Comparison of the polypeptides of lectin purified bovine viral diarrhea virus by three methods

by

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INTRODUCTION

Recent reports concerning the etiology of bovine viral diarrhea-mucosal disease complex have shown that two forms of bovine viral diarrhea virus are required for the manifestation of mucosal disease (MD). Mucosal disease is seen only when calves persistently infected with noncytopathic BVDV are superinfected with cytopathic BVDV. Involvement of two forms of BVDV in the manifestation of MD strongly indicate that distinct differences exist between cytopathic and noncytopathic isolates of BVDV. Studies employing serological and immunological methods have not shown distinct differences between these two forms. Limited attention has been given to the possibility that these differences may exist at a structural level.

The literature which deals with dissociated polypeptides of BVDV has been generally limited to cytopathic isolates. One report has been made as to the dissociated polypeptides present in a noncytopathic isolate and possible distinctions between it and other cytopathic isolates (Akkina 1982). Further studies are required to determine if a significant difference in structural polypeptides exists between these two types of BVDV.

To determine if significant differences between cytopathic and noncytopathic BVDV are present at the structural level, the various isolates to be compared must be purified. This has not been an easy task with BVDV, since many of the standard virus purification methods fail to yield high levels of infectivity for BVDV. Reduced levels of infectivity recovered for BVDV using physical methods of purification can be attributed

to disruption of the BVD viral envelope. In an effort to overcome this obstacle, a gentler method of purification was required.

A method was proposed by Kristiansen et al. (1979) for purification of BVDV using affinity chromatography to preserve the viral envelope. Several lectins were used in association with affinity chromatography for the purification of BVDV. These include concanavalin A, Lens culinaris, and Vicia ervilia lectins with an affinity for mannose and N-acetyl-glucosamine, and Crotalaria juncea lectin with an affinity for galactose. The V. ervilia and C. juncea lectins proved to be effective in the purification of BVDV, but neither of these lectins was readily available.

Shortly after these findings were published, another lectin, Ricinus communis agglutinin was found to be effective in the purification by affinity chromatography of hog cholera virus (Neukirch et al. 1981). Like C. juncea lectin, R. communis agglutinin has an affinity for galactose. Hog cholera virus and BVDV are both members of the genus Pestivirus within the family Togaviridae and are known to share similar antigens as measured by serological methods. Because of these similarities and the similarity of the two lectins, affinity chromatography with the lectin R. communis agglutinin was used in this study for the purification of BVDV.

The dissociated polypeptides of six purified isolates of BVDV were separated and compared by three methods, high performance gel permeation chromatography under nondenaturing and denaturing conditions, continuous and discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and Western blotting with subsequent labeling of the

polypeptides with specific immunoglobulins. By using these methods, specific distinctions which may exist between three cytopathic isolates, Singer, NADL, and TGAC; and three noncytopathic isolates, 7443, NY-1, and Tifton were studied.

LITERATURE REVIEW

History of BVD-MD

The bovine viral diarrhea-mucosal disease (BVD-MD) complex was first described in 1946 as separate disease entities. These entities presented very similar clinical pictures in that infected cattle had severe diarrhea, ulcerations of the oral mucosa, leukopenia, anorexia, severe lacrimation, and a mucopurulent nasal discharge (Childs 1946, Olafson et al. 1946, Ramsey and Chivers 1953). The major distinctions between these two entities were the mortality and morbidity rates.

Olafson et al. (1946) studied a disease in the New York area which often affected nearly 80% of a herd, but with a mortality rate of 4% to 8%. Upon necropsy of affected animals, ulcerations were observed on the oral mucosa, muzzle, nares, pharynx, larynx, esophagus, and cecum. Hemorrhages were observed on the omasum, subcutaneous tissue, epicardium, and the vaginal mucosa. The lesions observed were often less severe than the clinical signs would indicate. Abortions were also characteristic of this disease entity, occurring 10 days to 3 months after the initial outbreak.

The other element of this complex was first identified in Saskatchewan as X-disease of cattle by Childs (1946). Several years later, Ramsey and Chivers (1953) described mucosal disease (MD) in Iowa cattle, a disease similar to Childs' X-disease of cattle. Mucosal disease, as described by Ramsey and Chivers (1953) had a morbidity rate which ranged from 5% to 20% and a mortality rate which was often greater than 90%. Necropsy of these animals revealed that erosions were present in the buccal, pharyn-

geal and oral cavities, on the tongue and gingivae. Severe erosions could be seen on the esophageal epithelium and in the abomasum, stomach, over the Peyer's patches, and scattered throughout the small and large intestines. The lesions on the distal 2 to 3 feet of the large intestine would advance to such a degree that the mucosal surface was often entirely absent. Hemorrhages could be seen in the subepicardium and/or subendocardium. There was a conspicuous absence of leukocyte infiltration. Lymph nodes were only slightly edematous and rarely showed any significant enlargement or other lesions. Another feature of MD was the often occurring ulcerative skin lesions. These were seen near the oral labia, inside the ears, medial sides of arms and thighs, and perineal region (Ramsey 1956, Pritchard 1963). Lameness, which was also frequently present, was the result of interdigital necrosis and disfiguration of the coronary region (Pritchard 1963).

Another disease at this time had also been identified as X-disease. This second X-disease, or hyperkeratosis, had clinical signs similar to those of the BVD-MD complex, but especially to MD. The one feature which distinguished hyperkeratosis from BVD and MD was the thickened, dry, wrinkled skin of the affected animals (Olafson 1947).

