively). At 2 DPI, 98% of infected cells demonstrated the presence of BVDV-antigens in the cytoplasm (Figure 15). Offset by the fluorescence were dark, nonstaining cell nuclei, as well as small cytoplasmic vacuoles. The vacuoles closely resembled those observed in early C24V-infected BEC (contrast Figures 13 and 15). These observations remained consistent in NY1-infected BEC examined at 4 DPI (Figure 16), however, the level of cytoplasmic fluorescence and extent of vacuolation were less prominent and arrested, respectively.

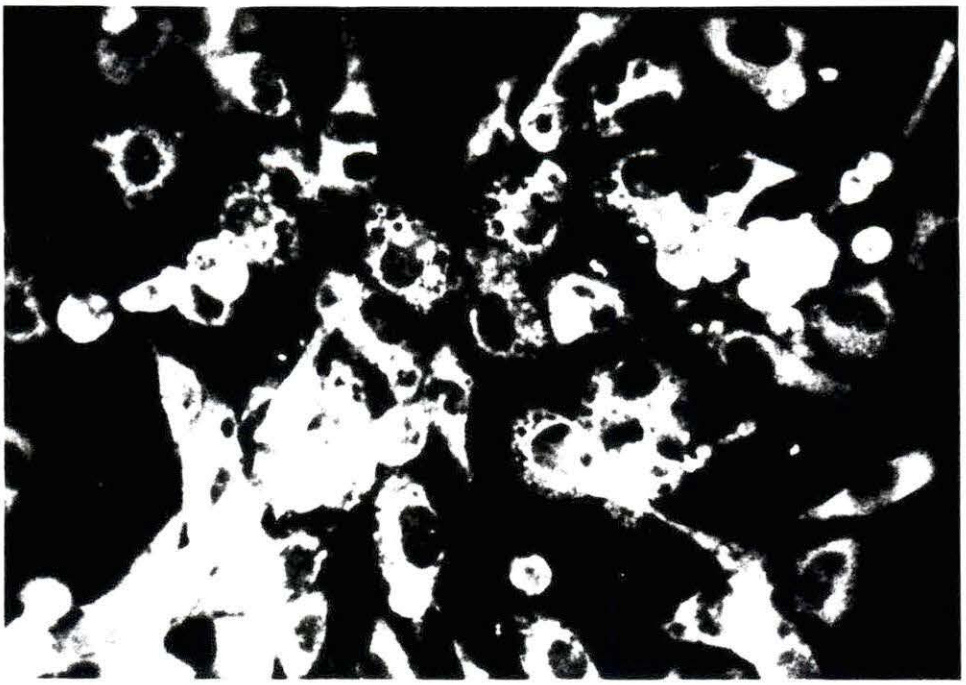
NY1-infected BEC observed at 6 DPI by direct FA-assay still demonstrated the presence of BVDV-antigens in the cytoplasm (Figure 17). The level of fluorescence, however, was markedly diminished compared to infected BEC observed at 2 and 4 DPI. No increase in cytoplasmic vacuolation was noted, and cells appeared to be reduced in cytoplasm volume compared to NY1-infected BEC observed early-on in the infection (contrast Figures 15-17).

Mock-infected BEC did not demonstrate BVDV-antigens following direct FA-assay (Figure 18). Further, monolayers of mock-infected cells did not show any structural changes or CPE suggestive of BVDV-infection throughout the time-frame of the experiment.

Monolayers of NY1-infected BTU were also examined by direct FA-assay at 2, 4, and 6 DPI (Figures 19-21). Similar to NY1-infected BEC, 98% of

Figure 13. Direct immunofluorescence assay of CP-BVDV-infected bovine BEC. One day post-infection, strain C24V. Magnification 1080X

Figure 14. Direct immunofluorescence assay of CP-BVDV-infected bovine BTU. One day post-infection, strain C24V. Magnification 1080X



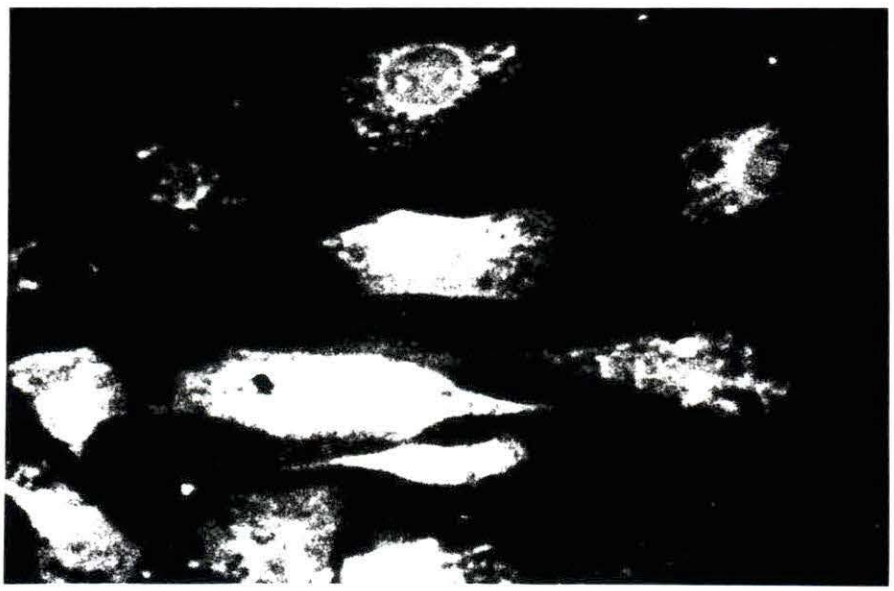
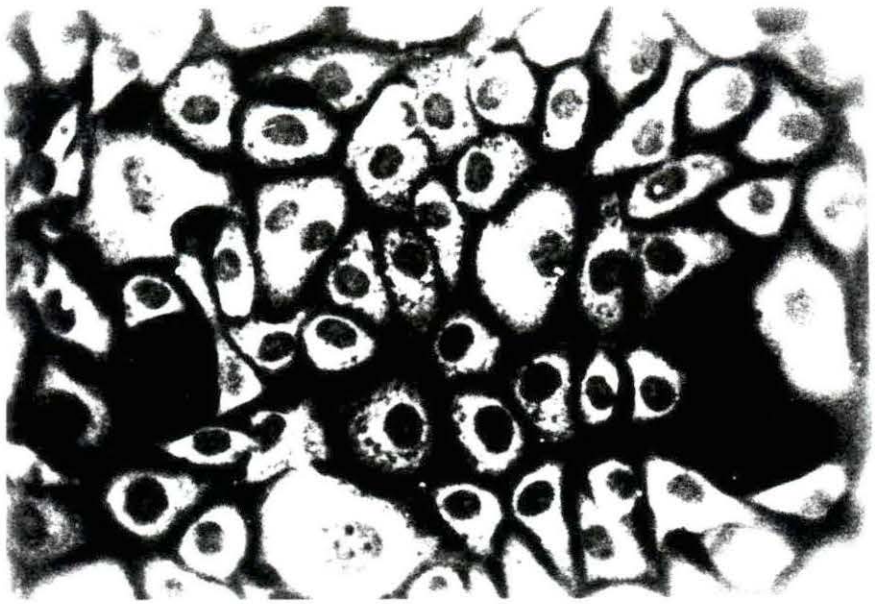


