Studies on viral polypeptide processing by bovine viral diarrhea virus

by

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INTRODUCTION

As a single-stranded RNA virus, bovine viral diarrhea virus (BVDV) is a member of the genus pestivirus, family Togaviridae. Other members of the pestivirus genus are hog cholera virus in swine and border disease virus in sheep. Therefore, those three viruses are structurally and serologically related (Horzinek, 1981).

Olafson (1946) in the United States and Childs (1946) in Canada reported previously unknown transmissible disease patterns related to BVDV infection in cattle. Clinical signs included anorexia, depression, salivation, pyrexia, leukopenia, a watery or hemorrhagic diarrhea, and abortion among pregnant cattle. Subsequently the disease was identified as BVDV infection.

Thereafter, BVDV has been recognized as a causative agent in respiratory, gastrointestinal, and reproductive disease among domestic cattle. This virus has been implicated in causing persistently infected animals through BVDV infection of the fetus during the first four months of gestation, and thus, asymptomatic, persistently viraemic calves are susceptible to superinfection with a heterogeneous strain of BVDV, resulting in mucosal disease (Horzinek, 1990). Recently, it has been shown that young children under 2 years old who suffered with gastroenteritis have been infected with an agent associated with BVDV (Yolken et al., 1989). Previous *in vitro* research on BVDV, characterizing strains of BVDV with respect to viral protein processing has used various bovine cell types including vascular endothelial cells. While providing information about the basic features of viral protein synthesis in bovine endothelial cells,

the early work has not adequately identified viral polypeptides of noncytopathic BVDV strains. Reports on the biological aspects of BVDV infected vascular endothelial cells have been scant in the literature to date.

This research was conducted to: 1) identify production of a vp2-like viral polypeptide of several strains of BVDV in bovine endothelial and turbinate cells, 2) provide information about influence of BVDV infection on polymorphonuclear (PMN) leukocyte adherence to the endothelial cell, and 3) examine the role that cells play in the expression of BVD virus-induced cytopathology.

LITERATURE REVIEW

Bovine viral diarrhea virus (BVDV) is a non-arthropod-borne pestivirus of the family Togaviridae. Pestiviruses are the smallest enveloped RNA viruses and are about 40 nm in diameter. They possess a nucleocapsid of icosahedral symmetry (Horzinek, 1981). However, because the virus has several genetic features similar to the Flaviviridae, the new idea that BVDV should be classified as a flavivirus holds much attraction to molecular genetists. Through the production of monoclonal antibodies and molecular clones of BVDV, the diversity of BVDV strains has been demonstrated, together with a better understanding of viral polypeptide expression and processing in host cells. Reflecting this diversity, BVDV infection may result in an acute or chronic enteritis (upper alimentary tract erosions and ulcers, necrosis of gut associated lymphoid tissue including Peyer's patches), congenital deformities, abortions, immunosuppression, and persistently infected animals. Further, bovine viral diarrhea virus has been associated with the development of the bovine respiratory disease complex or shipping fever pneumonia. This review has been focused on the molecular aspects, target cell cytopathology and respiratory pathogenesis of BVDV.

Molecular Characteristics of BVDV

Collett et al. (1989) updated previous reviews, emphasizing the molecular aspects of BVDV that have been studied. Purchio et al. (1983) found several features of the BVDV genome synthesized in bovine cells to be similar to the

flaviviruses. They observed that the resolved 8.2 kilobase (kb) viral genome did not contain a polyadenylated (polyA) 3'-end and that there was no apparent subgenomic RNA for the BVDV; one viral-RNA species serves as message for structural and nonstructural proteins.

Renard et al. (1985) confirmed Purchio's finding on the lack of the 3'-polyA tail, and further, molecular cloning techniques used in the analysis of viral genomic sequences demonstrated a viral genome representing 12.5 kb. Purifying the viral RNA from BVDV (Osloss strain) infected cells and using this RNA as a template for complementary DNA (cDNA) synthesis, they showed that the nucleic acid of BVDV was a single RNA-species of 12.5 kb.

The size discrepancy of BVDV-RNA was corrected by further detailed work. Determining the nucleotide sequence of cloned BVDV-RNA genome, Collett et al. (1988b) reported that the cloned sequence for the RNA of the NADL strain was 12,573 nucleotides in length, having a molecular weight of 4.3×10^6 daltons, and a base composition of 32.2% A, 25.7% G, 22.1% U, and 20.0% C. Further, the authors found that a single open reading frame of the viral-sense (positive polarity) sequence encoded 3,988 amino acids, representing 449 kilodaltons (kDa) of viral protein. Sequence comparison revealed a high degree of homology between hog cholera virus (HCV) and BVDV genomic RNAs. The HCV genome consisted of 12,284 nucleotides containing a single open reading frame (Stark et al., 1990).

Studies using cytopathic (CP), and noncytopathic (NCP)-strains of BVDV indicate that the expression or the lack of cytopathology serves as a genetically stable marker for a particular strain (Horzinek, 1981). Akkina (1982) observed

differences in protein profiles between two BVDV biotypes. For a NCP-BVDV, New York 1 (NY1) isolate, he noted a marked reduction in the quantity of the 80 kDa protein compared to protein profiles from CP-BVDV strains (NADL and Oregon C24V).

A host genome insertion representing an ubiquitin-like polypeptide was found in the sequence coding for the p120 polypeptide of CP-BVDV (Osloss strain) (Meyers et al., 1989). A different insertion of 270 nucleotides found in this same polypeptide of another CP-BVDV (NADL strain), was confirmed as a host cellular mRNA. Both insertions are in the genomic region coding for the nonstructural protein p120 which is cleaved into a p80 protein only in cells infected with CP-BVDVs (Collett et al., 1988a). Because ubiquitin itself acts as an efficient signal for protein processing, it is possible that cleavage of the p120 protein is influenced by the insertions. Meyers and co-workers suggested a novel model for pathogenesis of mucosal disease in which a NCP-BVDV in a persistently infected animal changes to a CP-BVDV by taking up cellular sequences during a recombination event. Altered viral pathogenicity may be linked to the presence of these insertions (Meyers et al., 1990). Particularly, an alteration from NCP-BVDV to CP-BVDV is one trait of expected pathogenic enhancement in BVDV infections (Horzinek, 1990). During adaptation of apathogenic influenza virus in the host cell, an infectious pathogenic variant was produced by insertion of the 28S host ribosomal RNA into the haemagglutinin (HA) gene. This insertion may affect increased cleavability of HA, infectivity, and pathogenicity of the adapted virus (Khatchikian et al., 1989).

Moerlooze et al. (1990) characterized the gene encoding the carboxy-terminal portion of the 120 kDa BVD viral polypeptide, using the polymerase chain

reaction (PCR). However, through this study they could not recognize notable insertions in this portion with 8 pestiviral strains employed, with exception of the two BVDVs mentioned previously. The amino acid sequences of the 120 kDa viral polypeptide contain a highly conserved, cysteine-rich region similar to binding domains of gene-regulatory proteins. Thus, Moerlooze et al. (1990) proposed that this peptide might be involved in viral RNA binding.

Synthesis of Specific BVDV Polypeptides

Dubovi (1990) has reviewed morphological and biochemical aspects of the synthesis of BVDV specific polypeptides. Chasey and Roeder (1981) observed budding tubules and tubular networks from distended regions of the rough endoplasmic reticulum in an electron microscopic study of BVDV-infected bovine turbinate cells and found virus-like particles within unmodified ER cisternae and vacuoles. Their findings were consistent with flavivirus morphogenesis. In electron microscopic observation of bovine tissues and cell cultures infected with a Danish strain of BVDV (UG-59), Ohmann and Bloch (1982) observed that virions were assembled in vesicles with smooth membrane; however, no evidence of a budding process during maturation of the virus was detected on cytoplasmic or surface membranes.

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). Gray and Nettleton (1987) reported different findings, regarding the classification of the BVDV. From their ultra-structural study of BVDV-infected cell cultures, infected cells displayed modified ER-tubules, and virus replication appeared to be associated with the ER. 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.

Using co-electrophoresis techniques to differentiate ^{3}H-labeled virus proteins from contaminating ^{14}C-labeled host cell proteins, Pritchett and Zee (1975) reported four major viral peptides of BVDV, NADL strain, with molecular weights of 93-110 kDa, 70 kDa, 50-59 kDa, and 25 kDa. Radiolabeled, sucrose-gradient purified, and immunoprecipitated BVDVs (NADL and Oregon C24V) were analyzed on sodium dodecyl sulfate-polyacrylamide gels. Peptides with molecular weights of 57 kDa, 44 kDa, and 34 kDa were resolved and further analysis with ^{3}H-fucose revealed that the 57 kDa and 44 kDa peptides were glycoproteins (Matthaeus, 1979).

Coria et al. (1983) identified four major viral proteins with the cytopathic Singer isolate of BVDV grown in bovine turbinate cells. Their techniques which concentrated and purified virus, kept viral envelope intact. Four major proteins with apparent molecular weights (Mr) of 75, 66, 54, and 26 kDa were detected, and further, the 75 and 54 kDa proteins were found to be glycoproteins by dansyl hydrazine staining.

Purchio et al. (1984) radioimmunoprecipitated BVDV-infected cell lysates and analyzed samples by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Viral peptides of BVDV, NADL strain migrated with Mr of 115, 80, 55, 45, and 38 kDa. This group used two-dimensional electrophoretic analysis of polypeptide digests to show structural relatedness between the 115 kDa and 80 kDa peptides which were likely to be nonstructural peptides. No precursor-product relationship between these two polypeptides was established with pulse-chase experiments.

Pocock et al. (1987) reported viral proteins of BVDVs grown in bovine testicle cells using radioimmunoprecipitation/polyacrylamide-gel-electrophoresis (RIP/PAGE) analysis. The RIP/PAGE of CP-BVDV (NADL strain) infected cell lysates resolved eight proteins with Mr of 120, 87, 69, 57, 49, 37, 33, and 23 kDa, while the two isolates of CP-BVDV showed similar polypeptide migration patterns with minor differences. Among the NCP-BVDV isolates the 87 kDa protein was absent. Further analysis with ^{3}H-glucosamine radio-labeling showed that the 69, 57, 49, and 23 kDa proteins were glycoproteins. They also revealed that bovine antiserum against a NCP-BVDV isolate could immunoprecipitate the 87 kDa protein from CP-BVDV-infected cell lysates. Conversely, antiserum against a CP-BVDV isolate did not immunoprecipitate the 87 kDa protein in the NCP-BVDV infected cells. Induction of the 87 kDa protein by NCP-BVDV has not been reported, however, the structural relatedness between the 120 and 87 kDa proteins suggests potential antigenic relatedness.

The hypertonic initiation block is a technique that establishes the direct visulalization of virus-specific proteins. Under hypertonic conditions, cellular protein synthesis is specifically inhibited while the viral translation machi-

nery in host cells remains intact (Nuss et al., 1975). Donis and Dubovi (1987a) studied specific BVDV polypeptides in infected cells using a hypertonic initiation block and SDS-PAGE. Twelve proteins with Mr of 165, 135, 118, 80, 75, 62, 56-58, 48, 37, 32, 25, and 19 kDa were identified from the Singer-strain of BVDV grown in infected fetal bovine testicle cells.

Further expanding their initial observations, Donis and Dubovi (1987b) confirmed the sizes of BVDV specific polypeptides with several CP- and NCP-strains of the virus. Both of the most abundant proteins, 80 kDa and 118 kDa, were detected in CP-BVDV-infected cells. However, the 118 kDa protein without a 80 kDa protein, was the most abundant protein recovered from NCP-BVDV-infected cell lysates. All NCP-BVDVs synthesized 75 and 90 kDa peptides in the study.

Examination of the published values for the Mr of vp2 shows a discrepancy between the authors utilizing RIP with CP-BVDV-infected cell lysates. Akkina (1982) and Donis and Dubovi (1987a, 1987b) have reported a vp2 with Mr of 80 kDa, while Pocock et al. (1987) resolved a vp2 of 87 kDa. As this vp2 is widely employed to differentiate between CP- and NCP-BVDV, an accurate Mr value has been defined as 80 kDa (Collett et al., 1989).

The BVD viral polypeptides are described as arising by co- and post-translational proteolytic processing (Collett et al., 1988a). The authors expressed short fragments of molecularly cloned BVDV genome (NADL strain) as β-galactosidase fusion peptides in Escherichia coli and prepared a panel of sequence-specific antisera against expressed peptides. Explaining about 83% of the virus coding sequence with the viral gene products, they described 3 precursor polypeptides (118, 125, and 133 kDa) and a 20 kDa peptide from a

single open reading frame of BVDV. Further, the 118 kDa peptide which is glycosylated is cleaved into two smaller polypeptides, gp62 with further cleavage to gp38 + gp25, and gp53. The BVDV glycoproteins gp53 and gp25 revealed a distribution of cysteine residues and a linkage of disulfide bridges which was identical to that of hog cholera virus (HCV) gp55 and gp33 (Weiland et al. 1990). The125 kDa precursor encompassed peptides of p54 and p80. The 133 kDa polypeptide consisted of the proteins, p58 and p75 (Collett et al., 1988a).

Applying site specific antisera, Akkina (1990) mentioned that two large unreported precursor polypeptides of Mr 170 and 172 kDa surround previously described precursor polypeptides of Mr 125 and 133 kDa, respectively. He identified three new proteins of 168, 96, and 72 kDa encoded by the last third of the genome, suggesting the presence of an alternative cleavage pathway of the precursor polypeptide 172 kDa in proteolytic processing of BVDV (Oregon C24V) infected cells.

On autoradiographs of RIP/SDS-PAGE from bovine endothelial cells (BEC) infected with BVDV, Johnson (1988) found that these host cells could induce a previously unreported viral protein processing. Eight BVDV-specific peptides were resolved from Oregon C24V (C24V)-infected BTU with Mr of 117.5, 109.6, 89.1, 76, 58-56.2, 52.5, 46, and 27.5 kDa. Seven peptides were identified in C24V-infected BEC, since the 46 kDa peptide was not detected. Lysates from NY1-infected BTU and BEC were obviously different. Seven NY1-specified peptides were resolved from infected BEC with Mr of 117.5, 109.6, 92.2, 76, 72.4, 56.2, and 52.5 kDa, however, five peptides were identified in NY1-infected BTU lysates with Mr of 117.5, 109.6, 76, 56.2, and 52.5 kDa. The author proposed that peptide differences of NY1 in BTU compared to BEC were suggestive of the presence of

proteases in BEC during virus infection (Johnson, 1988). These proteases would be responsible for the appearance of 92.2 kDa and 72.4 kDa peptides in NY1-infected BEC.