Attempts to transmit BVD and MD to other susceptible cattle yielded yet another difference. Olafson et al. (1946) found that the milder disease, BVD, could easily be transmitted by drenching susceptible cattle with fecal material or by the injection of blood or splenic tissue emulsion. The clinical signs shown by these infected cattle were similar to

those seen in the field. However, transmission studies with MD often produced little more than a mild fever, failing to reproduce the clinical signs seen in the field (Ramsey and Chivers 1953, Pritchard 1963). These differences in experimentally produced infections led to speculation that these two diseases resulted from infection with the same virus, but required a secondary infection for the production of MD (Baker et al. 1954); or that separate infectious agents were involved (Ramsey and Chivers 1953, Ramsey 1956).

Before the agent, or agents, could be identified from both diseases, each had to be isolated. Olafson et al. (1946) had isolated the agent of the milder disease by inoculating susceptible cattle with various materials taken from an ill animal. Further efforts to identify this agent led Olafson and Rickard (1947) to conclude that the agent responsible was a virus. (At their suggestion, this disease is now referred to as bovine viral diarrhea (BVD).) Several isolates obtained from disease outbreaks identified as BVD were compared to the New York-1 (NY-1) isolate of BVD, first identified by Olafson et al. (1946), by cross-immunization studies and were found to be related (Baker et al. 1954, Pritchard et al. 1956, Gillespie and Baker 1959).

In attempts to find a more convenient host for viral propagation, Olafson and Rickard (1947) inoculated rabbits, guinea pigs, sheep, and 10-day-old chick embryos. None of these proved to be a suitable host for the BVD virus (BVDV). Other workers were also unsuccessful in finding an alternate host which could manifest the clinical signs of BVD. However,

Baker et al. (1954) found that the virus could be propagated in rabbits; and when cattle were infected with the rabbit-passed virus, immunity to BVDV resulted.

In 1957, Lee and Gillespie (1957) had successfully isolated the viral diarrhea virus in embryonic bovine kidney cell culture. The isolated virus proved to be noncytopathic in cell culture; consequently, inoculation of cattle had to be used to show that the virus had been isolated.

Underdahl et al. (1957) isolated two cytopathic viruses in embryonic bovine kidney cell culture from separate outbreaks of MD. Cross immunization tests showed these two isolates to be immunologically related. The authors found neutralizing antibodies in several herds with no known history or clinical signs of MD. This evidence of widespread exposure of healthy animals to these isolates, and the failure to experimentally reproduce MD as seen in the field cast doubt as to whether this virus was responsible for MD (Ramsey and Chivers 1953, Pritchard 1963).

Gillespie et al. (1960) isolated a cytopathic virus from the spleen of a calf having clinical signs associated with BVD. This isolate, identified as Oregon C24V, was found to be related by cross-immunization to the noncytopathic isolates of BVDV. In further work, Gillespie et al. (1962) observed that noncytopathic BVDV infected cell cultures were refractory to infection by cytopathic BVDV. Presence of noncytopathic BVDV in cell culture prevented the characteristic cytopathic effect of superinfecting cytopathic BVDV from being demonstrated. Isolation and identification of a cytopathic form of BVDV and recognition of the interference

phenomenon provided researchers with a biological system in which work with BVDV did not require the continued need for cattle inoculations.

New York-1, the first noncytopathic isolate identified as being BVDV, and Oregon C24V were found to be antigenically related on the basis of cross-neutralization tests (Kniazeff and Pritchard 1960). These comparisons were soon expanded to include additional cytopathic isolates which were obtained from both BVD and MD (Gillespie et al. 1961). Findings that this wide range of isolates were antigenically related resulted in the recognition of these diverging clinical conditions as forming the bovine viral diarrhea-mucosal disease complex.

Clinical Syndrome

Bovine viral diarrhea-mucosal disease complex is recognized today as three distinct clinical entities, bovine viral diarrhea, mucosal disease, and the persistently infected animal. Each of these presents its own characteristic set of clinical signs.

Bovine viral diarrhea - acute infection

The most prevalent syndrome of BVD is the acute infection which occurs in cattle of all ages. Cattle undergoing an acute infection may demonstrate a variety of clinical signs; including transient fever, diarrhea, leukopenia, depression, anorexia, tachycardia, polypnea, nasal discharge, dry nonproductive cough, dehydration and abortion. Erosions and/or reddened areas may be seen on the lips, dental pad, buccal mucosa, nares, muzzle and vulva. Lameness, as a result of laminitis, can also be a common clinical sign (Pritchard et al. 1956, Pritchard 1963). The

extent with which these lesions are present vary from animal to animal and from herd to herd.

Serological evidence for the presence of viral neutralizing antibodies suggests that most cattle have experienced a BVD viral infection (Pritchard 1963). In the majority of these cases, the animal had a subclinical infection, evident only by a slight rise in body temperature, leukopenia, and mild transient diarrhea, and resulting in full recovery. Other cases of the acute infection were characterized by sudden onset of fever, depression, tachycardia, polypnea, anorexia, nasal discharge, nonproductive cough, lesions on the nasal and oral mucosa, and diarrhea persisting continuously or intermittently for 1 day to 4 weeks (Pritchard 1963).

An acute respiratory form of BVD was seen in association with shipping fever. Reggiardo (1979) reported the involvement of BVDV in severe pneumonia which was unresponsive to antibiotic therapy. From the lesions, BVDV and Pasteurella hemolytica type 1 were isolated. In contrast to other forms of BVD-MD, the typical gross lesions associated with BVD-MD were usually absent. In a few cases, microscopic lesions could be seen in the pharyngeal and esophageal mucosas.

Another syndrome associated with BVD is the chronic infection generally occurring in animals 6 to 14 months of age, after the colostral antibody has declined. The onset of the clinical signs associated with the chronic infection are ill-defined. These animals may not grow at a rate comparable to age-mates; or they may become cachectic and experience intermittent or continuous diarrhea, or no diarrhea. Histological changes

which occur in chronic BVD are very similar to those seen in acute BVD. The course of this syndrome may vary from 2 to 6 months and usually results in total recovery (Pritchard 1963).