Figure 15. Direct immunofluorescence assay of NCP-BVDV-infected bovine BEC. Two days post-infection, strain NY1. Magnification 1080X

Figure 16. Direct immunofluorescence assay of NCP-BVDV-infected bovine BEC. Four days post-infection, strain NY1. Magnification 1080X



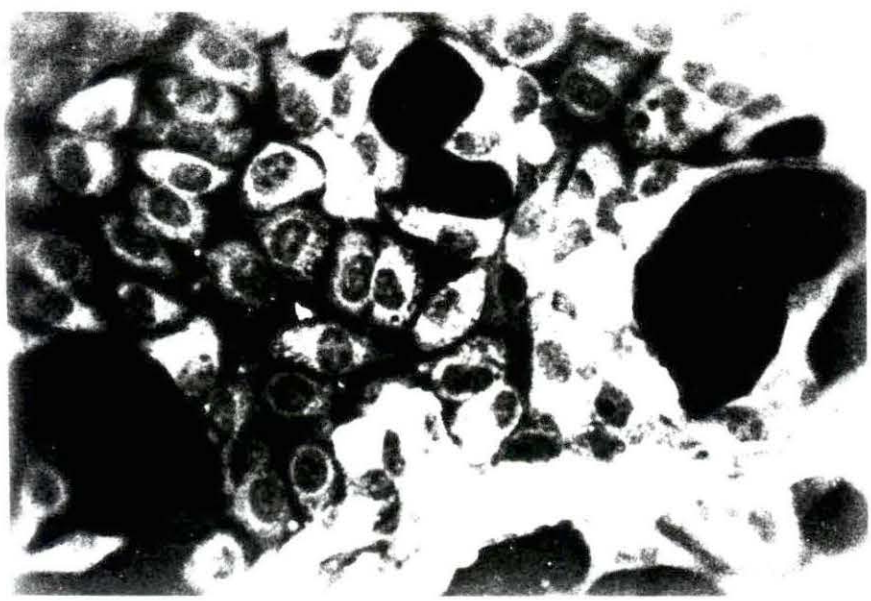
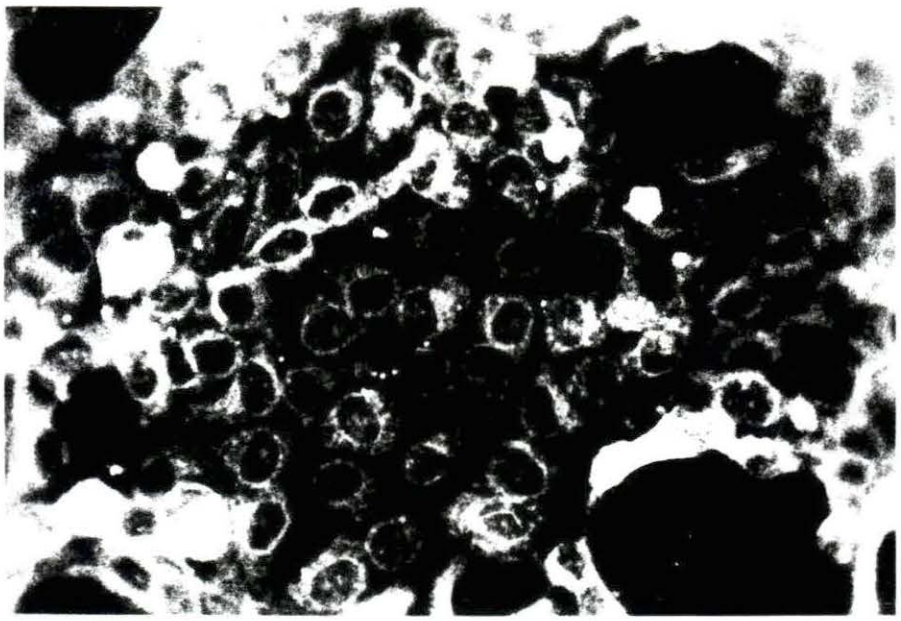
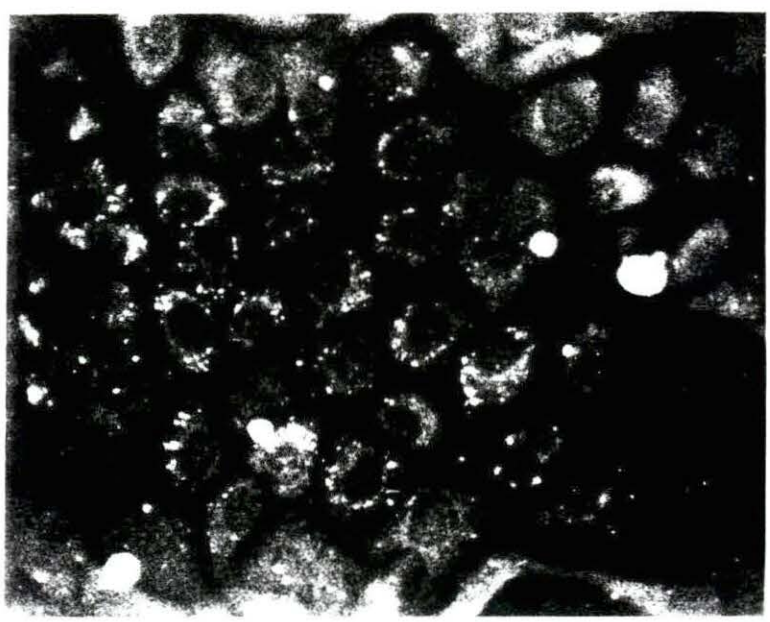