Virus-encoded glycoproteins on the outer surface of the virion play an important role in host infection and represent basic targets for host immunity because a specific cell surface receptor mediates entry of BVDV into bovine cells (Moennig, 1988). Bolin (1988) detected neutralizing and non-neutralizing antibodies in cattle persistently infected with NCP-BVDV after vaccination with modified live virus (MLV)-BVDV. Neutralizing antibodies reactive against the 53 kDa peptide were detected at 3 weeks after vaccination and non-neutralizing antibodies reactive with peptides of 115, 80, and 47 kDa at 7-12 weeks post-vaccination. However, in healthy cattle, precipitating antibodies to MLV-BVDV vaccine were induced to polypeptides of 115, 80, 56, 48, 39, and 25 kDa at 2 weeks after vaccination and to 61 and 37 kDa at 6 weeks post-vaccination (Bolin and Ridpath, 1989). The production of precipitating antibodies to the MLV-BVDV vaccine was delayed in PI cattle, when compared to healthy cattle, which was suggestive of suppressed immune response in the PI cattle.

Hybridomas secreting monoclonal antibodies (MAbs) specific for BVD viral peptides were used to characterize the antigenic diversity of the neutralizing epitopes on the virion (Dubovi et al., 1986). Moennig et al. (1987) raised MAbs against several strains of BVDV and HCV. Some neutralizing MAbs reacted with antigenic determinants on the nonstructural proteins p115/p80 and gp57 of BVDV. According to their reactivity, they categorized their MAbs into 3 groups: a) reactive with various isolates or strains of different pestiviruses without a distinct pattern, b) reactive with either BVDV or HCV, and 3) reactive with all

pestiviruses tested. Corapi et al. (1990) produced 40 MAbs specific for BVDV and determined the relatedness of viral-induced proteins in bovine testicle cells infected with the Singer strain of BVDV. Group-1 MAbs recognized proteins with molecular sizes of 80 and 118 kDa. Group-2 MAbs identified proteins with 54, 56, and 58 kDa, while Group-3 recognized proteins with 43 and 65 kDa. Through these works, they confirmed that a high degree of antigenic variation is found among the envelope glycoprotein.

Endothelial Cells and Virus Infection

The cardiovascular system of blood-transporting vessels is lined by the endothelium which is a single layer of mesodermal origin, called endothelial cells. Structural and functional differences exist between endothelial cells originated from various organs (Bar et al., 1982).

Macarak et al. (1977) observed that BEC obtained from bovine aortas show 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 (Factor VIII:RAg or Factor VIII). The Factor VIII is a glycoprotein complex and its major component is detected as a 200-225 kDa disulfide-linked structure (Jaffe, 1982). Most normal cultured mammalian endothelial cells, including those of bovine origin express this protein. The macrostructure of Factor VIII newly synthesized by endothelial cells varies from that found in plasma or culture medium. Newly synthesized Factor VIII is intracellularly detected as a 240 kDa glycoprotein precursor, while soluble Factor VIII is generally detected as a 225 kDa moiety (Lynch et al., 1982).

The factors which regulate the synthesis and release of Factor VIII are poorly understood. Under *in vivo* conditions, exercise, adrenergic stimulation, growth hormone, steroids, thyroid hormone, and several disease conditions are associated with rises in Factor VIII. Growth hormone, thyroid hormone, and steroids did not enhance Factor VIII expression *in vitro* (Jaffe, 1982). However, estradiol and thrombin were observed to increase the release and accumulation of Factor VIII by cultured human endothelial cells (Harrison and McKee, 1981; Levine et al., 1981).

Andrews et al. (1978) studied whether the human dengue virus, a flavivirus, and the human Junin virus, an arenavirus, could replicate in cultured rabbit and human endothelial cells. They reported that these two viruses replicated in endothelial cells without the induction of cytopathology. In contrast, both viruses produce severe hemorrhagic syndromes in humans, and the authors suggested that viral replication in endothelial cells triggered cellular and humoral effector mechanisms that would produce vascular pathology, including intravascular coagulation and capillary fragility. Examining endothelial cells of human umbilical veins and bovine aortas for permissiveness of infection by 11 human viruses, Friedman et al. (1981) reported that some viruses caused acute cytolytic changes while others produced chronic alterations or no apparent changes. They also suggested that viral replication within endothelium may play an important role in the pathogenesis of viral disease or initiation of vessel wall injury. Johnson (1988) described a transient cytopathology in BEC infected with a NCP-BVDV (NY1 strain), and confirmed the noncytopathogenic nature of this strain in bovine turbinate cells.

Focal adhesion of polymorphonuclear (PMN) leukocytes to the blood vessel lining is an important process in inflammation and certain vascular disease processes (Bevilacqua et al., 1989). Incubating PMN leukocytes with supernate from virus-infected endothelial cells increased PMN adherence to noninfected endothelial cells and this PMN adherence was independent of complement and antibody activity (MacGregor et al., 1980). Under *in vivo* conditions, the PMN cells adhered to the endothelium may diapedese, infiltrate at the perivascular area, degranulate, and release lytic enzymes. Virus-infected endothelium appeared to synthesize and release substances that may cause granulocytopenia. This fall in the clinical granulocyte count represents PMN redistribution rather than an actual loss of PMN (MacGregor et al., 1980).

Enhanced human peripheral blood PMN adherence to human umbilical vein endothelial cells (HEC) was mediated by lipopolysaccharide, tumor necrosis factor (TNF), and interleukin 1 (IL-1) (Pohlman et al., 1986). This effect was dependent upon synthesis of a surface epitope in primary and passaged HEC, and was not detected in bovine aortic endothelial cells or human dermal fibroblasts (Pohlman et al., 1986).

Endothelial-leukocyte receptors are classified in three groups: the selectins or LECCAMs (for their homology to lectins, epidermal growth factor, and complement regulatory proteins), the immunoglobulin (Ig) supergene family, and the integrin family (Osborn, 1990). Using monoclonal antibodies to identify a surface receptor for neutrophils on cytokine-stimulated endothelium (Pohlman et al., 1986; Bevilacqua et al., 1989), a cell surface glycoprotein of 96 kDa was described. This molecule mediates leukocyte adhesion, and was designated endothelial-leukocyte adhesion molecule 1 (ELAM-1) (Bevilacqua et

al., 1989). The primary ELAM-1 sequence represents an aminoterminal lectin-like domain, an epidermal growth factor domain, and 6 tandem repetitive motifs similar to those of complement regulatory proteins (Bevilacqua et al.,1989). Focal expression of ELAM-1 at endothelial cell activation sites promotes neutrophil adhesion and emigration (Bevilacqua et al., 1989). The granule membrane protein (GMP 140) found on platelets and endothelium, which also belongs to the LECCAM family, functions in adhesion of PMNs and monocytes to endothelium.

The second group of inducible endothelial molecules, intercellular adhesion molecules (ICAMs) of the Ig supergene family, which recognize β2 integrin, have been associated with both neutrophil and lymphocyte adherence *in vitro* (Bevilacqua et al., 1989; Osborn, 1990). Unlike ELAM-1, ICAM-1 is expressed on multiple leukocytic and non-leukocytic cell types, while ICAM-2 is expressed on endothelial cells. The ICAMs are associated with lymphocyte-fibroblast adhesion and lymphocyte aggregation (Bevilacqua et al., 1989; Osborn, 1990). Incubation of cultured endothelial cells with IL-1, TNF, and endotoxin results in an increased ICAM-1 expression. ICAM-1 expression is more constant over a 48 hour period than that recorded with ELAM-1, and this expression may explain why ICAM-1 plays a role in chronic inflammation processes (Bevilacqua et al., 1989). Vascular cell adhesion molecule 1 (VCAM-1), a member of Ig supergene family on activated endothelial cells recognizes its leukocyte ligand, β1 integrin, and enables the adhesion and migration of lymphocytes and monocytes (Osborn, 1990).

Increased PMN adherence may promote cellular damage and vasculitis due to PMN degranulation (MacGregor et al., 1980; Friedman et al., 1981). The

harmful activity of neutrophils which induce vascular injury and edema in acute inflammation has been described (Issekutz, 1984). The phagocytic and biodegradative mechanisms of the neutrophil can foster host defence, but create lung damage (Janoff et al., 1979; Harlan et al., 1981). As an example, neutrophils appear to be important in lung damage due to pneumonic pasteurellosis because neutrophil-depleted calves infected with Pasteurella haemolytica had reduced lung lesions (Slocombe et al., 1983).

In immunohistological studies of BVDV-infected bovine tissues, Meyling (1970) demonstrated BVD virus-infection of the endothelium, the tunica media, and adventitia of blood vessels. Studying the distribution of BVDV-antigens in virus-infected bovine cells, Ohmann (1983) observed that viral antigen was associated with endothelial cells lining the blood vessels, and the predominant antigen-positive cells were cells of the mononuclear phagocyte system. The distribution of BVDV-antigen in lymphoid tissues corresponded with the localization of "antigen-presenting-cells" (APC), suggesting that the pathogenesis of BVDV infection involves strong interaction of the virus with 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, control the proliferation of T-lymphocytes, and release immunoregulatory molecules, such as interleukin-1 and prostaglandin E-2 (Roska et al., 1984; Wagner et al., 1984; Cotran, 1987).

Lymphocyte migration into lymph nodes and into mucosa-associated lymphoid tissue is regulated by specialized postcapillary high endothelial venules (HEVs). In immuno-peroxidase assays using a monoclonal antibody specific to a human endothelial cell differentiation antigen, HEVs in lymphoid

organs were intensely stained while vascular endothelium in normal non-lymphoid tissue remained unstained. Inflammation is a stimulus for the differentiation of vascular endothelial cells into specialized postcapillary HEVs, and the development of HEVs may occur in inflammatory sites of increased lymphoid infiltration (Duijvestijn et al., 1988).

Clinical Aspects of BVDV Infected Cattle

Bovine virus diarrhea (BVD) in cattle is manifested by a variety of clinical presentations such as an acute ulcerative enteritis, subclinical infection, congenital defects, abortions, chronic diarrhea, persistent infection, acute and chronic mucosal disease (Ramsey and Chivers, 1956), mixed respiratory disease, and immunosuppression. Reviews on the pathogenesis, immunological features, and strain association in BVDV infections have recently been presented (Baker, 1990; Brownlie, 1990; Horzinek, 1990; Howard, 1990).

General features of BVDV infection

The neonatal calf passively acquires anti-BVDV-immunoglobulins through the ingestion of colostrum. As these antibodies normally decay within three to eight months after birth, calves become seronegative for BVDV and are then susceptible to infection. Infection typically occurs via inhalation or ingestion of infected saliva, oculonasal discharge, milk, semen, and urine (Baker, 1990). Acute disease generally occurs in cattle from 6-24 months of age and the clinical signs of infection include pyrexia, leukopenia, depression, anorexia, oculo-nasal discharge, occasionally a watery to hemorrhagic diarrhea, and oral ulcerations. Adult, lactating cows may show decrease of milk production and

occasionally, BVDV-infected cattle develop high temperature and ulcerations or erosions of the interdigital area of the hoof, the teat epithelium and the muco-cutaneous junctions of the vulva or prepuce (Baker, 1990).

In an acute BVDV infection may be usually observed on the palate, tips of the buccal papillae, and the gingiva. Stretching into the alimentary canal, linear erosions or ulcerations of the esophagus, ulceration of the nonvillous portions of the rumeno-reticulum, 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 (Jubb et al., 1985). As a feature of acute BVDV infection, thrombocytopenia which resulted in clinical signs of bleeding has been reported, occurring as a result of BVDV-induced myelosuppression or bone marrow necrosis (Baker, 1990).

Observing microscopic lesions in collected tissues of clinically healthy, persistently infected cattle, Cutlip et al. (1980) detected BVDV antigen by direct immunofluorescence assays. Viral antigen was detected in brain, spinal cord, lymph nodes, spleen, and kidney. Histologically, kidney tissue was characterized by irregular thickening of the glomerular basement membrane, suggestive of glomerulonephritis which resulted from the deposit of immune complexes. In the sections of brain tissue mononuclear cell cuffing was observed around blood vessels, suggestive of encephalitis.

Effects on the developing fetus following BVDV-infection are influenced by the cytopathogenicity of the infecting strain of virus (CP- or NCP-) and the gestational age of the fetus at the time of viral infection (Baker, 1990). Trans-placental transmission of the BVDV in the seronegative dam is the major route

of infection of the fetus (Van Oirschot, 1983). According to the pathogenicity of virus strains and the stage of pregnancy, manifestations of BVDV infection consist of fetal resorption, mummification, abortions, congenital malformations, persistent infection, or a normal, seropositive calf. Infection during the first trimester (0-100 days of gestation) may result in stillbirths, mummification, and abortion. BVDV-infections of the fetus from 100-150 days of gestation are associated with congenital defects such as microencephalopathy, cerebellar hypoplasia, hydranencephaly, retinal atrophy, optic necrosis, cataract, arthrogryphosis, brachygnathism, growth retardation, thymic aplasia, pulmonary hypoplasia, hypotrichosis, and alopecia (Baker, 1990). Fetuses have developed immunocompetence in the later stages of gestation and typically exert an immune response to the virus and are born normal, seropositive to BVDV prior to ingesting colostrum (Baker, 1990).

Persistently infected cattle

Persistent infection with BVDV is established in the fetus during early pregnancy through transplacental infection. These PI calves may have pneumonia and enteritis (Baker, 1987) and are at risk of superinfection with heterologous BVDV strains. Done et al. (1980) experimentally infected 15 pregnant, BVDV-seronegative heifers with a mixture of CP-BVDVs at 100 days of gestation, and observed the pathogenicity of developing fetuses to the virus. Although none of the cows showed clinical syndrome, six fetuses died in utero (5 aborted, 1 mummified), and 10 fetuses survived to term with growth retardation. The recovery of NCP-BVDV from calves suggested contamination of the mixture

of CP-BVDV, or dams were persistently infected with virus at the time of experimental challenge.

McClurkin et al. (1984) experimentally produced PI-calves from sero-negative, pregnant cows exposed to NCP-BVDV during days 42-125 of gestation. The development of immunocompetence in the bovine fetus has been described to occur from 150-200 days of gestation, but immunotolerance to BVDV is an uncommon outcome at the 100-125 days of gestation (Baker, 1990). Clinical reproduction of BVDV infection was reported by Brownlie et al. (1984) and Bolin et al. (1985c) superinfecting NCP-BVDV PI cattle with a heterologous CP-strain of BVDV. Bolin et al. (1985b) described persistent BVDV infection in selected cattle herds and isolated BVDVs from 6 among 66 herds. In these herds 54 of 3,157 cattle (1.7%) were detected as persistently infected.