Bovine viral diarrhea virus infection generally results in life long immunity, but only after the animal has experienced a brief period of immunosuppression, which has become the hallmark of the BVD-MD complex. Bovine viral diarrhea virus has a predilection for cells of the immune system. Leukopenia occurs during the time span between the two febrile responses, coinciding with peak viral titers in the blood stream (Pritchard 1963). In studies utilizing cattle infected with a cytopathic isolate, Bolin et al. (1985a) followed the fluctuating levels of leukocytes during leukopenia. Total numbers of leukocytes were decreased from preexposure levels by approximately 35% on the fourth day after infection. The percentage of T lymphocytes was reduced from preexposure levels by 52% at this time. Although the percentage of B lymphocytes remained at preexposure levels, the absolute number of B lymphocytes were reduced on the fourth day post infection. On the seventh day post infection, total number of leukocytes had returned to preexposure levels. At this time, absolute numbers of T and B lymphocytes remained decreased, along with decreased percentages of monocytes, eosinophils and basophils and increased percentage of neutrophils. By 17 days post infection, all cell counts had returned to preexposure levels. Roth et al. (1981) reported similar reductions in cell numbers of neutrophils, lymphocytes, and eosinophils following BVDV infection.

Lymphocytic depletion was also noted during gross and histological examination of BVD associated lesions. Gross observations of lymphatic tissues from cases of both BVD and MD appeared normal. Microscopically, partial to complete depletion of lymphocytes were seen in the lymph nodes, thymus and spleen. Necrotic foci were occasionally observed in the spleen and lymph nodes. Decreased or total lack of differentiation between the cortex and medulla was often seen in the thymus (Ramsey 1956, Carlson et al. 1957, Pritchard 1963). In conjunction with this, lymphocytic infiltration, normally associated with severe lesions in certain diseases, has been noted to be absent in some animals simultaneously infected with BVDV (Ramsey 1956). This failure of lymphocytic infiltration to occur may be associated with in vitro suppression of the chemotactic response, resulting from BVDV infection of monocytes (Ketelsen et al. 1979).

Truitt and Shechmeister (1973) demonstrated the in vitro ability of BVDV to replicate within nonimmune and immune leukocytes. A slower increase in extracellular virus and lower titers were seen in macrophage cultures obtained from immune animals as compared to those from nonimmune animals. If BVDV antibody was included in these cultures, extracellular virus was not present; however, viral titers returned to previous levels when the antibody was removed. Leukocyte and lymphocyte enriched cultures obtained from nonimmune and immune animals behaved similarly, in that they produced comparable viral titers.

A difference was observed when leukocyte and lymphocyte enriched cultures were stimulated with phytohemagglutinin (PHA). Phytohemagglutinin stimulated nonimmune leukocyte and lymphocyte enriched cultures to produce

significantly larger titers of BVDV than did nonstimulated cultures. In contrast, immune leukocyte and lymphocyte enriched cultures produced lower titers of BVDV when stimulated with PHA than the nonstimulated cultures. This may be explained by the ability of BVDV to inhibit PHA induced DNA synthesis (Muscoplat et al. 1973a). This inhibition of mitogenic stimulation by PHA in vitro has also been reported in naturally infected lymphocytes obtained from a calf chronically infected with a noncytopathic isolate (Johnson and Muscoplat 1973).

Calves infected with BVDV experience a generalized reduction in the cellular immune response. Experimentally infected calves experience an increased incidence of bacteremia, coinciding with PHA suppression of lymphocytes and leukopenia. This reduction in blood clearance efficiency has been attributed to BVDV induced impairment of macrophage and polymorphonuclear leukocyte (PMN) activities (Reggiardo and Kaeberle 1981). Reduction in phagocytic efficiency of macrophages may be related to the ability of macrophages to support BVDV replication (Truitt and Shechmeister 1973). Inhibition of the myeloperoxidase, hydrogen peroxide, halide system of PMN is evident by their reduced iodination ability as a result of BVDV infection (Roth et al. 1981).

Antibody response is also affected by BVDV infection. Studies conducted utilizing in vivo infected B lymphocytes and in vitro infected splenocytes have shown BVDV to suppress the release of cytoplasmic immunoglobulin. This suppression appears to be the result of failure in BVDV infected B lymphocytes to differentiate into mature plasma cells (Muscoplat et al. 1973b, Atluru et al. 1979).

Bovine viral diarrhea - fetal infection

Another aspect of BVD occurs when pregnant heifers and cows are infected with BVDV. Like other infections, clinical signs in the dams often are unnoticed except for an increase in reproductive failures. Reproductive failures are manifested as abortions, stillbirths, and the birth of calves with congenital defects. Pregnancy outcome is dependent on gestational age at which fetal infection occurs.

The fourth month of gestation appears to be the point at which fetal susceptibility to BVDV decreases (Casaro et al. 1971). Infections occurring prior to 100 days of gestation usually result in fetal death. The pregnancy may end in abortion, stillbirth or expulsion of a mummified fetus (Kahrs et al. 1970b, Casaro et al. 1971, Kendrick 1971, Scott et al. 1972). Lesions which have been reported in these fetuses include: alopecia due to follicular hypoplasia and cystic dilation of the adnexal glands, mild anasarcous, mandibular brachygnathism, cataracts, petechiae on conjunctiva, general nonsuppurative inflammatory changes, and necrosis with or without inflammation in skin, lung, and brain (Casaro et al. 1971, Kendrick 1971, Scott et al. 1972).