Figure 17. Direct immunofluorescence assay of NCP-BVDV-infected bovine BEC. Six days post-infection, strain NY1. Magnification 1080X

Figure 18. Direct immunofluorescence assay of mock-infected bovine BEC. Four days following inoculation with MM, minus BVDV. Magnification 1080X





infected BTU examined at 2 DPI demonstrated the presence of BVDV-antigens distributed in the cytoplasm, surrounding a dark, nonstaining nucleus (Figure 19). In contrast to BEC, however, NY1-infected BTU did not display any structural changes such as cytoplasmic vacuolation. The pattern of fluorescence was granular in appearance.

Similar results were noted in NY1-infected BTU stained at 4 DPI (Figure 20). Infected cells stained positive with the conjugate, fluorescence was granular, and no evidence of structural changes was observed in infected cells.

Direct FA-assay of NY1-infected BTU performed at 6 DPI demonstrated the presence of BVDV-antigens in the cytoplasm of infected cells; dark, nonstaining nuclei were offset by the cytoplasmic fluorescence (Figure 21). Fluorescence appeared to be reduced in 6-day infected cells compared to BTU at 2 and 4 DPI. Cytoplasmic volume remained consistent throughout the six-day observation period (contrast Figures 19-21).

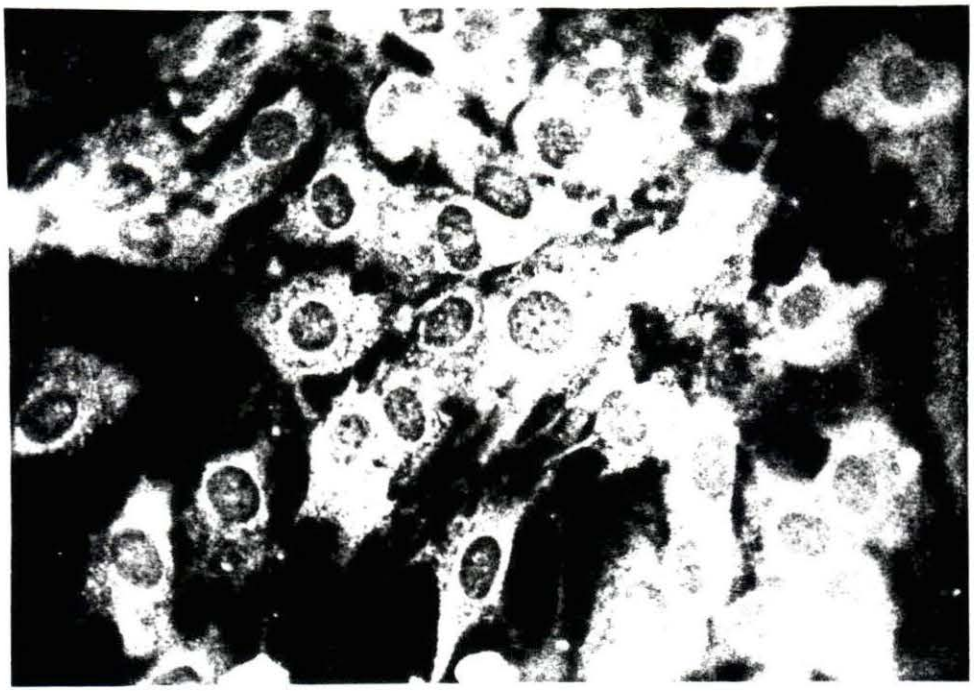
Mock-infected BTU did not display BVDV-antigens following direct FA-assay. In addition, monolayers did not show evidence of CPE suggestive of BVDV-infection in the time-frame of the experiment.