Virus isolations from cattle with acute and chronic forms of BVD revealed the presence of both NCP- and CP-BVD viruses in over 70% of spleens tested (McClurkin et al., 1985). The pathogenic steps of MD have been proposed as follow; 1)transplancental infection with NCP-BVDV, 2) the establishment of an immunotolerant fetus, 3) the birth of the PI calf, and 4) superinfection of the PI calf with CP-BVDV (Dubovi, 1986). Understanding the mode of exposure of the persistently infected bovine to CP-BVD viruses in the pathogenesis of the MD syndrome is a major objective of current study. In a supplementary study, persistently infected cattle inoculated with either MLV- or killed-CP-BVDV vaccine, detrimental findings were not manifested after vaccine inoculation. However, the vaccinated PI cattle were subsequently challenged with CP-BVDV six weeks after vaccination, and all steers developed clinical MD (Bolin et al., 1985d).

Persistently infected cattle are shown to be immunocompetent to infectious bovine rhinotracheitis (IBR) virus, parainfluenza type 3 (PI-3) virus and Pasteurella haemolytica. However, the affected cattle demonstrated a BVD-viremia and developed no or low levels of serum antibodies to the homologous strain of virus throughout their lives (McClurkin et al., 1984). Thus, the PI cow represents the major reservoir of BVDV for the bovine population (Dubovi, 1986; Baker, 1990).

Because the surveys on persistent infection of BVDV in bovine populations has been based on selected herds, the frequency of PI cattle reported may be inaccurate. A Danish slaughter survey showed a frequency of viraemic cattle of 0.9% and similar findings have been reported from the United Kingdom (0.8% for viraemic and 0.4% for persistent infection) and Germany (0.9%) (Meyling et al., 1990).

Mixed respiratory infection with BVDV

Bovine viral diarrhea virus has been noted as a causative agent of respiratory infections in cattle together with other pneumotropic organisms. Rossi and Kiesel (1977) observed that PI-3 virus, IBR virus, and BVDV readily destroyed ciliary function in tracheal ring organ cultures. In a study of bovine respiratory disease in feedlot cattle, BVDV was isolated from 21% of pneumonic lungs (Reggiardo, 1979). Simultaneous infection with BVDV and Pasteurella haemolytica was a typical finding. Seroconversion to both BVDV and P. haemolytica was found in 72.4% of the animals clinically diagnosed as pneumonic, and only in 11.2% of the control animals (Reggiardo, 1979).

Reggiardo and Kaeberle (1981) induced an endogenous bacteremia during the first 5 days after an experimental BVDV infection. Lopez et al. (1982) tested

whether preinfecting calves with BVDV or Mycoplasma bovis at the time interval of 3, 5, and 7 days before aerosol challenge with P. haemolytica impaired the pulmonary clearance of bacteria. Investigation was conducted on the mean clearance rate of P. haemolytica at three designated time intervals. No significant difference was observed on the effect of these two pathogens on the capacity of bovine lung to clear inhaled P. haemolytica. The role of BVDV in bovine respiratory disease remains unclear. Corstvet and Panciera (1983) reproduced clinical pneumonia and pulmonary lesions in BVDV inoculated calves, followed by an aerosol challenge of P. haemolytica at 12 days post-infection.

Potgieter et al. (1984a) also reported the effect of BVDV infection on the distribution of IBR virus in calves. Aerosol exposure with IBR virus increased the comparative distribution of recoverable IBR virus three to eight days after inoculation. In the control calves, IBR virus was recovered from the cranial and the caudal parts of the respiratory tract in much lower concentrations, while BVDV/IBR virus inoculated cattle showed a general distribution of IBR virus in tissues, with high concentrations (Potgieter et al., 1984a). From this work it was concluded that BVDV was synergistically involved with IBR virus in bovine respiratory disease.

Potgieter et al. (1984b) reproduced pneumonia in calves with endobronchial inoculation of BVDV, and sequential inoculation of P. haemolytica. After inoculation of BVDV alone or P. haemolytica alone, calves developed pneumonic lesions ranging from 2 to 7% of total lung volume and 15% of total lung volume, respectively. However, calves sequentially challenged with P.

haemolytica 5 days after BVDV inoculation developed severe fibrinopurulent bronchopneumonia and pleuritis ranging from 40 to 75% of total lung volume.

Barber et al. (1985) reported that during a two year clinical investigation of a BVDV outbreak in a dairy herd, the major cause of calf loss was pneumonia. The association of BVDV with bacterial bronchopneumonia was suggested by histological examination of pneumonic lungs. Further, calves born to cows infected with BVDV during gestation had a significantly higher death rate. Bovine viral diarrhea virus isolated from tissues of pneumonic calves and aborted fetuses was NCP-BVDV, whereas CP-BVDV was isolated from cases of clinical mucosal disease and from a hydrocephalic calf (Barber et al., 1985).

Potgieter et al. (1985) expanded their research to determine the difference of pneumopathogenicity with two biotypes of BVDV. Bovine viral diarrhea virus inoculated calves challenged sequentially by endobronchial inoculation with P. haemolytica showed impaired pulmonary clearance of bacteria. The CP-BVDV had more pneumopathogenic effects than the NCP-BVD virus isolate used. Lopez et al. (1986) examined the cellular inflammatory response in the lungs of calves exposed to BVDV, M. bovis, and P. haemolytica inoculation. Aerosols of P. haemolytica were given to calves at 3, 5, and 7 days post-inoculation with BVDV or M. bovis. Inoculation with BVDV or M. bovis did not have significant effects on the neutrophil/macrophage ratio in the bronchoalveolar lavage whereas P. haemolytica did. Inoculation with a cytopathic BVDV (Oregon C24V) did not induce gross or microscopic changes in pulmonary tissue. Histologic changes in small bronchi and bronchioles were well correlated with the cellular changes that follow exposure to P. haemolytica.

Interaction of BVDV with peripheral blood leukocytes and phagocytic cells of the respiratory system explains the potential synergism that could result from BVDV and other bovine pathogens. The exact role of BVDV in the pathogenesis of bovine respiratory disease remains unsolved due to the difficulties encountered in experimentally inducing clinical pneumonia after BVDV infection. Many diagnostic laboratories report that NCP-BVDV is usually recovered from bovine respiratory disease cases (the most frequent virus isolated) (Baker, 1990).

Immunosuppression by BVDV infection

Because of immunosuppressive effects, BVDV infection may increase the pathogenicity of co-infecting pathogens, such as IBR virus, PI-3 virus, coronavirus, rotavirus, Pasteurella spp., Salmonella spp., Actinomyces pyogenes, Coccidia, and helminths (Baker, 1990).

Affinity of the virus for leukocytes, even in subclinical infections, has been demonstrated by isolating BVDV from washed buffy coat leukocytes (Malmquist 1968). Truitt and Shechmeister (1973) showed that bovine peripheral blood macrophages and lymphocytes, from either immune or nonimmune animals, were permissive to BVDV infection and the addition of phytohemagglutinin (PHA) to BVDV-infected lymphocytes enhanced viral replication compared to unstimulated lymphocytes.

Bovine peripheral blood lymphocytes (PBL) from cattle infected with BVDV had inhibited blastogenic responses to PHA (Johnson and Muscoplat, 1973; Muscoplat et al., 1973a) and had impaired immunoglobulin secretion (Muscoplat et al., 1973b).

Rossi and Kiesel (1977) reported that macrophage alteration by BVDV infection was as important as impaired lymphocyte function during clinical infection because the macrophage is essential in the elicitation of immune response. Lymphocytes infected with BVDV have significantly depressed chemotactic responses (Ketelsen et al., 1979). 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.

In cattle challenged with BVDV, Roth et al. (1981) demonstrated a reduction in circulating PMN leukocyte populations and an inhibition of PMN degranulation was accompanied by functional defects in PMNs. Healthy, BVDV-seronegative cattle vaccinated with a MLV-BVDV showed similar changes in PMN numbers and function (Roth and Kaeberle, 1983). Toth and Hesse (1983) found that bovine alveolar macrophages would support BVDV infection and replication.

An immunosuppressive material which was released by bovine fetal lung cells inoculated with BVDV was reported. This material suppressed blastogenic response of PBL stimulated with concanavalin A. Because the addition of indomethacin to BVDV infected cells blocked this release, it was suggested that the immunosuppressive substances were probably involved in prostaglandin metabolism (Markham and Ramnaraine, 1985).

Bolin et al. (1985a) found that absolute numbers of circulating B- and T-lymphocytes declined in healthy cattle inoculated with a cytopathogenic isolate

of BVDV, and although the decrease lasted seven days post-exposure, all cattle recovered and neutralizing antibody was detected later.

It has been shown that PMN function of cattle persistently infected with BVDV differed from that of healthy cattle. Ingestion capability of PMN was significantly inhibited but random migration of these cells under agarose was normal (Roth et al., 1986).

Blood mononuclear leukocytes collected from PI cattle contained BVDV antigens. Infection with NCP-BVDV was demonstrated in about 4.4% of all mononuclear leukocyte cells, 5.4% of T lymphocyte-enriched, and 2.1% of B lymphocyte-enriched sub-populations of mononuclear leukocytes (Bolin et al., 1987). By analyzing the cellular association of BVD virus with a flow cytometer, the antigen was detected in the cytoplasm of mononuclear leukocytes (Bolin and Ridpath, 1990; Qvist et al., 1990).

MATERIALS AND METHODS

Bovine Endothelial Cells

Cells from aorta

Bovine endothelial cells (BEC) were obtained by enzymatic digestion of isolated segments of calf thoracic aorta as described by Macarak et al. (1977), with the following procedural modifications (Johnson, 1988). 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. A ten centimeter segment of thoracic aorta was aseptically removed and placed in a transport media of Ca^{2+}, Mg^{2+} free-Hanks' balanced saline solution (CMF-HBSS) containing 1% PKS (penicillin 100 units/ml, kanamycin 10 µg/ml and streptomycin 10 µg/ml), 1% gentamycin (82 µg/ml), and 1% amphotericin B (10 µg/ml). Aortas were processed within one hour of harvest.

Following a rinse for 20 seconds in transport media plus 0.2% hypochlorite (Clorox), the aorta was rinsed again with transport media. The aortic 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 in parallel with the vessel opening. A volume of sterile 2.5% agar (56 ^{o}C) was carefully poured around the aorta and allowed to seal small vessels coming off the aorta. The 10 ml pipette was removed when the agar was firm.

The enzyme solution for endothelial cell removal was CMF-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 digestions were sufficient to obtain cells. Pooled 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% fetal bovine serum (FBS), F-15 MEM (minimal essential media, GIBCO) containing 1% PKS, 1% gentamycin, and 1% amphotericin B supplemented with 0.5% lactalbumin hydrolysate (LAH) and 50 µg/ml of endothelial cell growth supplement (ECGS; Collaborative Research, Inc.).

Prior to BEC seeding, the tissue culture flasks were precoated with bovine fibronectin (Sigma) 45-60 minutes before the initiation of the digestion. Supplier's recommendations were followed for proper product reconstitution and storage at -20 ^{o}C. After thawing, stock fibronectin was added to serum-free MEM (SF-MEM) at 10 µl/ml and flasks were precoated for 45 minutes at room temperature (RT). Fibronectin-MEM was discarded and precoated flasks were rinsed with SF-MEM prior to seeding with resuspended cell digests.

When BEC approached near confluency in flasks, cells were briefly treated with a solution of 0.05% trypsin and 0.02% sodium ethylenediaminetetra-acetate (EDTA) in calcium and magnesium-free saline. Cells were then dispersed with a disposable cell scraper. Cell suspensions were pooled and centrifuged at 200 x g for 5 minutes and cell pellets were resuspended in growth media and seeded into tissue culture flasks. Following the second to third passage, BECs were adequately grown in growth media without supplementing LAH or ECGS. Cultured BECs were determined to be endothelial cells by their

characteristic "cobblestone" morphology in tissue culture flasks and by positive staining in an indirect fluorescent antibody (IFA) assay for Factor VIII:von Willebrandt factor-related-antigen (VIII:vWF-RAg) with rabbit anti-Factor VIII serum undiluted (Calbiochem) and fluorescein isothiocyanate (FITC) conjugated sheep anti-rabbit serum (1:30 dilution, Cappel). Cells were screened for the presence of BVDV by direct FA assay. The protocol will be described under direct FA assay for BVDV contamination.

Cells from the pulmonary microvascular system

The procedure of Ryan et al. (1982) was employed with a modification, to isolate and culture pulmonary microvascular endothelium. Briefly, one calf was used as a source of endothelial cell. Heparin (10,000 units) was administered intravenously. The animal was killed by intravenous barbiturate overdose, and the skin resected over the thorax. The ribs were retracted, the lungs and the heart were removed, and then a ligature was placed around the pulmonary artery as far from the heart as possible and was tied tightly.

A short incision was made in the pulmonary artery just proximal to the ligature and a catheter (1/3 inch in diameter) was gently inserted into the artery and secured with a rubber band ligature. A small incision was made in the left auricle, through which a second catheter (1/3 inch in diameter) was inserted and was secured with a rubber band ligature. Calcium and magnesium free-Dulbecco's phosphate buffered saline (CMF-PBS), with penicillin (100 unit/ml), streptomycin (100 µg/ml) and gentamycin (50 µg/ml) at 37 ^{o}C was perfused through the pulmonary artery for approximately 20 minutes with proper flow rate (100 ml/minute) so that most red blood cells might be removed. It was

essential to reverse the flow through the catheters every 2-3 minutes to eliminate trapped blood. The PBS container was kept in a warm water bath while perfusing. Two peristaltic pumps were used for each direction of flow. Most blood cells were purged from the lung. The perfusion pump was then connected to a reservoir containing CMF-PBS with 0.02% EDTA and microcarriers (Cytodex I, Sigma, St. Louis, MO) at approximately 600 beads/ml. The suspension was maintained in an ice bath and shaked gently. The bead suspension was pumped via the pulmonary artery for 3 minutes at 30 ml/minute. Collections from the reverse flow were begun immediately at the same flow rate, and the direction of flow was reversed every 250 ml of harvest, using Dulbecco's PBS with EDTA. Containers for the bead suspension were siliconized prior to use (Sigmacote, Sigma).

The tubes containing the bead-cell suspension were centrifuged at 200 x g for 10 minutes. The resulting pellets were resuspended in 10 ml of Medium 199 (GIBCO) containing 20% FBS and 1% PKS, 1% gentamycin (82 µg/ml), 1% amphotericin B (10 µg/ml), 0.5% LAH, and 50 µg/ml ECGS. Bead-cell suspensions from each tube were transferred to fibronectin-precoated 48 well clusters (0.5 ml/well). Medium was not changed for 5 days so that most of the cells would migrate off the beads. Thereafter the cells were fed with medium 199 containing 10% FBS twice weekly and observed under the microscope. A monolayer was screened for Factor VIII antigen and BVDV contamination.