If infection occurs between 100 and 180 days of gestation, these fetuses may survive to birth, but are born with a large number of congenital defects. Most notable is cerebellar hypoplasia. Clinically, these animals may be unable to stand at birth, have an abnormal stance, ataxia, dysmetria, and/or tremors (Wilson et al. 1983). Along with cerebellar hypoplasia, other central nervous system defects can be seen, including microencephaly, internal hydrocephalus, cerebellar dysgenesis,

spinal cord hypoplasia and spinal dysmyelination, microglial myelin deficiency, and hypomyelination of the optic nerve. Other lesions seen were cortical hypoplasia of thymus, vascular endothelial hyperplasia, perivascular mononuclear cuffing, complete loss of spermatogenic epithelium, eye and optic nerve lesions resulting in blindness, and intrauterine growth retardation (Kahrs et al. 1970c, Scott et al. 1972, Brown et al. 1974, Brown et al. 1975, Done et al. 1980, Binkhorst et al. 1983, Duffell et al. 1984).

When fetal infection takes place in the last trimester, viral specific antibodies are produced by the fetus. Immunologic competence is reached by the bovine fetus between 100 and 200 days of gestation (Braun et al. 1973). This immunologic response, as observed in the fetus, follows the expected pattern with the first immunoglobulin seen as IgM, followed by IgG_1 and IgG_2. Immunoglobulin M has been seen in fetuses at 95 days of gestation, but specific viral neutralization activity was not detected until 200 days of gestation (Braun et al. 1973, Brown et al. 1974). This response enables the fetus to recover in utero from BVDV infection with no apparent adverse effects and to possess, at birth, a life long immunity.

Another facet of fetal BVDV infection is seen in the "weak calf syndrome". These calves appear normal at birth, but within hours of birth develop diarrhea of varying severity. Death may result from severe dehydration within 18 to 96 hours or chronic diarrhea may persist for several weeks. Clinical signs associated with this syndrome are reproducible by infecting calves within the time period of 25 days prenatally

to 3 days postnatally. Severity of clinical signs is dependent on the timing of infection; if infected prenatally, the diarrhea is less severe than if the calf was infected at birth or within 3 days postnatally (Lambert et al. 1974). The severity of this infection could be directly related to the immunosuppression resulting from BVDV infection and the stress associated with birth (Roth and Kaeberle 1983).

Persistent infection

A fifth outcome of an in utero infection has recently been recognized in a small proportion of the cattle population. This infection results in apparently healthy, persistently infected adult cattle (Coria and McClurkin 1978). Persistently infected cattle may appear to be fully recovered from neurological disorders present at birth or to be clinically normal (Straver et al. 1983, McClurkin et al. 1984). Preliminary surveillance indicates the incidence of persistently infected cattle may occur in nearly 2% of the cattle population within the United States (Bolin et al. 1985b).

McClurkin et al. (1984) succeeded in experimentally producing persistently infected calves by infecting the fetus prior to day 123 of gestation with several isolates of BVDV. In these experiments, only the noncytopathogenic isolates could establish persistence; the cytopathogenic isolate, NADL, could not establish persistence. Not all of the experimentally infected fetuses resulted in persistently infected calves. As expected, stillbirths, abortions, and weak calves also occurred since the time interval for production of all four events coincide (McClurkin

et al. 1984). It is generally believed that the majority of the persistently infected calves found in the field are the offspring of persistently infected cows or first calf heifers undergoing a BVDV infection during the first trimester (Straver et al. 1983, McClurkin et al. 1984).

Persistently infected cattle lack the clinical signs which indicate the continued presence of virus. Similarly, the gross lesions associated with viral infections are not seen at necropsy of persistently infected animals. It is only upon microscopic examination that lesions indicating the presence of a glomerulonephritis and encephalitis can be seen. In spite of the wide distribution of viral antigens in the spleen, lymph nodes, epithelial cells of the small intestine and seminiferous tubules, and vascular endothelium of the lung, liver, adrenal gland, heart, and aorta; lesions are not observed (Cutlip et al. 1980).

The immunological status of persistently infected cattle has not fully been evaluated. Limited studies have shown levels of serum proteins to be lower than average, but immunoglobulins were within the normal range of healthy cattle (Coria and McClurkin 1978, Coria et al. 1983a). Persistently infected cattle are immunocompetent to antigens other than BVDV. A persistently infected bull responded with production of antibodies to a chlamydial infection; but when given a triple dose of BVDV vaccine, it failed to produce a significant anti-BVDV titer (Coria and McClurkin 1978). This immunologic tolerance of BVDV seen in persistently infected cattle may not be absolute. The glomerulonephritis present in these cattle appears to be very similar to that found in murine lymphocytic choriomeningitis. If this similarity is confirmed,

then the glomerulonephritis seen in cattle persistently infected with BVDV would be due to deposition of immune complexes on basement membranes of the glomeruli (Cutlip et al. 1980).

Mucosal disease

Clinical signs seen in young cattle with mucosal disease (MD) are fever, anorexia, suspension of rumination, depression, nasal discharge which is at first watery but becomes more viscid as the clinical signs advance. Often a sero-mucoid exudate is seen hanging from the nares and muzzle. Erosions and ulcers develop on the muzzle, nares, within the mucosa of the oral cavity, including the tongue and gingivae, pharynx, esophagus, and intestinal tract. Severity of these lesions often advance to such a degree that the mucosal surface is almost entirely absent from the affected tissues. As the lesions advance, profuse and violent diarrhea occurs first as watery feces, and later becoming more scanty, consisting largely of blood and mucus. In terminal stages, the calf experiences violent tenesmus (Childs 1946, Ramsey and Chivers 1953, Ramsey 1956, Pritchard 1963, Peter et al. 1967).