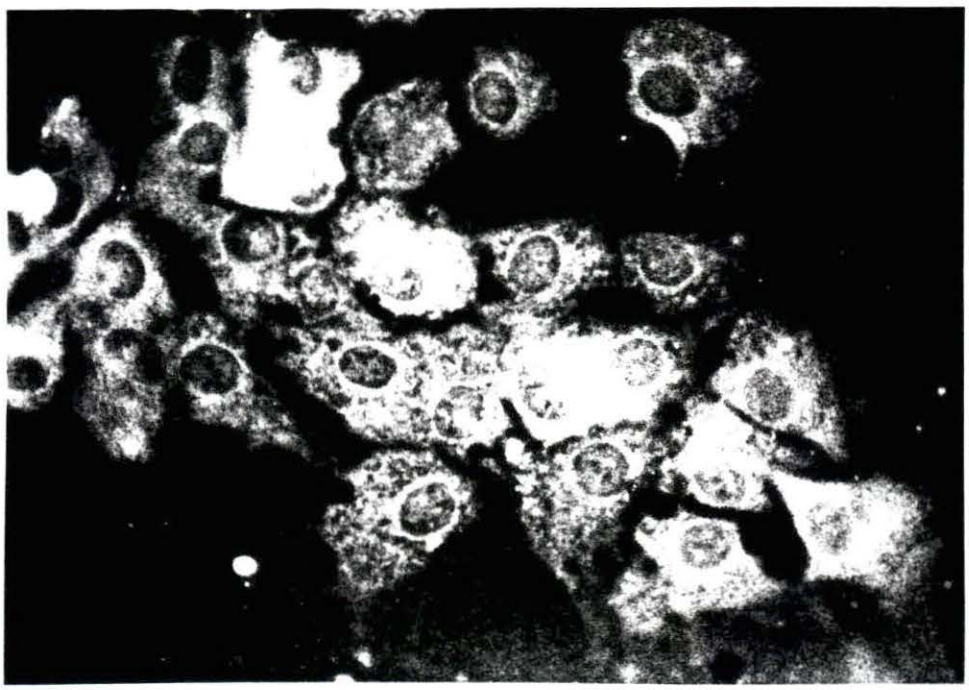
### Radioimmunoprecipitation(RIP)/Sodium-dodecyl-sulfate polyacrylamide-gel-electrophoresis (SDS-PAGE) Analysis

Following BVDV- or mock-infection and radiolabelling of polypeptides with L-(35-S)-methionine, cell lysates were immunoprecipitated and analyzed by gel electrophoresis. Polypeptides were resolved from BVDV-infected cells (BTU and BEC) and the number and relative molecular mass (Mr) were consistent with published findings (Pocock et al. 1987, Donis and Dubovi 1987a).

Figure 19. Direct immunofluorescence assay of NCP-BVDV-infected bovine BTU. Two days post-infection, strain NY1. Magnification 1080X

Figure 20. Direct immunofluorescence assay of NCP-BVDV-infected bovine BTU. Four days post-infection, strain NY1. Magnification 1080X





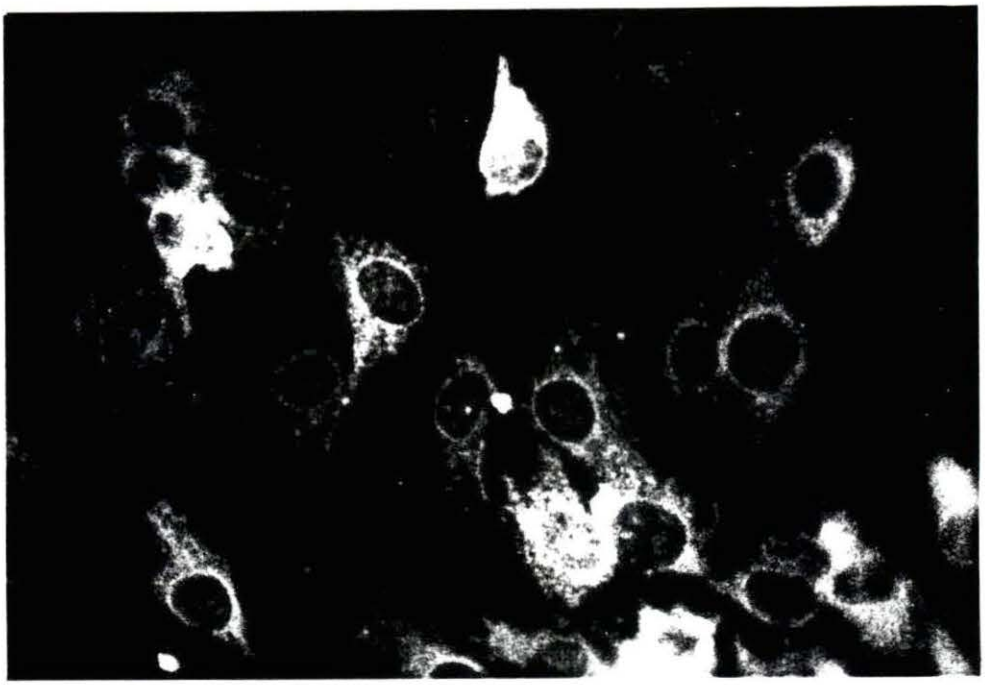


Figure 21. Direct immunofluorescence assay of NCP-BVDV-infected bovine BTU. Six days post-infection with strain NY1. Magnification 1080X

Resolving gels were autoradiographed for variable lengths of time (24, 72, and 120 hours) to maximize the detection of virus-specific polypeptides (Figures 22, 23, and 24, respectively). The immunosorbent was specific for BVDV-polypeptides, as SDS-PAGE lanes used to analyze mock-infected cell lysates did not demonstrate radiolabelled immunoprecipitates (Lane A, mock-infected BTU; Lane H, mock-infected BEC in Figures 22-24).

Eight BVDV-specific polypeptides were resolved from C24V-infected BTU with Mr in kilodaltons (kD) of 117.5, 109.6, 89.1, 76, 58-56.2, 52.5, 46, and 27.5 kD (Figures 22-24, Lane B). Seven polypeptides were identified in C24V-infected BEC; the 46 kD polypeptide was not resolved, however, the

others were identical in Mr to polypeptides found in C24V-infected BTU (Figures 22-24, Lane C).