Bovine Turbinate Cell

Bovine turbinate (BTU) cells were kindly provided by the National Animal Disease Center, Ames, Iowa at passage level seven and grown in F-15 Eagle's

MEM, supplemented with 10% fetal bovine serum (McClurkin et al., 1974). Cells were screened for BVDV by direct FA, and for bovine Factor VIII by IFA.

The growth media for BTU consisted of F-15 Eagle's MEM, 1% PKS, and 10% v/v serum. Adventitious viruses had been screened or ultrafiltered by the supplier, and FBS was heat-inactivated for 30 minutes at 56 ^{o}C prior to use.

Viral Strains and Virus Titration

The CP-BVDV strain Oregon C24V (C24V) was grown in BTU cells in maintenance media (MM) consisting of Eagle's F-15 MEM with 1% PKS and 3% FBS. Virus was quantified in 96-well plates by observing the highest virus dilution that caused cytopathic effect (CPE) in 50% of target cells (BTU) (Truitt and Shechmeister, 1973). Titers of virus were calculated as 50% tissue culture infective doses per ml ($TCID_{50}/ml$) (Reed and Muench, 1938).

The NCP-BVDV strain New York 1 (NY1) was grown in MM in BTU cells. The 13 NCP-BVDVs isolated from commercial fetal bovine sera or respiratory disease cases are presented in Table 1. These isolates were further passaged twice in BTU cells to assure that no CPE was observed. The NCP-BVDVs were quantified as follows: After ten-fold dilutions of the virus (in 50 µl MM) were placed into microtiter wells, BTU cells (in 100 µl MM) were added. Plates were incubated at 37 ^{o}C in a humidified, 5% CO_2 atmosphere for 4 days, and then infected cells were challenged with 50 $TCID_{50}/ml$ of stock C24V virus. Uninfected 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 CPE typical of C24V at 3, 4, and 5 days post-challenge (Johnson, 1988). Titers were calculated using the 50%-endpoint method of Reed and Muench (1938) by

Table1. Strains of bovine viral diarrhea virus

Strain	Passage No.	Biotype	Year of isolation	Origin
16787	5	ncp*	1980	BRD***
12824	5	ncp	1980	BRD
14917	5	ncp	1980	BRD
80-14182	7	ncp	1980	BRD
80-14049	6	ncp	1980	BRD
NY1	16	ncp	1948	Acute
099	13	ncp	1983	FBS****
FCS031	4	ncp	1979	FBS
C23	4	ncp	1979	FBS
Hic154	4	ncp	1981	FBS
FC28	4	ncp	1979	FBS
A20	4	ncp	1979	FBS
FC21	4	ncp	1979	FBS
118GBK	4	ncp	1980	FBS
C24V	9	cp**	1959	Acute

*noncytopathic

**cytopathic

***bovine respiratory disease

****fetal bovine serum

observing the highest virus dilution that prevented CPE in 50% of the microtiter wells.

Indirect Fluorescent Assay for BVDV Contamination

Both cell types (BEC and BTU) were trypsinized from confluent cultures, resuspended and seeded into four-chambered slides (Labtek, Miles Laboratories) in appropriate growth media (Johnson, 1988) to test BVDV contamination. Virus control for each cell type was prepared as follows. Growth media was discarded when the cells formed a monolayer. Each chamber was rinsed with a 0.5 ml volume of warm SF-MEM. Cells were made to adsorb NY1-BVDV in a 0.5 ml volume of MM for 1 hour on a rocking platform at 37 ^{o}C. Multiplicity of infection for the virus was calculated to be approximately 1.3. 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 4 days after infection, slides were fixed in acetone for 7 minutes at 25 ^{o}C. Normal and infected cells were overlaid with anti-BVDV immune serum conjugated to FITC (anti-BVDV-reagent 8802, 1:40 dilution, NADC, Ames, Iowa) and incubated at 37 ^{o}C for 30 minutes in a humidified atmosphere. Slides were then treated with two, 5 minute rinses in phosphate buffered saline (PBS) and then dipped in distilled water and air-dried. Slides were mounted with 90:10 v/v, glycerol:PBS (pH 7.4) and viewed under a coverslip using a Leitz fluorescence microscope.

RIP/SDS-PAGE Analysis

Immunosorbent preparation

Johnson (1988) has established the procedure as follows. Briefly, cell lysates from BTU and BEC were prepared by growing cells in 75 cm^2 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 re-incubated 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 µl phenylmethyl-sulphonyl fluoride (PMSF)/10 ml HNE-buffer and 10 µl leupeptin/10 ml HNE-buffer.

Two 25 ml volumes of Affi-Gel 10 agarose beads (Biorad) were prepared according to 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 Affi-Gel slurry and incubating overnight at 4 ^{o}C on a rocker platform. Following binding, each gel-ligand was washed extensively with 0.1 M 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 ^{o}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, 0.01M Tris, pH 7.9, with 0.15 M NaCl and 0.02% NaN_3). 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 25 ^{o}C. Globulins were purified through each cell lysate by eluting with TBS and the eluant was recovered in 0.5 ml volumes for absorbance readings at 280 nm. Volumes of eluant 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 ^{o}C until used in the RIP procedure.

Virus lysate preparation

A modification of the method outlined by Akkina (1982), and Donis and Dubovi (1987a) was followed. Cells were seeded into 25 cm^2 tissue culture flasks in their respective growth media and allowed to reach confluency (48 hours) prior to infection; flasks were then rinsed three times with warm, SF-MEM. Cells were infected with BVDV 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 ^{o}C. At the end of 1 hour, infective medium 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 labeling, medium was removed from each flask and cells were rinsed three times with warm methionine-free MEM (met-free-MEM) and incubated for 30 minutes in a fresh volume of met-free-MEM. Each flask was added with L-^{35}S-methionine (15.0 mCi/ml, specific activity 1489 Ci/mmol; Amersham) in a 3 ml volume of met-free-MEM at a final concentration of 50 µCi/ml, and cells were incubated for 4 hours. At the end of labeling, 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 1,000 x g 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 0.5 ml of TNE-lysis-buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 5 mM EDTA) containing 1% Triton X-100, 20 µl PMSF (100 mM) per 10 ml of TNE buffer, and 10 µl leupeptin (1 mM) per 10 ml of TNE-buffer. This volume of TNE-lysis-buffer containing cells was aspirated and expelled through a disposable syringe with a 26G needle, and incubated for 3 minutes on ice. Resulting lysates were centrifuged for 30 minutes, at 14,000 x g and 4 ^{o}C to remove large molecular mass material. Supernatants were harvested and used immediately for immunoprecipitation.

Immunoprecipitation and SDS-PAGE

For immunoprecipitation of radiolabeled 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 (200 x g, 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 Affi-Gel 10 beads (BioRad) (Johnson, 1988).

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 mixture was incubated for at least 90 minutes at 4^{o}C on a shaker (Labquake, Labindustries, Berkeley, CA). At the end of incubation, immunosorbent was pelleted in a microcentrifuge for 30 seconds at 14,000 x g and rinsed three times with a 200 µl volume of TNE-lysis-buffer with 1% Triton X-100, PMSF and leupeptin. Immunosorbent was re-suspended in a final volume of TNE-lysis-buffer to yield 100 µl of beads per treated lysate.

A 50 µl volume of immunosorbent was then added to 150 µl of labeled lysates in microcentrifuge tubes. This mixture was incubated overnight at 4 ^{o}C on a shaker. Immunosorbent-bound immune complexes were pelleted by micro-centrifugation at 14,000 x g for 2 minutes and extensively washed 6 times with 200 µl of TNE-lysis-buffer with 1% Triton X-100, PMSF, and leupeptin. After the final wash, pellets were resuspended with 50 µl 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 3 minutes. Tracking dye (2 µl of 1% bromphenol blue) was added to each sample prior to loading in separate wells of the polymerized resolving gel. Samples loaded on lanes of a given gel were adjusted to contain equivalent activity (in cpm).

For peptide resolution by SDS-PAGE, a discontinuous SDS-PAGE buffer system (a 4% stacking and 10% resolving gel) was used as described by Laemmli (1970). A 10% resolving gel was prepared with 20 ml of 30% acrylamide, 15 ml of resolving buffer (36.3g Tham in 200 ml of distilled water, pH8.8), 0.6 ml of 10% SDS, and 24.1 ml of distilled water, degassed, and then polymerized with 0.3 ml

of 10% ammonium persulfate (APS) and 20 µl of N,N,N',N'-tetramethylethylene-diamine (TEMED). A 4% stacking gel was prepared with 2.7 ml of 30% acrylamide, 5.0 ml of stacking buffer (3.0g Tham in 50 ml distilled water, pH6.8), 0.2 ml of 10% SDS, 12.2 ml of distilled water, 0.1 ml of APS, and 10 µl of TEMED. Protein molecular weight markers were electrophoresed simultaneously for size determination and included myosin (Mr=200 kDa), phosphorylase B (Mr=92.5 kDa), bovine serum albumin (Mr=69 kDa), ovalbumin (Mr=46 kDa), carbonic anhydrase (Mr=30 kDa), trypsin inhibitor (Mr=21.5 kDa), and lysozyme (Mr=14.3 kDa) (RAINBOW^{TM} Molecular Weight Markers; Amersham).

Following electrophoresis at 30 mA of constant current for about 3 hours, resolving 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 for over 2 hours, and destain II with 30% methanol, 0.3% acetic acid, and 0.3% glycerine for 30 minutes. 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 ^{o}C with typical exposure times of 1-3 days for detection of peptide bands. Radiographic film was developed according to manufacturer's specifications.

Antiserum preparations

A 5 month old calf, BVDV-free and originating from a BVDV-negative herd, was inoculated intravenously with $10^{7.8}$ $TCID_{50/ml}$ of BVDV, strain NY1. Convalescent serum was collected 40 days after virus inoculation. The preparation of antiserum against BVDV, strain C24V was described previously (Johnson, 1988).

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% NaN_3. Globulins were dialyzed against the 1% tris-tricine-saline at 4 ^{o}C with three changes of dialyzant every 24 hours. Globulins were harvested from dialysis tubing and stored at 4 ^{o}C.

Polymorphonuclear (PMN) Leukocyte Adherence Assay

Preparation of PMN

Venous blood was collected from normal, BVDV-negative adult Holstein steers into Alsever's solution for PMN preparation. A method for PMN isolation established by Roth et al. (1981) was followed. Briefly, after centrifugation at 1,000 x g for 20 minutes the plasma, buffy coat layer, and upper portion of the red blood cell (RBC) layer were removed by aspiration. The remaining RBCs were lysed by the addition of 2 volumes of cold phosphate buffered (0.0132 M, pH7.2) deionized water and isotonicity was restored to the red blood cell lysate after 45 seconds by the addition of 1 volume of cold phosphate buffered (0.0132 M, pH7.2) 2.7% NaCl. The PMNs were pelleted by centrifugation and washed twice (200 x g

for 10 minutes) with CMF-HBSS. The washed cells were suspended by vortexing in M199 with 5% FBS and standardized to a concentration of 2.5 x 10^7 cells/ml in Medium 199 with 5% FBS.

Labeling of PMNs

Polymorphonuclear leukocytes were labeled with ^{51}Cr as Na^2CrO^4 (Amersham Corp., Arlington Heights, IL) using a previously described method (Hoien-Dalen, 1989). The PMNs were labeled with ^{51}Cr (2 µCi/10^6 PMNs) and the mixture incubated at 37 ^oC with 5% CO_2 for 30 minutes. The PMNs were sedimented at 200 x g for 10 minutes and washed three times in PBS (pH7.4). The PMNs were resuspended in M199 with 5% FBS to a concentration of 2.5 x 10^7 cells/ml.

The assay and statistics

This assay was set up as a modification of two previously reported methods (MacGregor et al., 1980; Paulsen et al., 1990). To establish a confluent monolayer, BTU and BEC were seeded in parallel in 96-well, flat-bottom tissue culture clusters and grown at 37 ^oC for 48 hours. The resulting monolayers contained approximately 1.3 x 10^4 cells per well. The medium was removed at 48 hours after cell seeding, and NCP-BVDV isolates (1.0 moi) were inoculated into wells with MM. Inoculated BTU and BEC were incubated for 24, 48, and 72 hours at 37 ^oC to observe differences among strains or between host cells. Medium was aspirated from each well, and 2.5 x 10^6 ^{51}Cr-labeled-PMNs were added to each well and coincubated for 30 minutes at 37 ^oC. Medium and non-adherent PMNs were removed and each well was rinsed 3x in M199 with 5% FBS. Wells were lysed with 100 µl of 1.0% Triton X-100 for 15 minutes at room

temperature to reach complete lysis. A 50 µl aliquot from each well was counted on a gamma counter (Packard). The mean sample count per minute (cpm) was determined for each tetraplicate sample, and the mean control cpm was calculated from tetraplicate wells of mock-infected and labeled PMN-coincubated wells. The percent ^{51}Cr binding was calculated using the following formula;

$$\% \text{ binding} = \frac{\text{mean sample cpm}}{\text{mean control cpm}} \times 100$$

The numerical data were subjected to analysis of variance procedures and the significance was determined by F variables.

RESULTS

Harvest of Pulmonary Microvascular Endothelial Cells

To obtain bovine endothelial cells, two techniques were employed in this experiment. These were enzymatic digestion for endothelial cells from aorta and microcarrier harvest for those from the pulmonary microvascular system. In the microcarrier system, harvested beads (approximately 30% of perfused beads) contained 3-6 cells on their surface. Pulmonary endothelial cell was selected by its cobblestone morphology. A monolayer from the microcarrier method was established much slower (20 days) (Figure 1) than the enzymatic method (7 days), and only those wells with characteristic endothelial morphology were kept. At the passage level 4, endothelial cells were functionally screened to Factor VIII:vWF-RAg. About 95% of both cells were indirectly stained. Pulmonary microvascular endothelial cells were morphologically and functionally maintained as a uniform cell population for up to passage 15. More than 90% of these cells gave positive staining for Factor VIII:vWF-RAg using an indirect FA assay (Figure 2).