Cattle afflicted with MD possess similar immunologic capabilities as normal, healthy age-mates, except the ability to respond immunologically to BVDV. The incidence and levels of neutralizing antibodies to other common bovine viruses and the ability to respond immunologically to ferritin are similar in both healthy and fatally affected cattle. This corresponds with the relatively normal levels of immunoglobulins found in the MD affected cattle. These animals have normal levels of IgG, IgM,

and IgA; however, when the IgG classes are determined individually, IgG_2 is considerably lower than that found in healthy age-mates (Steck et al. 1980). These cattle may be able to respond to superinfection by a cytopathic BVDV with the production of neutralizing antibodies, but only if the superinfecting isolate is antigenically distinct from the noncytopathic isolate producing the viremia and only after a considerable length of time (Peter et al. 1967, Steck et al. 1980, Bolin et al. 1985a, Bolin et al. 1985c).

Lymphocytes of cattle fatally affected with MD have a reduced ability to respond to PHA and concanavalin A (Con A) (Steck et al. 1980). This aspect of the cellular immune response of cattle affected with MD is similar to that of BVD affected cattle (Johnson and Muscoplat 1973); however, MD differs from BVD in the occurrence of leukopenia. In MD, leukopenia occurs prior to the appearance of marked clinical signs and is followed by leukocytosis (Bolin et al. 1985a); while leukopenia occurs between the two febrile responses in BVD (Pritchard 1963).

Cattle succumbing to MD had remained BVDV seronegative in spite of originating from a BVDV seropositive herd (Thomson and Savan 1963, Malmquist 1968, Kahrs et al. 1970a, Liess et al. 1974, Steck et al. 1980, Brownlie et al. 1984, Roeder and Drew 1984, Bolin et al. 1985c). Since cytopathic BVDV was routinely isolated from these outbreaks of MD (Underdahl et al. 1957, Gillespie et al. 1961, Thomson and Savan 1963, Kahrs et al. 1970a); the possibility of noncytopathic BVDV infection playing a role in MD was not readily recognized until persistent BVDV infections of seronegative cattle had been established (Coria and McClurkin

1978). This involvement has been further confirmed by McClurkin et al. (1985) with the isolation of both cytopathic and noncytopathic BVDV from 16 of 17 spleens obtained from cattle diagnosed as having MD. Many of the field cases of MD result from vaccinating apparently healthy persistently infected cattle (Peter et al. 1967, McKercher et al. 1968, McClurkin et al. 1985). Clinical signs of MD have now been experimentally reproduced, which are identical to the clinical signs seen in the field, by superinfecting persistently infected calves with cytopathic BVDV (Brownlie et al. 1984, Bolin et al. 1985c).

Characteristics of Bovine Viral Diarrhea Virus

Serological and cultural characteristics

The fact that a noncytopathic and a cytopathic isolate of BVDV are required for the manifestation of MD indicates that distinct differences may exist between the isolates of BVDV. During the last several decades, various isolates have been compared and significant differences between them have not been demonstrated (Gillespie et al. 1961, Fernelius et al. 1971, Horzinek 1981). Limited in vivo studies concerning the pneumopathogenicity of BVDV in susceptible calves have indicated that the cytopathic isolates may result in the expression of more severe clinical signs than those of the noncytopathic isolates (Potgieter et al. 1985).

The most pronounced difference between the various isolates is the degree to which a particular isolate is cytopathic in cultured bovine cells. The first isolates identified as BVDV were noncytopathic. In 1960, Gillespie et al. (1960) reported the isolation of a cytopathic

agent, Oregon C24V (C24V), from a calf with clinical signs very similar to those associated with noncytopathic BVDV. Soon after this report, other cytopathic isolates were obtained, including C1K, C56K, and NADL (Gillespie et al. 1960, Gutekunst and Malmquist 1963).

Another difference associated with the cytopathogenicity of the isolates is the viral multiplication rate. In vitro growth rate of the cytopathic isolates is much higher than that of the noncytopathic isolates. Since viral replication of the noncytopathic isolates does not destroy the cell, a longer period of time exists in which virus can be released. This fact results in total virus yields per cell for both the cytopathic and noncytopathic BVDV to be comparable (Mahnel and von Moreau 1984). However, the cytopathic and noncytopathic isolates respond differently when actinomycin D is included in the cell culture medium. The replication rate for cytopathic isolates appears to be reduced by 30% to 100%, contrasted by that of the noncytopathic isolates which is increased 20% to 100% (Nuttall 1980).

Oregon C24V and NADL isolates each produce a cytopathic effect (CPE) which is distinct. The CPE produced by C24V is characterized by the formation of vacuoles of various sizes, the nuclei are pyknotic and often eccentric, with a few cells rounding-up and detaching from the growth surface. The prominent feature of the CPE produced by NADL is the pyknotic nuclei prior to rounding-up and detachment of the cell from the growth surface. The NADL isolate does not produce the vacuolation which is so evident in C24V (Fernelius and Ritchie 1966). Fernelius et al. (1971) proposed a classification system for BVDV based on the CPE pro-

duced. The 3 categories, or biotypes, proposed in this system are characterized by the CPE produced by NADL and C24V and the absence of CPE.

Slight antigenic differences have been observed between the various isolates. These differences can be demonstrated by immunofluorescence and viral neutralization. Fernelius (1964) reported that noncytopathic isolates fluoresced with the greatest intensity when the conjugate used was prepared with antiserum produced against a noncytopathic isolate. However, the cytopathic isolates fluoresced to equal intensity with either conjugated antiserum, anti-NADL (cytopathic) or anti-CG1220 (noncytopathic).

A greater variation is seen in the abilities of the various isolates to be neutralized by heterologous antiserum. Gillespie et al. (1961) compared the ability of a single anti-BVDV antiserum to neutralize six isolates from various sources. All of the isolates were neutralized by the antiserum but the neutralization indexes varied from $10^{2.5}$ to $10^{5.0}$ $TCID_{50}$. Fernelius et al. (1971) pursued this difference in degree of viral neutralization and proposed 3 BVDV serotypes, C24V, NADL, and noncytopathic. Fernelius et al. (1971) compared these serotypes in viral neutralization tests, and found each serotype to be neutralized to a greater degree with its homologous antiserum than with heterologous antisera. In immunization experiments, this group also found that when an immunized animal was given a hyperimmunizing dose of a heterologous serotype, antibodies specific for the heterologous serotype were not formed. However, the immunized animal was protected from challenge with the heterologous serotype illustrating that though various BVD isolates may

differ serologically, they are still immunologically very similar (Castrucci et al. 1975).