In contrast to the isolation of 12 BVDV-specific polypeptides by Donis and Dubovi (1987a), the resolution of 7-8 polypeptides from C24V-infected cells may be indicative of the anti-BVDV globulins used in the preparation of immunosorbent. Radioimmunoprecipitations performed in this research used anti-BVDV bovine immunoglobulins specific to the C24V-strain of BVDV. Immunoglobulins were prepared from convalescent serum collected after three live-BVDV inoculations. Immunoglobulins used for RIP by Donis and Dubovi (1987a) were collected from bovine serum following two immunizations with a killed BVDV vaccine (Singer strain) and a third exposure to the virus by inoculation with live Singer-strain BVDV.

To establish consistency with published reports using other CP-BVDV strains, such as NADL and Singer, monolayers of BEC were also infected with these two strains, immunoprecipitated, and resolved by SDS-PAGE (Figures 22-24, Lanes D and E). Differences in the number of resolved polypeptides were noted, compared to the C24V/BEC lysate and compared to published observations (Pocock et al. 1987, Donis and Dubovi 1987a). Those polypeptides resolved from NADL- and Singer-infected cell lysates, however, compared favorably in Mr to the major C24V-specific polypeptides. The 117.5, 89.1, 58-56.2, and 52.5 kD bands were identified in NADL- and Singer-infected BEC (Figures 22-24, Lanes D and E).

Interestingly, the heaviest polypeptide (vp 1) identified in the NADL-infected BEC-lysate demonstrated a slightly larger Mr compared to the 117.5 kD bands resolved from C24V- and Singer-infected BEC-lysates. This

Figure 22. Autoradiograph following 24-hour exposure. RIP/SDS-PAGE analysis of virus-specified proteins following BVDV-infection of bovine BTU and BEC. In lane G, ( ○ ) denotes 92.2 kD polypeptide and ( ● ) denotes 72.4 kD polypeptide

Lane A - BTU, mock-infected control
Lane B - BTU, C24V-infected
Lane C - BEC, C24V-infected
Lane D - BEC, NADL-infected
Lane E - BEC, Singer-infected
Lane F - BTU, NY1-infected
Lane G - BEC, NY1-infected
Lane H - BEC, mock-infected control