Viral Polypeptide Profiles by RIP/SDS-PAGE Analysis

Analyses of RIP/SDS-PAGE were conducted to compare the viral polypeptide processing by BVDVs on two different hosts. Fourteen different noncytopathic bovine viral diarrhea isolates including the reference NY1 strain were compared in bovine aortic endothelial cells (Figure 3) or bovine turbinate cells (Figure 4). Strains of NCP-BVDV could be differentiated by viral polypeptide

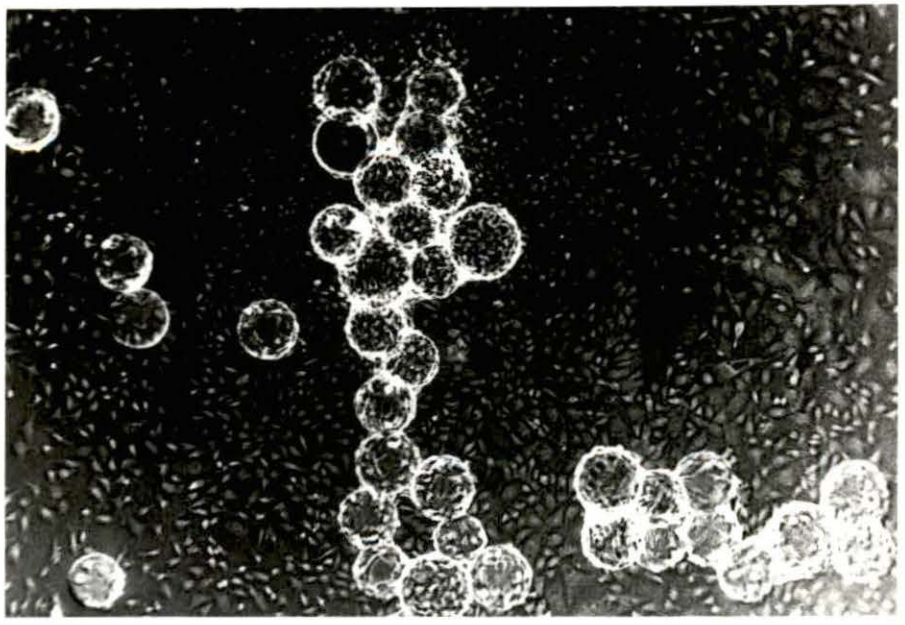

Figure 1. Bovine pulmonary endothelial cells collected by microcarrier beads 20 days after cultivation 50X

Figure 2. Indirect immunofluorescence specific to Factor VIII in bovine pulmonary endothelial cells 270X

processing patterns in BEC. All 14 strains produced a prominent 125 kDa peptide. Eight of the 14 strains produced 4 proteins of 125, 92, 76, and 66 kDa (lanes A, B, C, D, E, F, H, M, in Figure 3), while one strain (lane G) produced these same 4 proteins plus proteins of 72 and 34 kDa. Five other strains were divergent from the above, and did not produce the 92 kDa peptide (lanes I, J, K, L, N in Figure 3). Several other differences were noted among these 5 strains: a peptide of 89 kDa was produced by strains in lanes I, K, and N, a 54.5 kDa peptide was produced by the strain in lane I, and a 34 kDa peptide was present in strains of lanes I and L.

Viral polypeptide processing in BTU was similar to that observed in BEC, with most of the differences observed in the 5 divergent strains. Nine of the 14 strains (lanes A, B, C, D, E, F, G, H, M, in Figure 4) produced 5 proteins of 125, 92, 76, 66, and 46 kDa. These strains were the same ones that had high degree of similarity in BEC. All 5 divergent strains (lanes I, J, K, L, N, in Figure 4) produced proteins of 125, 115, 76, and 54.5 kDa. In addition, the strain in lane I produced a 66 kDa protein, the strain in lane J produced a 60 kDa protein, and the strains in lanes I, K, L, and N produced a 46 kDa protein.

Paired analyses of BVDV-infected BTU and pulmonary microvascular BEC against anti-NY1 BVDV serum (Figures 5, 6, 7, and 8) were performed to observe the detectability of the 92 kDa peptide and the differences of viral polypeptide processing between two hosts. Each gel contained mock-infected control, C24V, NY1, and 3 isolates of NCP-BVDV. The 125, 82, 76, 60, and 54.5 kDa proteins were identified in immunoprecipitates of C24V-infected BEC and BTU against anti-NY1 BVD immunoglobulin, with exception of the additional resolution of a

Figure 3. RIP/SDS-PAGE profile of 14 NCP-BVDVs replicated in bovine aortic endothelial cells. Cell lysates were immunoprecipitated with anti-C24V BVDV antiserum. Molecular weight standards are shown on the left. A; Hic154, B; A20, C; FC21, D; 80-14182, E; C23, F; FC28, G; 118GBK, H; 80-14049, I; FCS031, J; 16787, K; 14917, L; 12824, M; 099, N; NY1, O; mock-infected

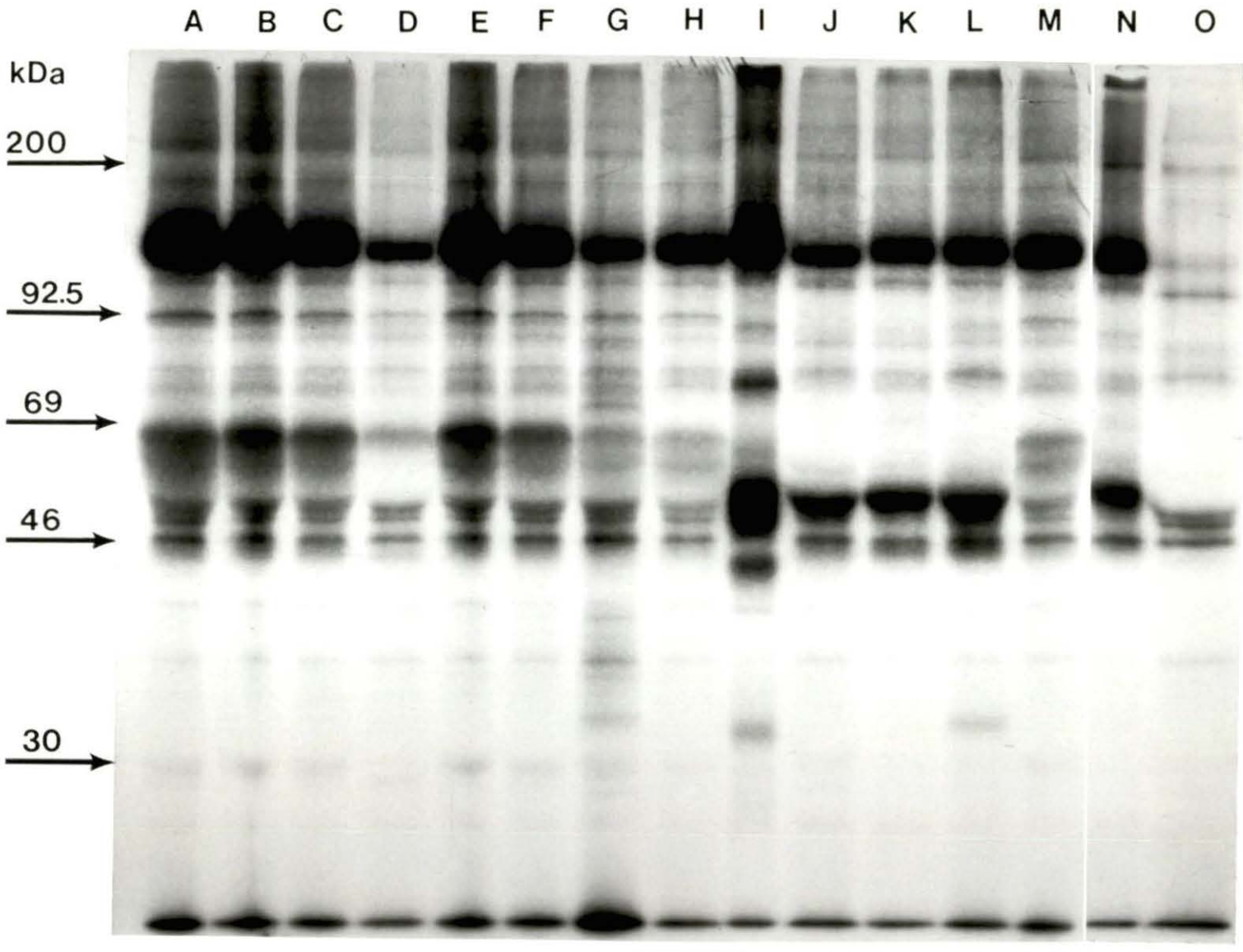
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Figure 4. RIP/SDS-PAGE profile of 14 NCP-BVDVs replicated in bovine turbinate cells. Cell lysates were immunoprecipitated with anti-C24V BVDV antiserum. Molecular weight standards are shown on the left. A; Hic154, B; A20, C; FC21, D; 80-14182, E; C23, F; FC28, G; 118GBK, H; 80-14049, I; FCS031, J; 16787, K; 14917, L; 12824, M; 099, N; NY1, O; mock-infected

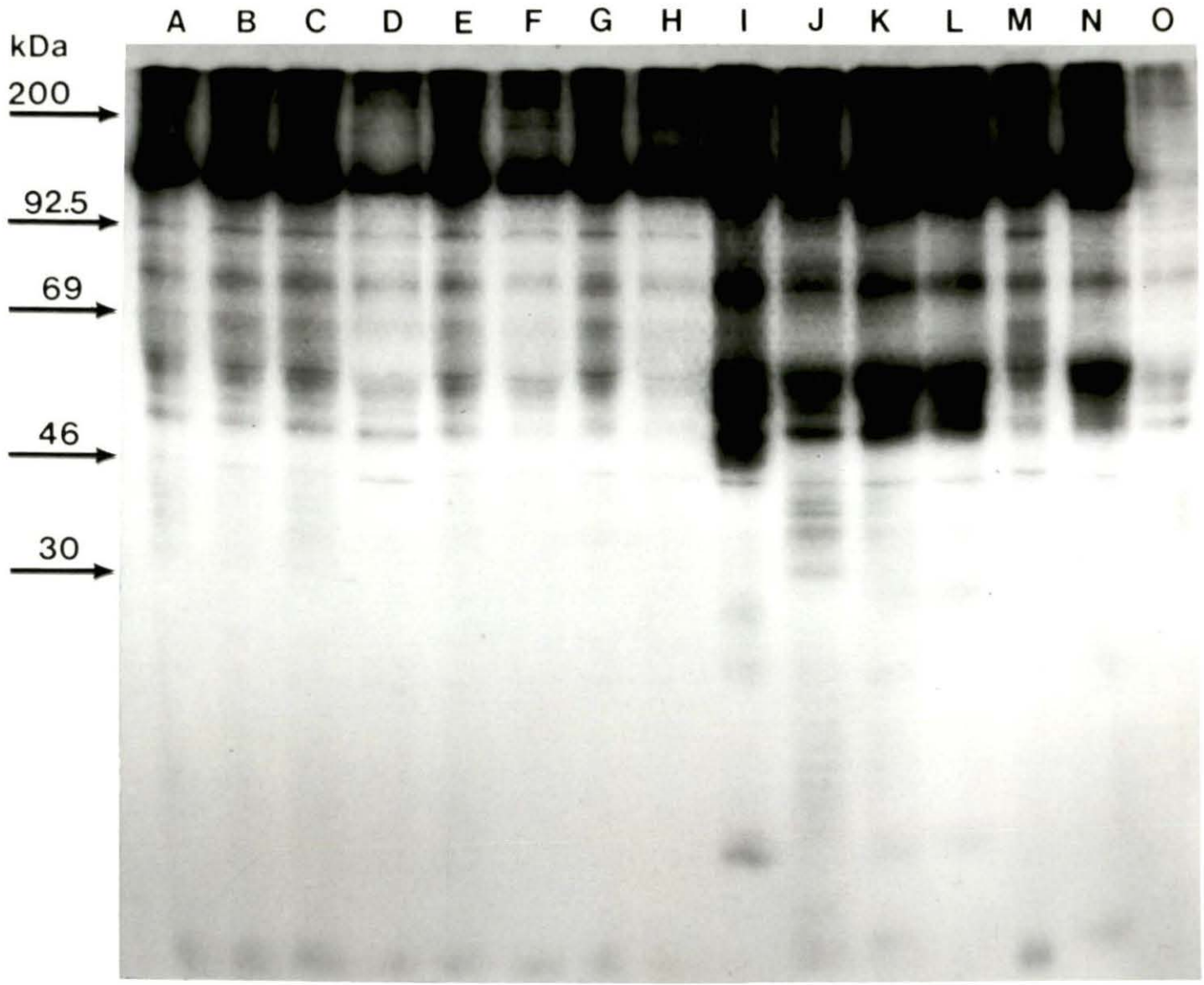
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Figure 5. RIP/SDS-PAGE profile of BVDV strains immunoprecipitated with anti-NY1 BVDV antiserum. The virus strains were grown in pulmonary microvascular BEC (lanes indicated with capital letters) or BTU (lanes indicated with lower case letters). Molecular weight standards are shown on the right. Mock-infected; A, a, C24V; B, b, NY1; C, c, 16787; D, d, 118GBK; E, e, 80-14182; F, f