Fernelius et al. (1969a, 1969b) were successful in changing the biotypes of C24V and NADL by serial passages in the PK-15 cell line, embryonic bovine kidney cell line (EBK) and rabbits. The altered biotypes resulted in PK-15 cell passaged C24V producing NADL type CPE; PK-15 and EBK passaged C24V resembling noncytopathic BVDV in culture, and rabbit passaged NADL producing C24V type CPE. With the change in biotype, the serological specificity changed to that of the new biotype. Thus, PK-15 passage C24V had the highest neutralization titers with anti-NADL, PK-15 and EBK passaged C24V with anti-CG1220, and rabbit passaged NADL with anti-C24V. This group speculated that the characteristics of CPE are genetically linked to the serological specificity (Fernelius et al. 1971).

Coria et al. (1984) have reported the failure of BVDV to be neutralized by antiserum to heterologous soluble antigen (sAg). The antiserum used was produced against sAg protein isolated by high performance gel permeation chromatography. Coria and coworkers demonstrated that this antiserum, produced against specific proteins of the NY-1 sAg, would neutralize the NY-1 isolate, but not the Singer or NADL isolates. Similarly, the antiserum produced against proteins of the Singer sAg would neutralize both Singer and NADL, but not the Ny-1 isolate. This fact seems to indicate the differences lie not with the whole virion, but rather with the sAg which is produced by the infected cell.

Replication

The method of replication which characterizes members of the Togaviridae family is illustrated by the Semliki Forest and Sindbis viruses, members of the Alphavirus genus. The polyadenylated 42S genome of these viruses is replicated in two segments on membrane bound cytoplasmic polyribosomes. After uncoating within the infected host cell, one large polypeptide is translated. This polypeptide represents the first 1/3 of the 5' terminus of the genome. From this polypeptide, 4 nonstructural proteins are cleaved, two of these compose the RNA polymerase which transcribes the viral +RNA to the -RNA template. From the -RNA template, two +RNA groups are transcribed: a full length RNA to serve as mRNA, template for -RNA and RNA for encapsulation; and a 26S length which represents the other 1/3 of the genome. This 26S length contains the code for the 4 structural proteins cleaved from the 130 kilodalton (kd) precursor (Joklik 1980, Joklik et al. 1984).

Replication of BVDV differs from that of the segmented genome of the alphaviruses. Bovine viral diarrhea virus appears to replicate similar to Uganda S and West Nile viruses, members of the Flavivirus genus of the Togaviridae family. Extracted RNA from these viruses does not bind to oligodeoxythymidylate cellulose indicating its lack of a polyadenylated tail and resulting independence of membrane-associated replication. A single large polypeptide precursor has not been detected, suggesting that the genome of these three viruses serves directly as mRNA for the viral-coded proteins. Direct coding in the viral RNA for the structural proteins implies the existence of multiple functional initiation sites on

the viral RNA (Porterfield et al. 1978, Purchio et al. 1983, Purchio et al. 1984a, Joklik et al. 1984).

In vitro translation of the BVDV genome in message-dependent reticulocyte lysates resulted in the synthesis of a broad spectrum of BVDV specific proteins. Only when polysome-associated RNA was isolated from infected cells and added to the reticulocyte lysate, did the two resulting proteins comigrate in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with the previously identified viral 115 kd and 80 kd proteins (Purchio et al. 1984b). This suggests that factors are present on the polysome-associated RNA which allow for the appropriate termination of the synthesized polypeptides.

Assembly of the BVD virion and maturation occurs in cellular vesicles (Ohmann and Bloch 1982, Mahnel 1984). During viral replication, smooth bi- and multilayered membrane fragments are formed. The viral specific proteins, produced either free in the cellular cytoplasm or in association with the membraneous structures, accumulate in a fibrillar material. As the membrane fragments interact to form vesicles, the fibrillar material and viral proteins are internalized. Bovine viral diarrhea virus has not been observed budding through the cytoplasmic membranes (Ohmann and Bloch 1982, Mahnel 1984) which is in contrast to the morphogenesis of other togaviruses, including hog cholera virus (Scherrer et al. 1970). Mature BVD virions appear to be released from viable cells by exocytosis as would be the case with noncytopathic BVDV, and by disintegration of the vesicle after cell death as with cytopathic BVDV.

Structural characteristics

Bovine viral diarrhea virus is a member of the genus Pestivirus of the family Togaviridae. Other viruses belonging to this group of non-arthropod-borne togaviruses include hog cholera virus, border disease of sheep, equine arteritis virus, lactic dehydrogenase virus of mice and simian haemorrhagic fever virus (Horzinek 1981). Of the viruses listed above, BVDV, hog cholera, and border disease are known to be related, at least on a serological basis (Darbyshire 1962, Osburn et al. 1973, Plant et al. 1973, Horzinek 1981).

The diameter of the pleomorphic BVD virion has been reported to be between 40 and 60 nanometers (nm) (Horzinek 1981). A recent report (Chu and Zee 1984) indicated the viral particle to be much larger, measuring 120 (+/-30) nm in diameter. Surrounding the nucleocapsid was a 5 to 7 nm thick membrane-like envelope with distinct knob-like projections, 4 to 5 nm in diameter. Chu and Zee did find particles in their preparations which matched the 40 to 60 nm particles reported earlier. These particles found in the noninfective fractions did form aggregates with BVDV-specific antiserum, indicating their relatedness to the BVD virion. The authors speculated that these particles may possibly be the viral cores or fragments of disintegrating virions.