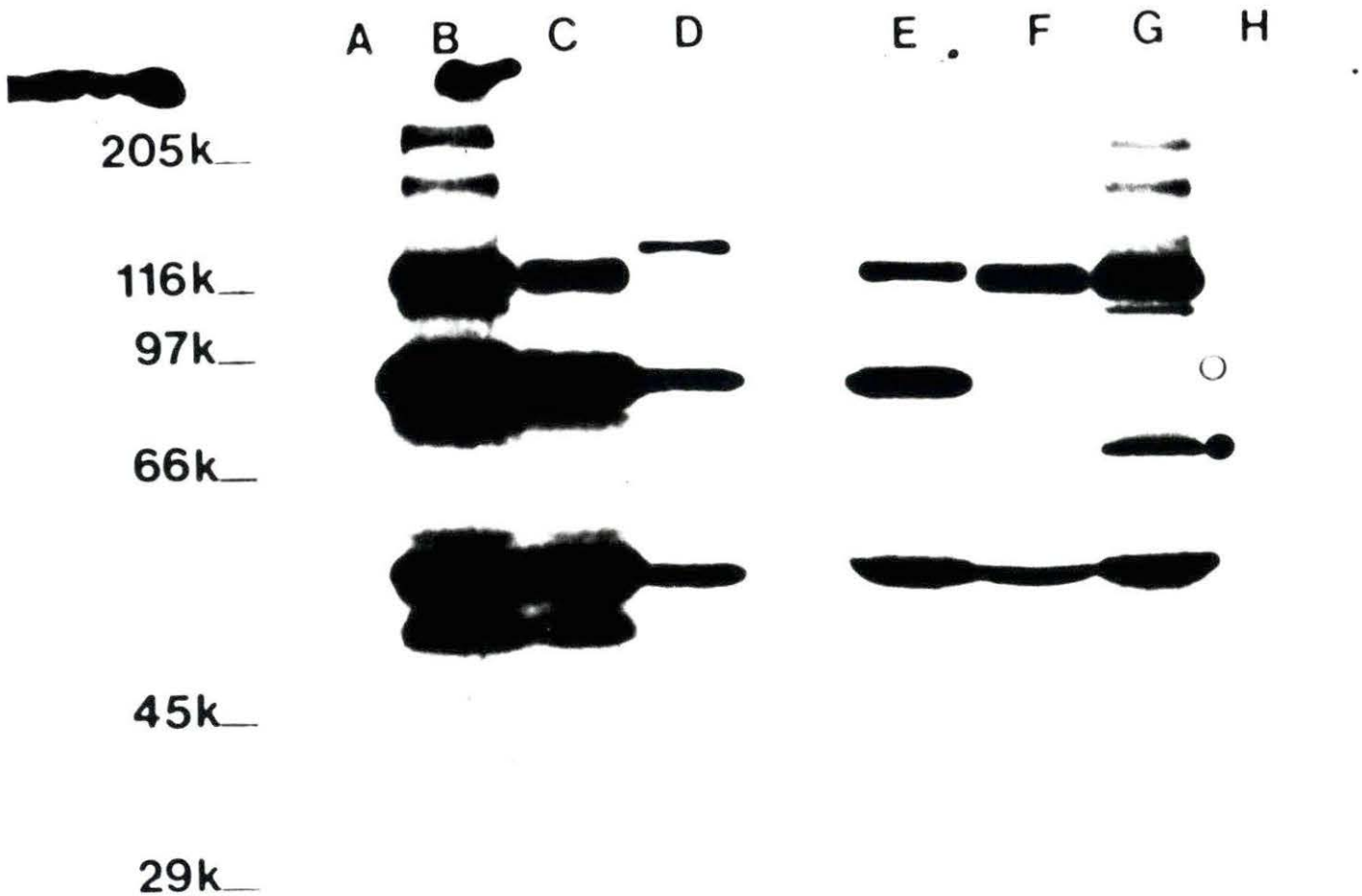

A
B
C
D
E
F
G
H
205k
116k
97k
66k
45k
29k

Figure 23. Autoradiograph following 72-hour exposure. RIP/SDS-PAGE analysis of virus-specified proteins following BVDV-infection of bovine BTU and BEC. In lane G, ( ○ ) denotes 92.2 kD polypeptide and ( ● ) denotes 72.4 kD polypeptide

Lane A - BTU, mock-infected control
Lane B - BTU, C24V-infected
Lane C - BEC, C24V-infected
Lane D - BEC, NADL-infected
Lane E - BEC, Singer-infected
Lane F - BTU, NY1-infected
Lane G - BEC, NY1-infected
Lane H - BEC, mock-infected control

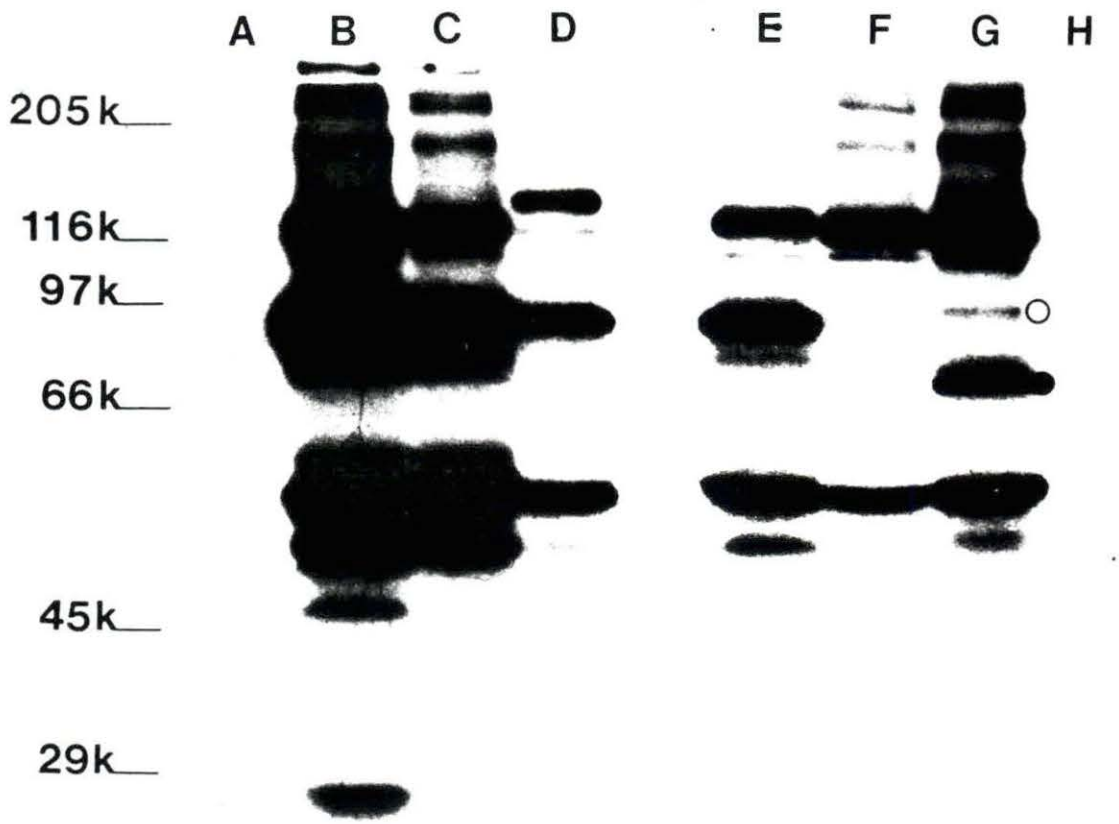
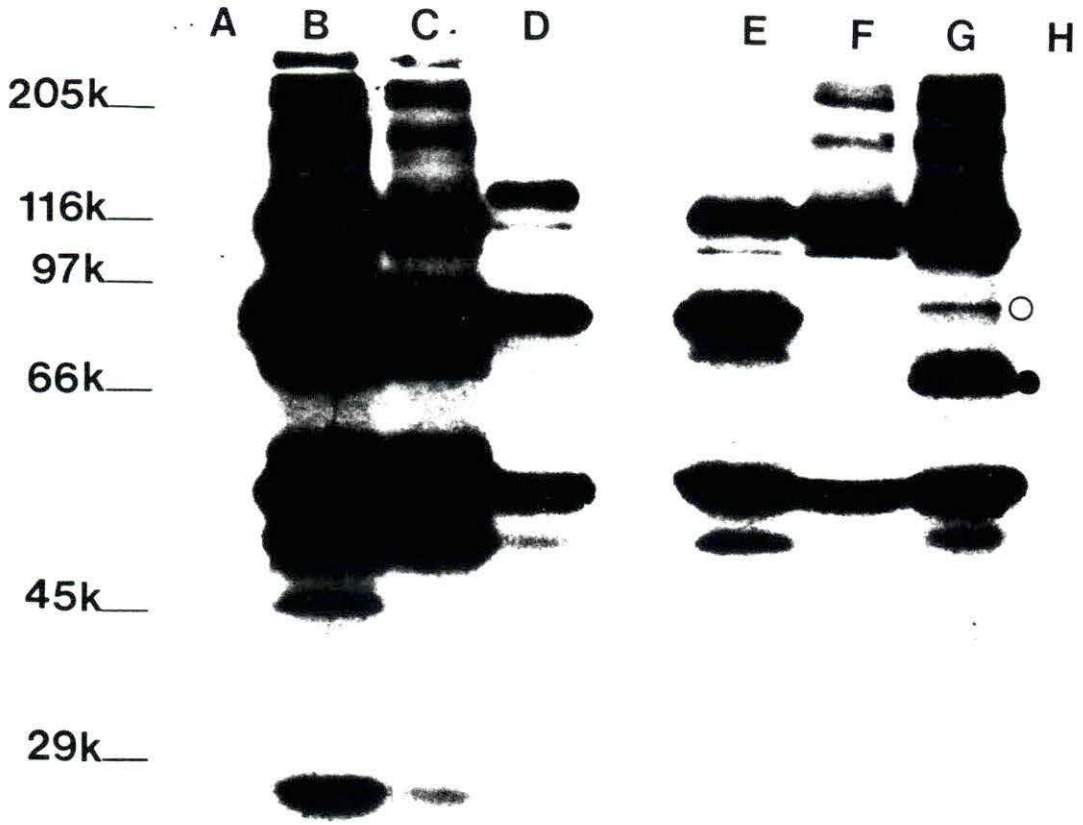