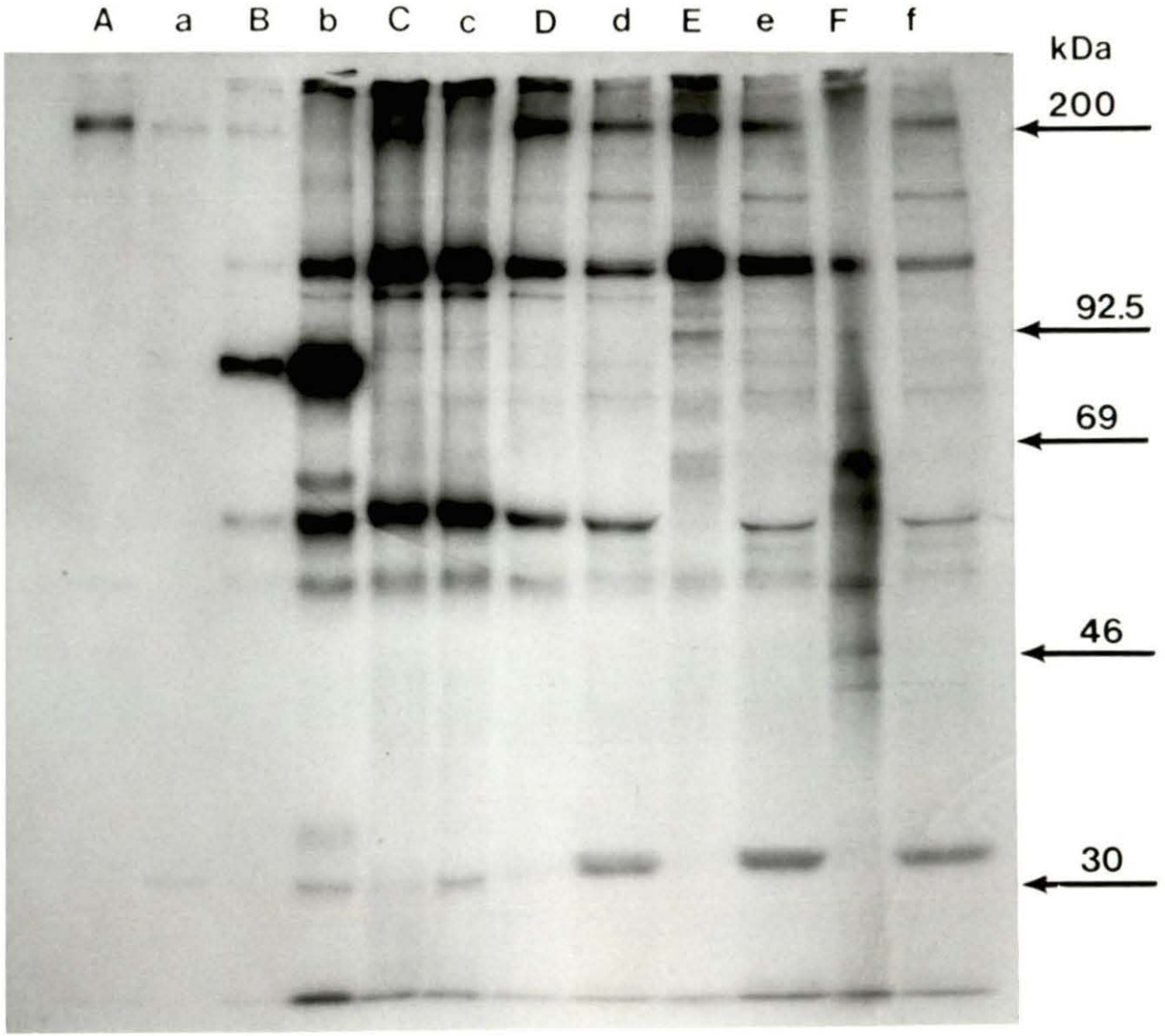
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Figure 6. RIP/SDS-PAGE profile of BVDV strains immunoprecipitated with anti-NY1 BVDV antiserum. The virus strains were grown in pulmonary microvascular BEC (lanes indicated with capital letters) or BTU (lanes indicated with lower case letters). Molecular weight standards are shown on the right. Mock-infected; A, a, C24V; B, b, NY1; C, c, FC21; D, d, Hic154; E, e, 12824; F, f

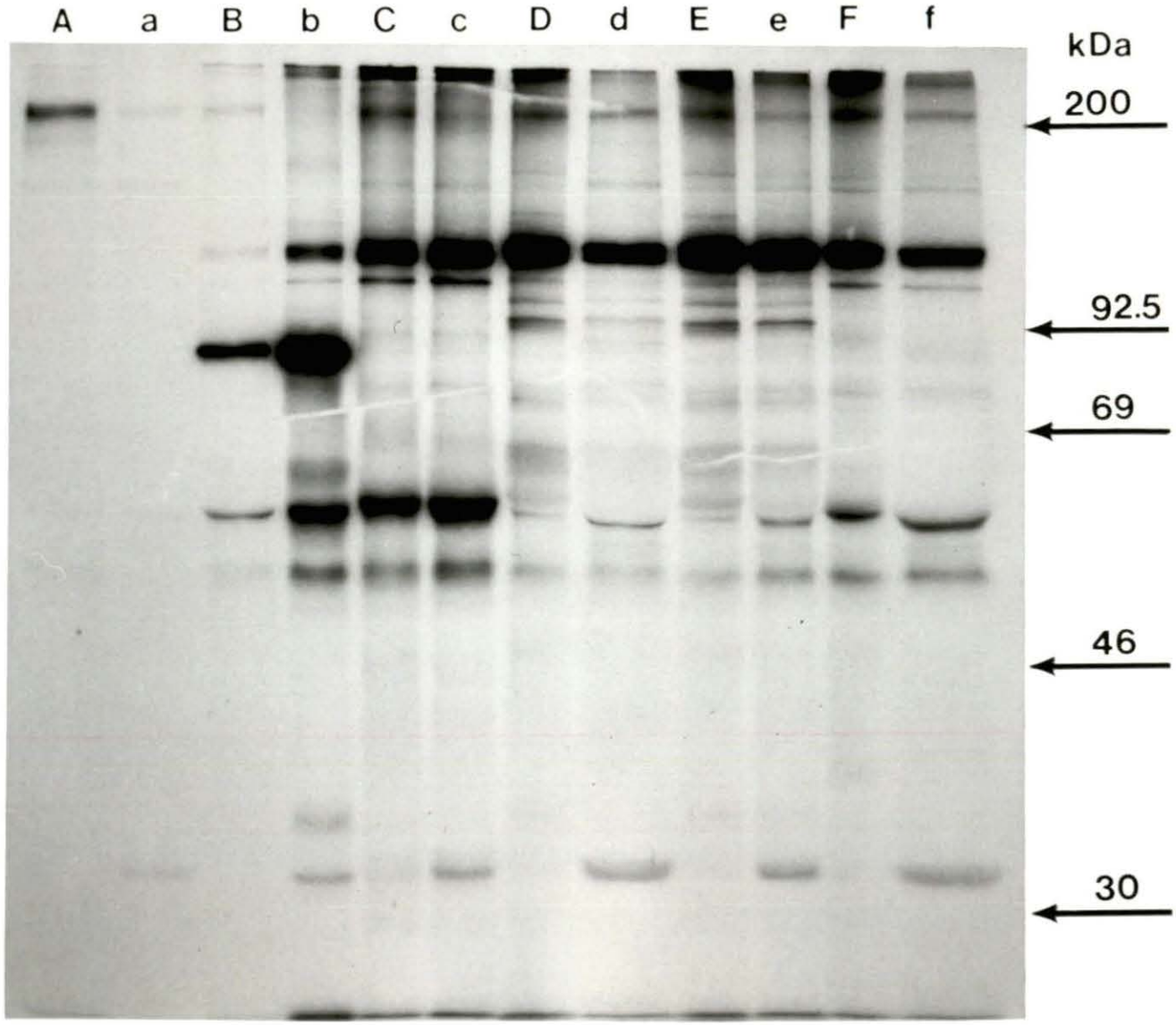
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Figure 7. RIP/SDS-PAGE profile of BVDV strains immunoprecipitated with anti-NY1 BVDV antiserum. The virus strains were grown in pulmonary microvascular BEC (lanes indicated with capital letters) or BTU (lanes indicated with lower case letters). Molecular weight standards are shown on the right. Mock-infected; A, a, C24V; B, b, NY1; C, c, 14917; D, d, A20; E, e, FC28; F, f

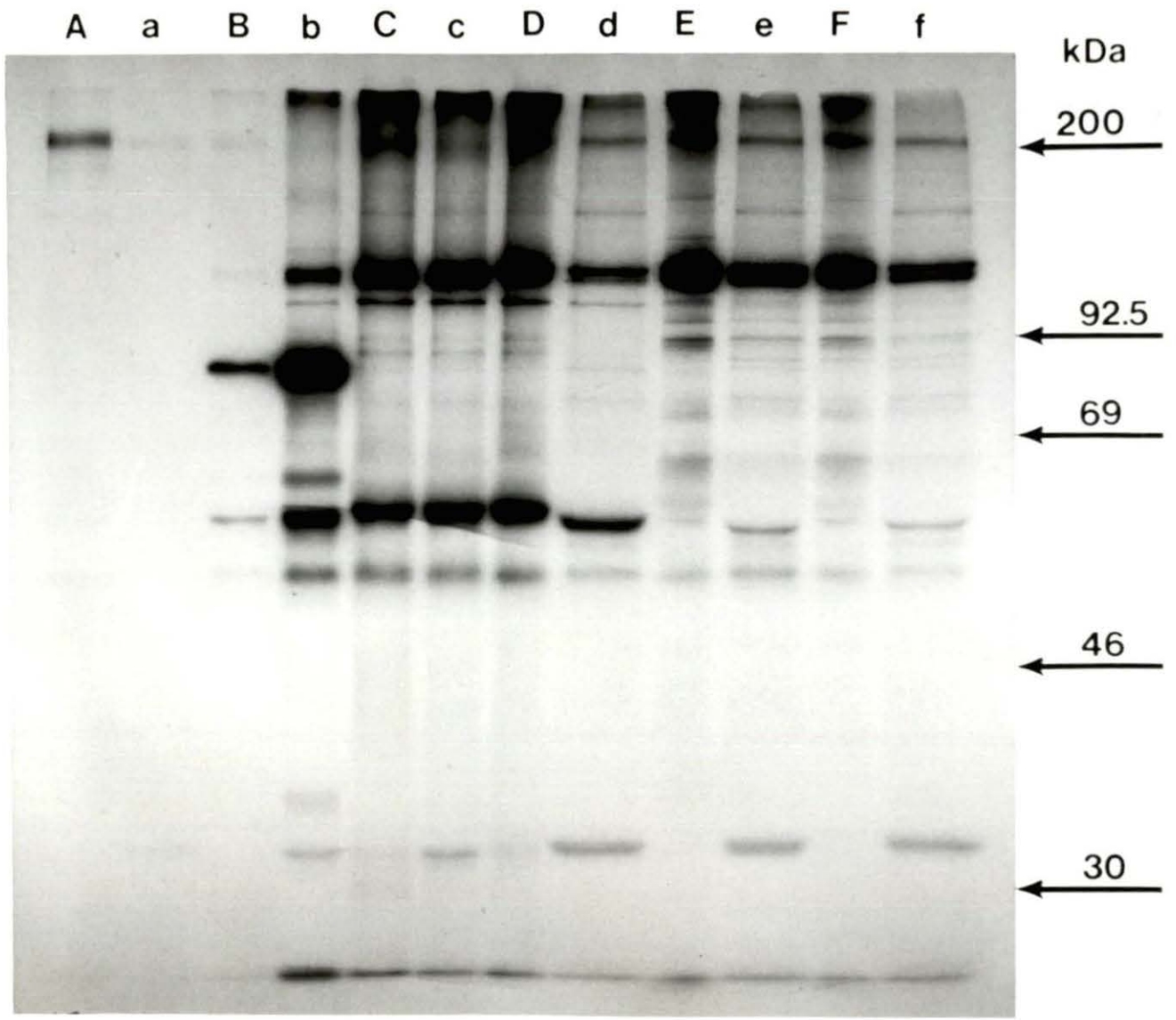
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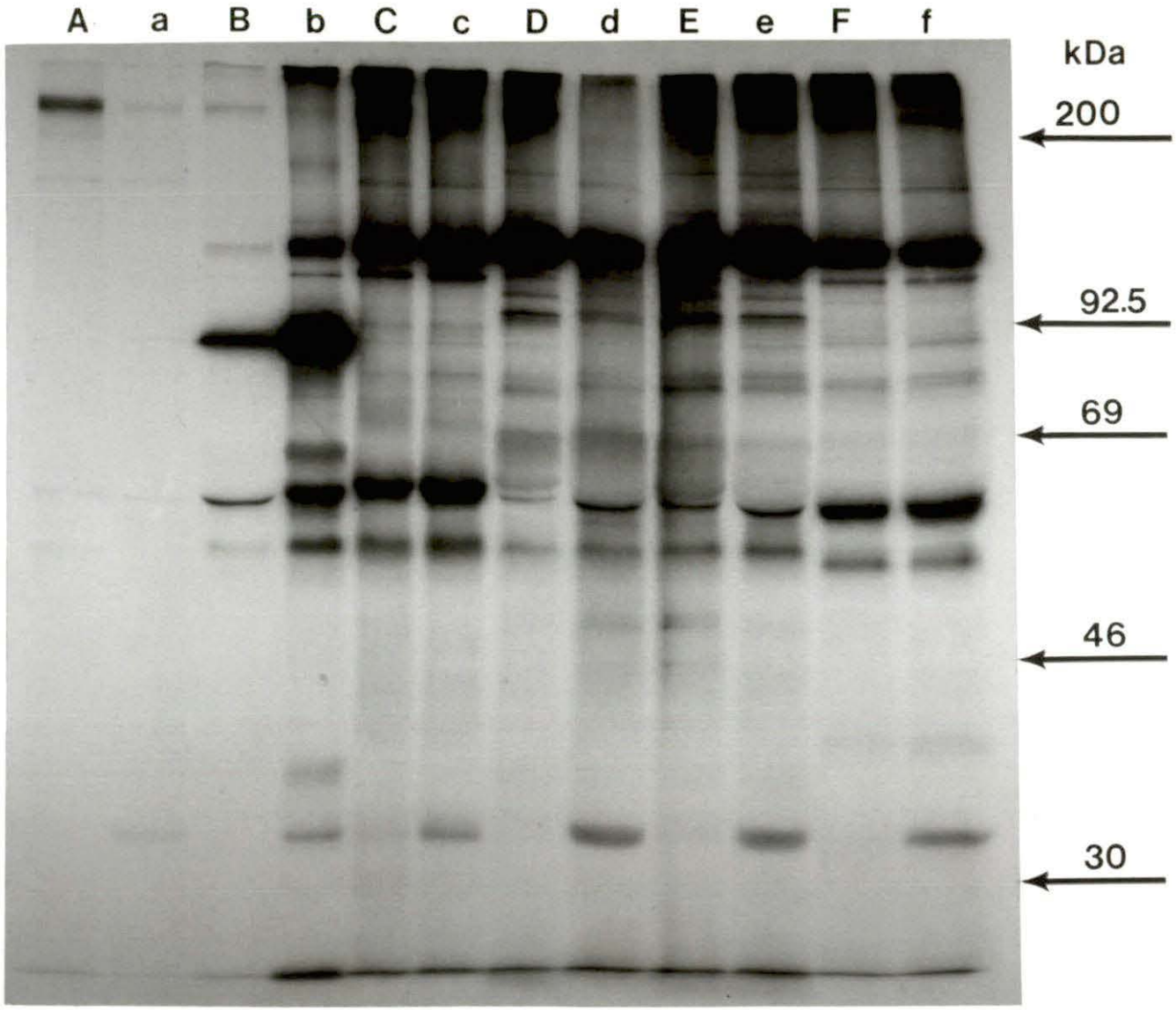
Figure 8. RIP/SDS-PAGE profile of BVDV strains immunoprecipitated with anti-NY1 BVDV antiserum. The virus strains were grown in pulmonary microvascular BEC (lanes indicated with capital letters) or BTU (lanes indicated with lower case letters). Molecular weight standards are shown on the right. Mock-infected; A, a, C24V; B, b, NY1; C, c, C23; D, d, 099; E, e, FCS031; F, f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72 kDa peptide in BTU (lanes B, b in Figures 5, 6, 7, and 8). The 125, 76, 60, and 54.5 kDa proteins were observed in immunoprecipitates of NY1-infected BTU and pulmonary microvascular BEC against anti-NY1 BVDV immunoglobulin (lanes C, c in Figures 5, 6, 7, and 8). The 92 kDa peptide production from NY1 strain was not seen, either. Eight of the 12 isolates produced 5 proteins of, 125, 92, 76, 60, and 54.5 kDa (lanes E, e and F, f in Figures 5 and 7; D, d and E, e in Figures 6 and 8), but the 60 kDa was less or not present at E, F of Figure 5. The 125 kDa polypeptides of BVDV in these gels were observed as "doublets". Four other strains were differentiated by the lack of 92 kDa protein (lanes D, d in Figures 5 and 7; F, f in Figures 6 and 8). Difference among the NCP-BVDV strains was substantiated with the selective induction of the 92 kDa protein.