Bovine viral diarrhea virion has been determined to contain RNA by its ability to replicate in the presence of 5-iododeoxyuridine. Upon extraction, the RNA was infectious, resistant to DNase, but sensitive to RNase (Diderholm and Dinter 1966). Initial characterization indicated

the BVD viral genome consisted of 3 single-stranded segments. The largest segment having a sedimentation coefficient of 38S with a corresponding molecular weight of 3.22×10^6 daltons (d). The two smaller segments observed had sedimentation coefficients of 31S (2.19×10^6 d) and 24S (1.22×10^6 d) (Pritchett et al. 1975). The significance of the various segments of RNA isolated from BVDV was not determined by these authors; however, they did propose several explanations which included the existence of a polyploid genome, random degradation of the genome, and a segmented genome. Others have shown the BVD genome to be a single segment (Felmingham and Brown 1977, Purchio et al. 1983). Felmingham and Brown (1977) were successful in isolating two forms of viral RNA, a single-stranded form and a double-stranded replicative form as determined by increased resistance of the double-stranded form to hydrolysis by RNase. The most recent study of Purchio et al. (1983) found the BVD virion to be composed of a single 33S length of RNA with a molecular weight of 2.9×10^6 d, representing 8.2 kilobases (kb).

Identification of the structural proteins has not been fully determined. Frost and Liess (1973) did initial studies which identified 3 proteins within the virion; however, determination of the molecular weights was not included in this report. Pritchett and Zee (1975) reported the occurrence of 5 polypeptides within the BVD virion. These identified polypeptides were reported to have molecular weights of 93-110 kd, 70 kd, 50-59 kd, 39 kd, and 27 kd. Other reports on the numbers of polypeptides composing the BVD virion and their estimated molecular weights include: Felmingham and Brown (1977) observing polypeptides of

144 kd, 117 kd, and 100 kd; Matthaeus (1979), 57 kd, 44 kd, and 34 kd; and Coria et al. (1983b) 75 kd, 66 kd, 54 kd, and 26 kd.

Akkina (1982) and Purchio et al. (1984a) reported the presence of 5 polypeptides in dissociated BVDV. The reported molecular weights of these 5 polypeptides are 115 kd, 80 kd, 54 kd (55 kd, Purchio et al.), 45 kd, and 35 kd (38 kd, Purchio et al.). Akkina (1982) had also included a noncytopathic isolate of BVDV in his study. The noncytopathic isolate studied possessed the 115 kd polypeptide, but not the 80 kd. Akkina speculated that noncytopathic isolates were unable to further process the 115 kd polypeptide into the 80 kd polypeptide. Recently, Purchio et al. (1984a) demonstrated a structural similarity between the 115 kd and the 80 kd polypeptide, but a precursor-product relationship was not shown.

MATERIALS AND METHODS

Virus

Six isolates of BVDV are included in this study; three cytopathic, NADL, Singer, and TGAC; and three noncytopathic, 7443, NY-1, and Tifton. Bovine viral diarrhea isolate 7443, was isolated from a persistently infected bull (Coria and McClurkin 1978), and TGAC and Tifton were isolated from a clinical case of MD (Bolin et al. 1985a). The isolates were propagated on monolayers of bovine turbinate cells (BT). The culturing fluid consisted of Eagles minimum essential media with Earles salts (EMEM) supplemented with 0.25% lactalbumin hydrolysate and 15% adult ovine serum known to be free of BVD, border disease, parainfluenza type 3 (PI-3), and infectious bovine rhinotracheitis (IBR) viruses and antibodies. Four-day-old cultures were inoculated with virus at high multiplicity of infection. The virus was allowed to adsorb for 1 hour at 37° C. Culturing fluid was replaced with EMEM without serum supplement. Cytopathic isolates were harvested when cytopathic effect had involved 80 to 90% of the cell sheet. Noncytopathic isolates were harvested after 7 days.

Reagents

Anti-BVDV antiserum was prepared in a calf known to be free of BVD, IBR, PI-3, and bovine parvovirus and also free of antibodies to these viruses. The calf was given an initial inoculation of 10 ml of BVDV-NADL intravenously. One year later, a second intravenous inoculation of 50 ml of BVDV-NADL was administered. The antiserum obtained 3 weeks post-infection had a serum neutralization titer to BVDV of 1:8192.

Bovine anti-BT antiserum was prepared against BT cultures maintained in EMEM with 10% bovine serum in a known BVD virus and antibody free calf. Three 20 ml intravenous injections were given at intervals of 9 and 11 days. The antiserum obtained 10 days post-inoculation had remained free of BVDV serum neutralization antibodies.

Normal bovine serum was obtained from calves housed in isolation and known to be free of BVD virus and antibody.

Immunoglobulins were precipitated with ammonium sulfate from the various bovine sera as described by Garvey et al. (1977).

Goat anti-bovine immunoglobulin was purchased from Nordic Immunological Laboratories (Tilburg, Netherlands).

Rabbit anti-bovine immunoglobulin antiserum was purchased from Cooper Biomedical (Malvern, PA).

Biotinylated Protein A (BPA) and horseradish peroxidase conjugated streptavidin (SA-HRP) were purchased from Zymed Laboratories, Inc. (So. San Francisco, CA).

Iodine [125] labeled Protein A (^{125}IPA) with a specific activity of greater than 30 mCi/mg was purchased from Amersham (Arlington Heights, IL).

Purification of Virus

The infected cultures for each isolate were harvested and pooled. An equal volume of uninfected cells and culture medium was also subjected to the viral purification procedures to serve as a BT cell control.