A
B
C
D
E
F
G
H
205k
116k
97k
66k
45k
29k

Figure 24. Autoradiograph following 120-hour exposure. RIP/SDS-PAGE analysis of virus-specified proteins following BVDV-infection of bovine BTU and BEC. In lane G, ( ○ ) denotes 92.2 kD polypeptide and ( ● ) denotes 72.4 kD polypeptide

Lane A - BTU, mock-infected control
Lane B - BTU, C24V-infected
Lane C - BEC, C24V-infected
Lane D - BEC, NADL-infected
Lane E - BEC, Singer-infected
Lane F - BTU, NY1-infected
Lane G - BEC, NY1-infected
Lane H - BEC, mock-infected control


A
B
C
D
E
F
G
H
205k
116k
97k
66k
45k
29k

has been observed in NADL-infected fetal bovine testicle cells (FBT) (Donis and Dubovi 1987b) and reflects the stability (across laboratories) of the BVDV genotype and the reliability of the RIP protocol used in this research.

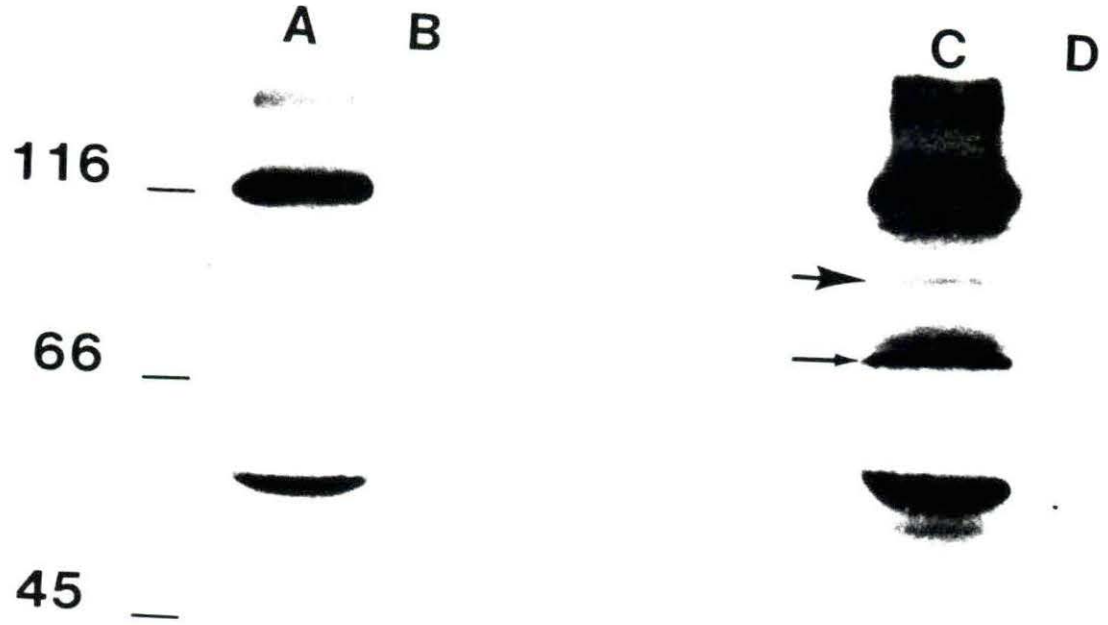
The 89.1 kD vp 2 identified in CP-BVDV-infected cell lysates is within the approximate range of 87 kD reported for vp 2 resolved from NADL-infected calf testis cells (Pocock et al. 1987). This Mr, however, is not within the acceptable Mr range of 80 kD observed by others (Akkina 1982, Purchio et al. 1984, Donis and Dubovi 1987a, 1987b). This disparity may be a function of virus strains, immunosorbents, cell types, and potentially, virus-cell interactions.

Lysates from NY1-infected BTU and BEC, following RIP and resolution by SDS-PAGE, were markedly different (Figures 22-24, Lanes F and G; Figure 25, Lanes A and C). Seven NY1-specified polypeptides were resolved from infected BEC with Mr of 117.5, 109.6, 92.2, 76, 72.4, 56.2, and 52.5 kD. Out of these seven NY1-polypeptides, five were identified in NY1-infected BTU lysates and appeared to be identical in Mr, with 117.5, 109.6, 76, 56.2, and 52.5 kD bands resolved by SDS-PAGE.