Adherence of PMN

The PMN adherence to pulmonary microvascular BEC and BTU infected with 14 NCP-BVDVs was recorded (Figures 9-13). Infected BEC and BTU cells appeared to have enhanced ability to adhere PMNs. Seven of the 14 strains of NCP-BVDV gave significant adherence increase at all three time intervals tested (Figures 9 and 11, and FC21 in Figure 12). In most BEC and BTU infected with NCP-BVDVs, a peak of PMN adherence to both infected cells was reached at 48 hours postinfection (HPI). The maximum counts ranged to 133%-186% of control in the infected BECs and 133%- 203% of control in the infected BTUs. Differences between host cells were observed with 2 of 14 strains of NCP-BVDV at 24 HPI (118GBK of Figure 12; A20 of Figure 13), 6 strains at 48 HPI (Figures 11 and 13; 80-14049 in Figure 12), and 1 strain at 72 HPI (FC21 in Figure 12). Two

strains among 14 NCP-BVDVs, 14917 and A20 in Figure 13, caused infected BECs to be more adherent to PMN cells than the infected BTUs at 48 HPI (186% vs 152% of control; 143% vs 107% of control, respectively). Conversely, 4 strains caused the infected BTUs to be more adherent to PMN cells than the infected BECs at 48 HPI (Figure 11; 80-14049 in Figure 12). The values were 199% vs 165% of control for the strain 12824, 203% vs 173% of control for the strain Hic154, 193% vs 169% of control for the strain FC28, and 181% vs 155% of control for the strain 80-14049. The remaining 8 strains were observed with no significant differences in PMN adherence to host cell (Figures 9 and 10; 118GBK and FC21 in Figure 12). Correlation between 92 kDa protein induction, PMN adherence, and propensity to cause bovine respiratory disease could not be shown among the strains of BVDV tested (Table 2).

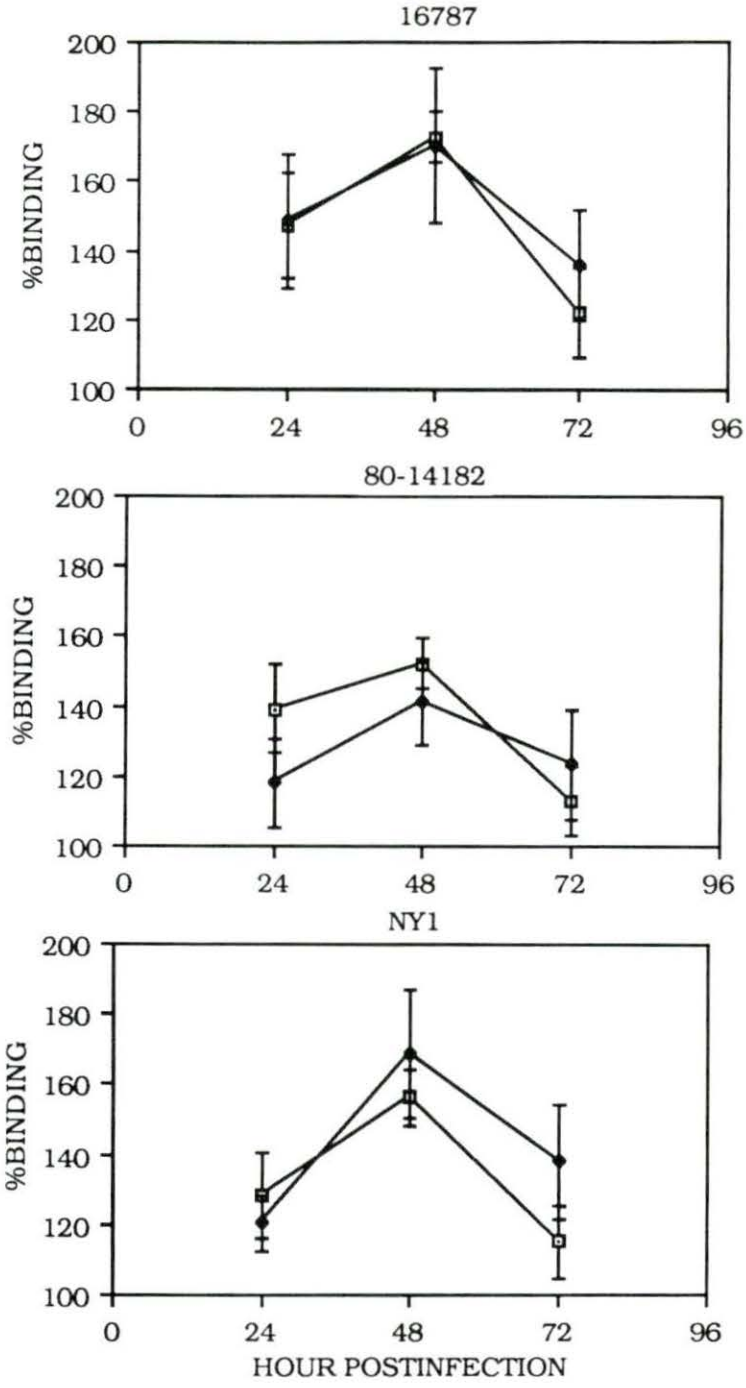

Figure 9. Percent adherence of PMNs to pulmonary microvascular BEC (open squares) or BTU (closed diamonds) after infection with BVDV strains (16787, 80-14182, and NY1)

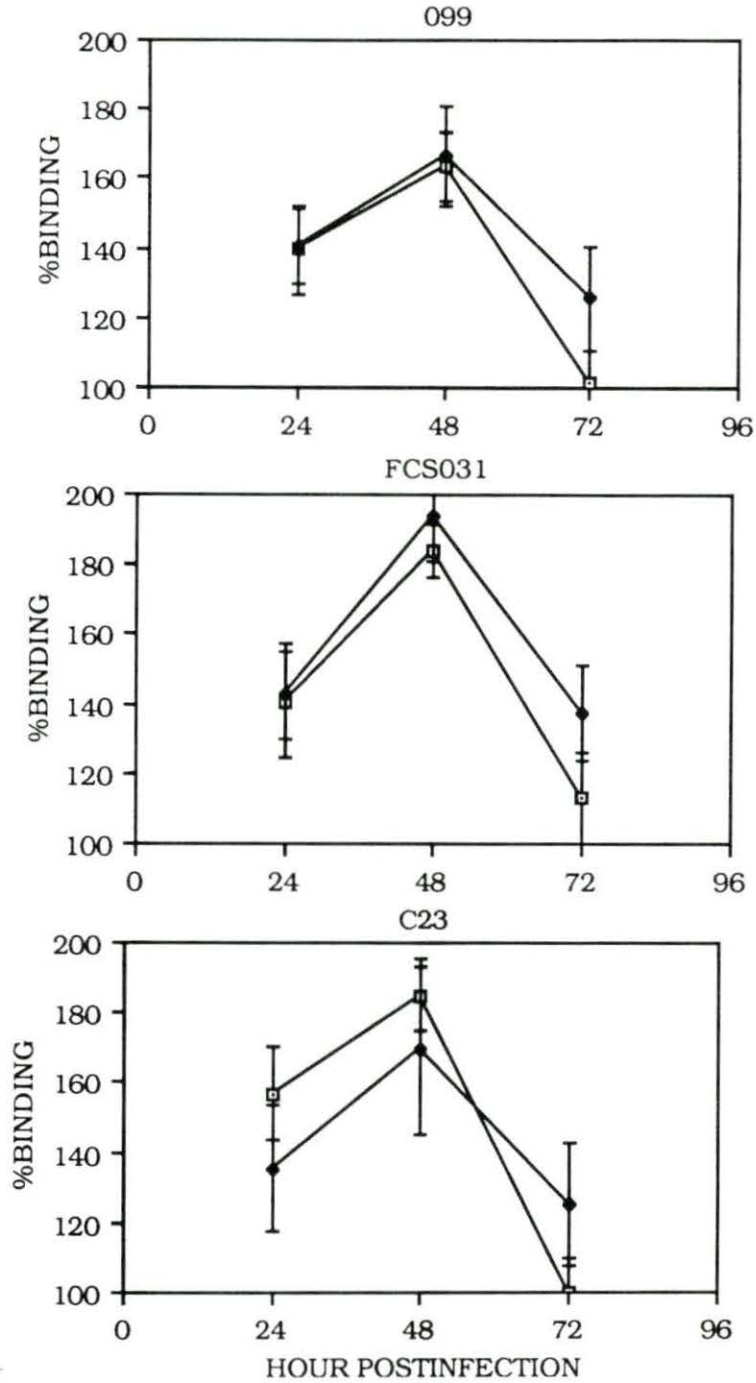

Figure 10. Percent adherence of PMNs to pulmonary microvascular BEC (open squares) or BTU (closed diamonds) after infection with BVDV strains (099, FCS031, and C23)

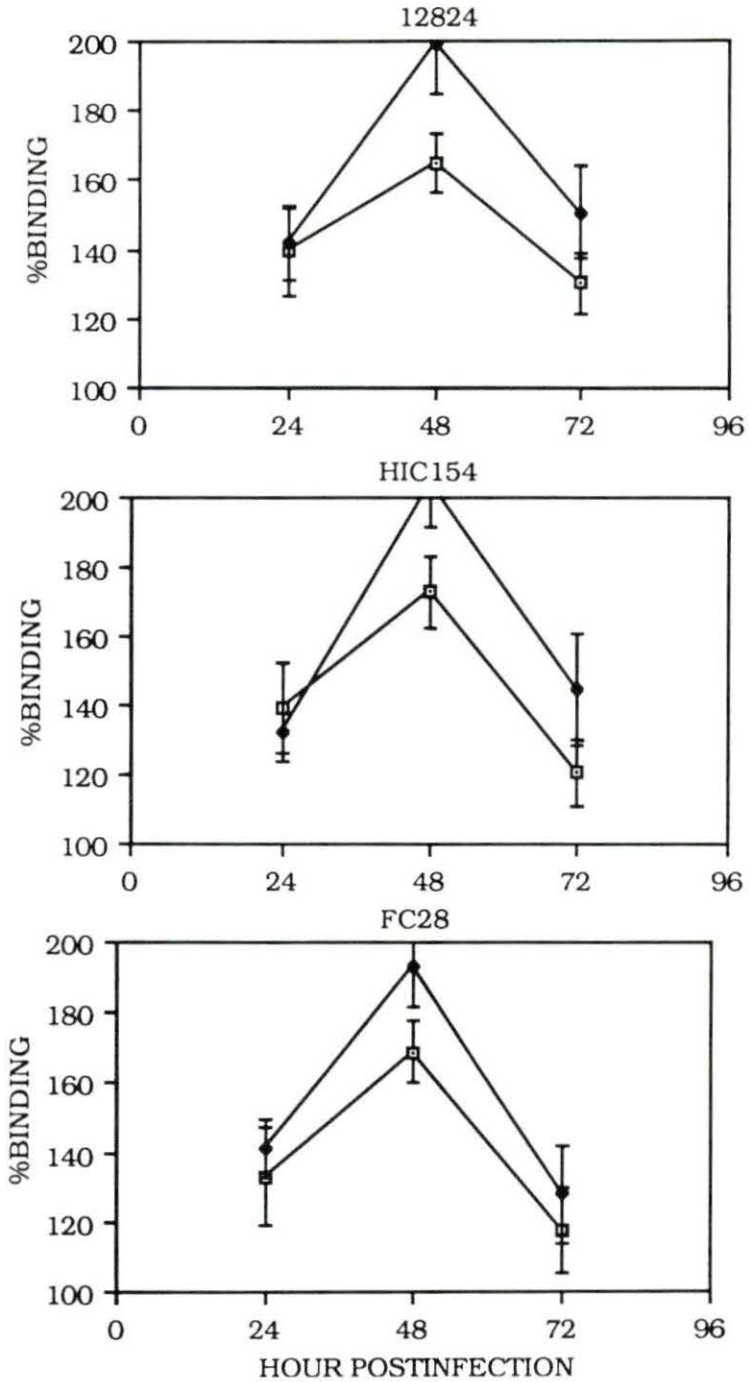

Figure 11. Percent adherence of PMNs to pulmonary microvascular BEC (open squares) or BTU (closed diamonds) after infection with BVDV strains (12824, Hic154, and FC28)