The 92.2 kD and 72.4 kD polypeptides peculiar to NY1-infected BEC (highlighted in Figures 22-25) were not resolved or were present in minute quantities in NY1-infected BTU. A separate RIP/SDS-PAGE was performed to confirm this observation (Figure 25). The differences between similarly infected cell types were obvious after a 24-hour autoradiograpahic exposure of the gel (Figure 25). Further, overexposure of an RIP autoradiograph for 120 hours did not dramatically improve the resolution

Figure 25. Autoradiograph following 24-hour exposure. RIP/SDS-PAGE analysis of virus-specified proteins following BVDV-infection of bovine BTU and BEC. In lane C, large arrow (➡) denotes 92.2 kD polypeptide and small arrow (⟶) denotes 72.4 kD polypeptide

Lane A - BTU, NY1-infected
Lane B - BTU, mock-infected control
Lane C - BEC, NY1-infected
Lane D - BEC, mock-infected control


A
B
C
D
116
66
45

of these two proteins from NY1-infected BTU lysate (Figure 24, Lane F).

The presence of 92.2 and 72.4 kD polypeptides in NY1-infected BEC-cell lysates has not been reported. Donis and Dubovi (1987b) observed a 90 kD polypeptide specific for NCP-BVDV isolates grown in FBT. This 90 kD protein was not observed in CP-BVDV isolates used in their study, and further, the polypeptide resolved from NY1-infected FBT-lysate migrated as a "doublet" (Donis and Dubovi 1987b). These observations were not confirmed from the RIP analyses conducted in this research. The 92.2 kD polypeptide migrated as a distinct "singlet" (Figure 25, Lane C).

The strength of the signal generated by the 72.4 kD polypeptide (Figure 25, Lane C) suggests a significant incorporation of L-(35-S)-methionine during virus replication. Whether this polypeptide was synthesized de novo or represents a product from post-translational processing of the larger polypeptides remains open to speculation.

Behaviorial differences of NY1 in BTU compared to BEC is suggestive of the presence of proteases in BEC, either peculiar to the cell type or specified and induced during virus infection. The consistency observed between polypeptide profiles of C24V-infected BTU and BEC, suggests that virus-induced changes are strain dependent. The disparities in polypeptide profiles in this research compared to published observations may also be due to differences in immunosorbents and cell types used in the various published RIP protocols.

The stability of the virus genotype should preclude speculation on strain variation in comparing RIP data for NY1. The resolution of a 92.2 kD polypeptide from infected BEC, compared to the resolution of a 90 kD

band in NY1-infected-FBT (Donis and Dubovi 1987b), suggests that the similar polypeptide profile resolved from heterogeneous cell types may be the result of a significant sub-population of endothelial cells in primary FBT cultures.

The Mr of the NY1-vp 2, 92.2 kD, within reasonable limits, approximates the 89.1 kD vp 2 resolved from the CP-BVDV isolates. The presence of both polypeptides in NY1-infected BEC-lysates suggests a virus-cell interaction not occurring in BTU following NY1-infection.

A summary of the results from the various protocols is presented in Table 2.

Table 2. Results

| Cell/ Virus | Extracellular mean virus titer from 8-12 HPI | Cytotoxicity assays[a] | | | RIP/SDS-PAGE polypeptides | |
|---|---|---|---|---|---|---|
| | | MTT | 51-Cr | FA | Number | Unique kD |
| BTU/C24V | 2.6 TCID-50/ml | -[b] | +[c] | + | 8 | 89.1 |
| BEC/C24V | 3.3 TCID-50/ml | + | + | + | 7 | 89.1 |
| BEC/NY1 | 3.0 TCID-50/ml | - | - | + | 7 | 92.2<br>72.4 |
| BTU/NY1 | 2.1 TCID-50/ml | - | - | - | 5 | - |

[a]Cytotoxicity assays: MTT = tetrazolium ring cleavage assay, 51-Cr = chromium-uptake assay, and FA = direct immunofluorescence assay for BVDV antigens.

[b]indicates assay did not detect cytotoxicity.

[c]indicates assay did detect cytotoxicity.

# CONCLUSIONS

Growth curves, cytotoxicity assays, and RIP/SDS-PAGE techniques for the resolution of BVDV-specified polypeptides were used to determine the permissiveness of bovine cell cultures to cytopathic and noncytopathic strains of BVDV. From the results summarized in Table 2, the observations from this research indicate that the NY1-strain of NCP-BVDV grown in bovine endothelial cells did not parallel the virus' behavior in bovine turbinate cells. Further, the observations suggest that NY1 showed tendencies of inducing CPE in endothelial cells that were similar to the characteristic CPE associated with CP-BVDV, strain C24V.

These findings suggest that this noncytopathic strain of BVDV may be interacting with the endothelial cell in such a manner that culminates in the expression of viral cytopathogenicity.

While previous research has provided valuable information on BVDV replication behavior and polypeptide production, this research suggests that the observations need to be documented and interpreted for well-defined cell populations. The advances made in tissue culture techniques, enabling the harvest and propagation of specific cell types, should permit the conduct of research to specifically define virus behavior.

The logical progression in the continuation of this research should be to define the behavior of other BVDV strains, both CP- and NCP- in specialized cell populations, such as the endothelial cell.

Secondly, examination of the role of BVDV (particularly NCP-strains) in the pathogenesis of bovine reproductive and respiratory disease should be renewed with emphasis on virus-cell interactions within target systems.

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