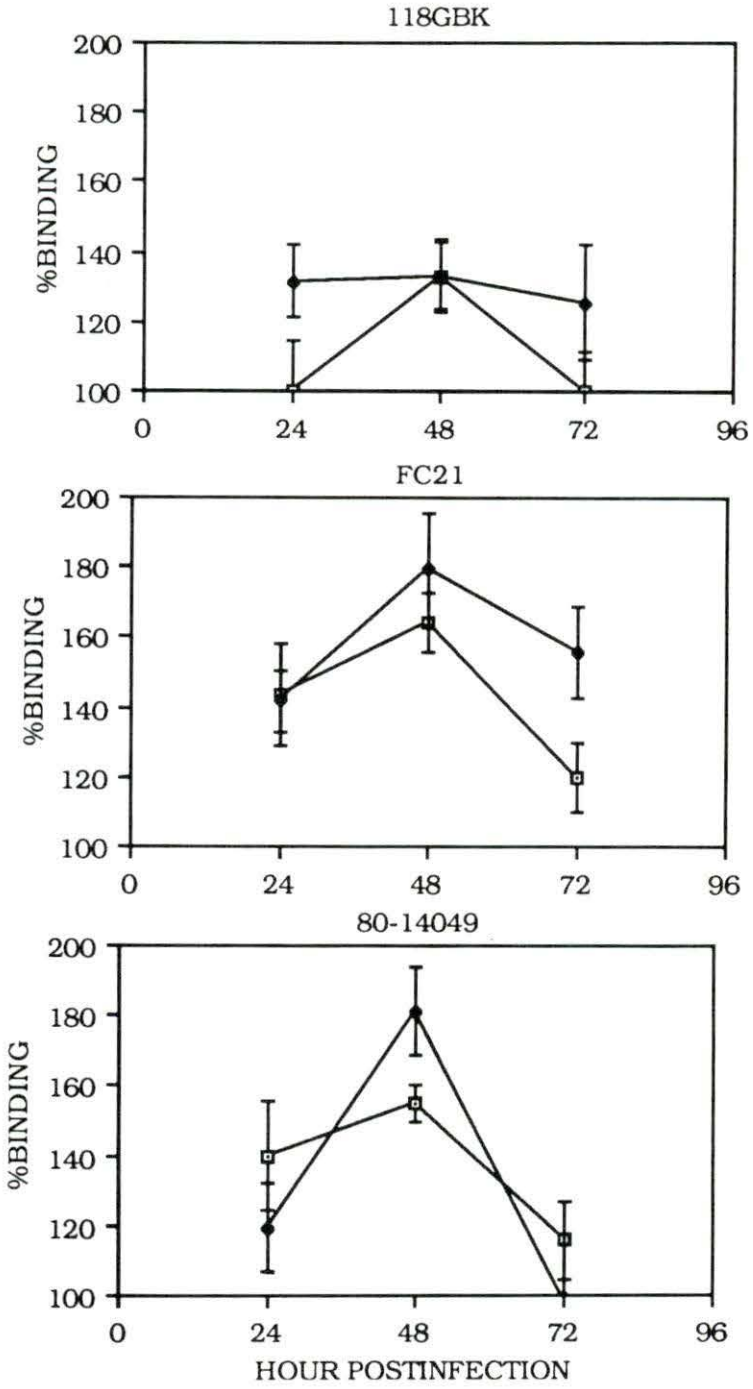

Figure 12. Percent adherence of PMNs to pulmonary microvascular BEC (open squares) or BTU (closed diamonds) after infection with BVDV strains (118GBK, FC21, and 80-14049)

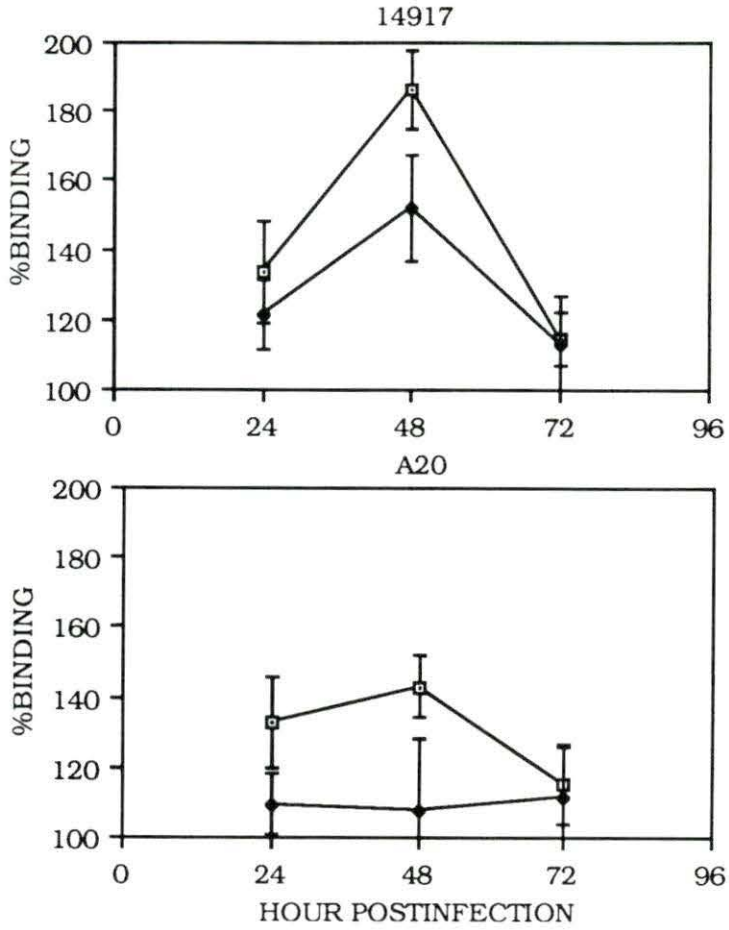

Figure 13. Percent adherence of PMNs to pulmonary microvascular BEC (open squares) or BTU (closed diamonds) after infection with BVDV strains (14917 and A20)

Table 2. Summary of protein expression and PMN adherence observations

Strain	Induction of 92 kDa	PMN adherence		
		No difference	BEC more	BTU more
16787	-	+*		
NY1	-	+		
FCS031	-	+		
80-14182	+	+*		
099	+	+		
C23	+	+		
12824	-			+*
80-14049	+			+*
Hic154	+			+
FC28	+			+
118GBK	+			+
FC21	+			+
14917	-		+*	
A20	+		+	

* Strain isolated from respiratory disease case.

DISCUSSION

Morphologically uniform bovine pulmonary microvascular endothelial cells could be obtained using a microcarrier system. Microcarrier systems have been used to harvest uniform and site-specific endothelial cells depending upon the size of the microcarrier beads used (Ryan et al., 1982).

Approximately 30% of the harvested beads used for perfusion contained 3-6 cells on their surface. Cold shock and EDTA caused pulmonary microvascular endothelial cell to detach from the vessel wall under conditions such that the cells remain attached to the surface of the bead. The number of cells attached to each bead was also low in the harvested microcarriers (3-20) from rabbit pulmonary pre-capillary vessels (Ryan et al., 1982). Cell attachment and subsequent proliferation were dependent on the precoating agent as were reported previously for bovine aortic endothelial cells (Johnson, 1988). By indirect FA-assay, Factor VIII:vWF-RAg was detected in about 95% of BECs with passage level 4. A similar finding has been reported with 85-95% of BEC from passage 3 through 11 (Johnson, 1988). Aortic endothelial cell up to passage 6 and pulmonary microvascular BEC up to passage 15 were shown to maintain characteristic morphology and over 90% Factor VIII positive cells by indirect FA.

The 92 kDa protein which was induced by lysates of NCP-BVDV infected BEC and BTU was resolved with 9 strains of 14 isolates. Even though there were two previously reported findings (Donis and Dubovi, 1987b; Johnson, 1988) about this protein, general comparison with several isolates of NCP-BVDV has not been performed to date.

Radioimmunoprecipitations performed in this research used anti-BVDV bovine immunoglobulin specific to the C24V or NY1 strains of BVDV. Anti-C24V immunoglobulins were prepared from convalescent serum collected after three inoculations with live virus (Johnson, 1988). Anti-p 92 antibodies were not exhausted in these old antisera because strong p92 bands were seen in some strains. Anti-NY1 immunoglobulins were prepared from convalescent serum harvested 40 days after a single injection of live virus. This protocol helped in reducing anti-host cell components in anti-NY1 immunoglobulin and in inducing anti-p92 activity. Immunoglobulins used for RIP by Donis and Dubovi (1987a) were prepared from bovine serum following two immunizations with a killed BVDV vaccine (Singer strain) and a third exposure by inoculation with live Singer-strain of BVDV.

The resolving conditions of proteins which are influenced by the concentration of the separating gel, resolving buffer, pH, current, and time may be critical factors in the size determination of this protein. Radiolabeled cell lysates from infected BEC and BTU yielded a 92 kDa protein on the 4% stacking/10% resolving gel. Similar findings with a lysate of strain NY1-infected BEC were made under the same resolving conditions (Johnson, 1988). However, this protein may migrate with a minor difference of molecular weight on a gradient gel (Donis and Dubovi, 1987b).

The immunosorbents were specific for BVDV-polypeptides, as SDS-PAGE lanes representing mock-infected cell lysates did not reveal viral specific bands. There appeared to be differences in induction of the 92 kDa peptide among NCP-BVDV strains with a consistent pattern of peptide migration. These results were comparable to those of Donis and Dubovi (1987b), except for the fact that these

investigators used primary bovine testicle cells as hosts, and these cells can be presumed to be a mixed population containing endothelial cells. When heterogeneous cell populations of BTU are passaged several times, a specific cell population may become predominant in the cultured cells. In our hands, the 92 kDa peptide was produced even with BTU cells free of Factor VIII antigen at passage level 8, even though BTU cells were observed with about 50% positive reaction to Factor VIII antigen at passage level 14 (data not shown). Even though different in size, the 89 kDa protein shown in NY1 infected BEC against anti-C24V globulin should be noted because this protein was not seen against anti-NY1 BVDV serum in NY1 infected BTU. Viral proteins of 117.5, 109.6, 92.2, 76, 72.4, 56.2, and 52.5 kDa were resolved in bovine aortic cells infected with the NY1 strain of BVDV (Johnson, 1988). The 92 kDa and 72 kDa polypeptides peculiar to NY1-infected BEC were not seen or were present in minute quantities in NY1-infected BTU (Johnson, 1988). The 92.2 kDa peptide of the NCP-BVDV strain NY1 migrated as a distinct "singlet". The strength of the signal generated by the 72.4 kDa peptide suggested a significant incorporation of L-^{35}S-methionine within this peptide 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 (Johnson, 1988).

The 82 kDa protein identified in C24V-infected cell lysates was within the acceptable Mr range of 80 kDa observed by others (Akkina, 1982; Purchio et al., 1984; Donis and Dubovi, 1987a, 1987b). The 89.1 kDa identified in CP-BVDV infected BEC lysates (Johnson, 1988) was within the approximate range of 87 kDa reported for vp 2 resolved from NADL-infected calf testis cells (Pocock et al.,

1987). The disparity (80 vs 87 kDa) may be a function of separating gel systems, virus strains, immunosorbents, cell types, and potentially, virus-cell interactions (Johnson, 1988).

While a 92 kDa and a 90 kDa peptides in NCP-BVDV isolates grown in bovine cells were observed with no comparable findings in the CP-BVDV isolates used in their study, Corapi et al. (1990) detected a similar shift in minor proteins estimated at 96 kDa in the Singer strain and 100 kDa in the NADL strain, suggesting an intermediate cleavage product of the 118 kDa protein (Singer) or 125 kDa (NADL).

The consistency observed between polypeptide profiles of 12 NCP-BVDVs, and including C24V and NY1 in BTU and BEC, suggests that virus-induced changes are strain-dependent. The similarities in polypeptide profiles in this research compared to published observations may also be due to the stability of BVD viruses, even though there are the differences in immunosorbents and cell types used in the various published RIP protocols.

In most BEC and BTU infected with NCP-BVDVs, PMN adherence was increased over the period of virus incubation with a peak at 48 HPI (133-186% of control for BEC and 133-203% of control for BTU). Adherence of PMNs was enhanced in both virus-infected BEC and BTU cells, suggestive of the association of BVDVs. As a similar observation, significantly increased adherence of PMN was produced with polio virus infected human endothelial cells (185.4% of control at 24 HPI) and herpes simplex type I infected bovine aortic endothelial cells (213.7% of control at 6 HPI) (MacGregor et al., 1980). Neutrophil adherence to subconfluent BEC monolayers was larger than that to confluent cells (Paulsen et al., 1990). Since the numbers of BEC and BTU cells were adjusted to establish

similar numbers at 48 hours after cell seeding, the disparity in counts per minute between BTU and BEC, either mock- or BVDV-infected, could be attributed to differences in PMN attachment. The virus association of PMN adherence may be due to the synthesis of substances or receptor expression from the infected BEC (MacGregor et al., 1980; Osborn, 1990). Lipopolysaccharide of P. haemolytica increased adherence of bovine neutrophils to bovine endothelial cells, emphasizing neutrophil-mediated effects in pneumonic pasteurellosis (Paulsen et al., 1990). However, this speculation remains to be proven.

No discernible correlation could be seen between induction of enhanced PMN adherence in a given host cell and field origin of the NCP-BVDV strain. In other reports, it was found that PMN adherence to fibroblasts, epithelial, and kidney cells was significantly less than endothelial cells (MacGregor et al., 1980). In our experiments, only two strains, 14917 and A20, showed more PMN adherence to infected BEC than to infected BTU, while most others showed less adherence or no difference between infected BEC and infected BTU. Multiplicity of virus input may be a critical factor for the kinetics of PMN adherence. Johnson (1988) observed that extracellular titers of virus replicated within BEC surpassed virus titers obtained in BTU. This may be a result of greater numbers of infected BEC reaching equilibrium in adsorbing/releasing virions, or virus replication was enhanced in BEC (Johnson, 1988). Typically, BEC forms a stable monolayer which is contact-inhibited at confluence. When cultures are maintained at high densities for periods of 1-2 weeks, a second growth pattern in focal areas termed "sprouting" is observed (Schwartz, 1978). This secondary growth is only partially contact-inhibited, and is represented by host cells that may have altered viral replication and PMN adherence characteristics.

CONCLUSIONS

The advances made in tissue culture techniques allow the harvesting and propagation of specific cell types and make possible the study of virus replication in and biological effect on specialized cell types.

The analysis of RIP/SDS-PAGE of viral proteins was used to characterize viral polypeptide processing of BVDV strains during replication in aortic and pulmonary microvascular BEC. Viral polypeptide processing of NCP-BVDV grown in bovine endothelial cells paralleled to those in bovine turbinate cells. Differences were noted with anti-C24V immunoglobulin, but not with anti-NY1 serum. Viral polypeptide processing of NCP-BVDVs included a 92 kDa protein which was produced by most but not all strains of NCP-BVDV tested.

A polymorphonuclear leukocyte adherence assay was conducted to observe virus-host interactions which might relate to bovine respiratory pathogenesis. Adherence of PMN was increased by NCP-BVDV infection and behavioral difference of the viruses was observed in PMN adherence to host cells. However, these experiments could not uncover any correlation between 92 kDa protein induction and PMN adherence to host cells or uncover any *in vitro* markers that could be used to identify NCP-BVDV strains that would be specially involved in bovine respiratory disease.

While this research has provided valuable information on viral polypeptide processing of BVDVs in BEC and BTU and PMN adherence, further observations need to be made using well-defined target cell populations. Examination of the role of BVDV (particularly NCP-strains) in the pathogenesis of bovine reproduc-

tive and respiratory disease should be conducted with emphasis on virus-cell interactions within target systems.

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