The pooled culture fluids were subjected to two cycles of freezing and thawing and clarified by centrifugation at 10,000 x g for 1 hour at

4° C. Each pool was made 10 mM with respect to ethylenediaminetetraacetic acid (EDTA). The volume of each pool was reduced to approximately 1/15th of the original volume by hydroextraction with polyethylene glycol (PEG) 20,000 through 10,000 MW exclusion dialyzing tubing at 4° C (Coria et al. 1983b) or by ultrafiltration (Pellicon Cassette System, Millipore, Bedford, MA) over a membrane with an exclusion limit of 1×10^6 daltons.

The virus and BT cell control concentrates were further purified by affinity chromatography using the lectin Ricinus communis agglutinin (RCA) coupled to CH Sepharose 4B (US Biochemical, Inc., Cleveland, OH). The coupled sepharose was packed in a 1.6 x 30 cm chromatography column and kept at 4° C. The concentrates were placed on the RCA lectin column and allowed to adsorb for 18 hours. Following extensive washing of the column with 0.1M phosphate buffer saline pH 7.1 (PBS), the bound material (eluate) was eluted with 0.2M lactose in PBS. The eluates were then collected and concentrated by hydroextraction as previously described or by ultrafiltration with Immersible CX-30 Filters (Millipore, Bedford, MA). Portions of the eluates were lyophilized in 100 µl aliquots.

Protein content of the cell culture stock, cell culture concentrate, and eluate, were determined by the BioRad Protein Assay (BioRad, Richmond, CA) (Table 1).

Virus titers were determined for each fraction by inoculating consecutive 10-fold dilutions onto BT cells (Table 1). The BT cells were either grown in microtiter plates for the detection of cytopathic effect or on coverslips and subsequently examined for BVDV specific immunofluorescence.

High Performance Gel Permeation Chromatography

Lyophilized RCA lectin eluates were subjected to high performance gel permeation chromatography (HPgPC) (Millipore, Bedford, MA) in both native and denatured states (Montelaro et al. 1981). For determination of native polypeptides, the eluates were reconstituted in 100 µl in 10mM sodium phosphate, 50mM sodium chloride buffer pH 7. Denaturing conditions were achieved by including 1.0% sodium dodecyl sulfate (SDS) in the reconstituting buffer and subsequent heating to 100° C for 3 minutes. The mobile phase for the nondenaturing system was 10mM sodium phosphate, 50mM sodium chloride pH 7; for the denaturing system, 0.1% SDS was included in the mobile phase. Both systems were run isocratically at 0.7 ml/min. on tandem 7.5 x 300 mm TSK 3000 SW columns (Beckman Instruments, Inc., Berkeley, CA). This column has a separation range of 30 kilodaltons (kd) to 500 kd under nondenaturing conditions and 10 kd to 100 kd under denaturing conditions. Chromatographs were determined by ultraviolet absorbance at 280nm.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Horizontal sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (LKB-Produkter AB, Bromma, Sweden) was performed in 7.5% acrylamide gels (Fehrnström and Moberg 1977). Prior to being placed on the slab gels, lyophilized eluates were reconstituted to 100 µl in 0.1% SDS, 1.0% 2-mercaptoethanol, 0.1M phosphate buffer pH 7.1 and solubilized by heating to 100° C for 3 minutes. Ten microliters of each eluate and commercial standards (BioRad, Richmond, CA) having a range of 14.4 kd

to 92.5 kd were placed on the gel and electrophoresed at 5 v/cm for 5 hours. The resulting protein bands were stained with Coomassie-blue R-250.

Discontinuous SDS-PAGE was performed as described by Laemmli (1970) with the following modifications: eluates were diluted 1:2 in sample buffer which did not contain 2-mercaptoethanol and the resolving gel was composed of 7.5% acrylamide. After solubilizing by heating at 100° C for 3 minutes, 100 µl of each eluate and 50 µl of both high (40 kd to 250 kd) and low (14.4 kd to 92.5 kd) molecular weight commercial standards (Bio-Rad, Richmond, CA) were placed on the gel and electrophoresed at 30 mAmp per gel for 2.5 hours or until the bromphenol blue tracking dye had migrated off the end of the gel.

Western Blotting

Gels used for Western blotting were run in a vertical slab gel apparatus (LKB Produkter AB, Bromma, Sweden) and soaked in a renaturing buffer composed of 50mM sodium chloride, 10mM Tris, 2mM EDTA, and 4M urea pH 7.0 for 30 min. at room temperature (V. Moennig, Department of Veterinary Microbiology, College of Veterinary Medicine, Iowa State University, Ames, Iowa, Personal Communication). Nitrocellulose replicas of the gels were made by electrophoretic transfer in a Trans-blot Cell (BioRad, Richmond, CA). Electrophoretic transfer was conducted under cooling conditions at 85V for 2 hours in 20% methanol, 25mM Tris base, 192mM glycine, pH 8.3 buffer (Towbin et al. 1979).

Nitrocellulose replicas were blocked in 5% fraction V bovine serum albumin (BSA), 20mM Tris buffered saline pH 7.5 for 1 hour on a rocking

platform. Prior to blocking, the nitrocellulose replicas of the molecular weight standards were stained with India ink as described by Hancock and Tsang (1983).

Detection of Proteins on Nitrocellulose Replicas

For the BPA/SA-HRP system, the nitrocellulose replicas were treated consecutively with each of the following reagents for 2 hours at room temperature on a rocking platform: a 1:5 dilution of the primary antiserum or 1:10 dilution of primary immunoglobulin, a 1:1000 dilution of the rabbit anti-bovine antiserum, a 1:1000 dilution of BPA, and 1:1000 dilution of SA-HRP. The diluent was 20mM Tris buffered saline pH 7.5 (TBS) with 1.0% BSA (TBS-BSA). Three 10 min. washes with 0.05% Tween-20 TBS (TTBS) on a rocking platform followed each incubation period. The horseradish peroxidase was developed with BioRad HRP color development reagent (BioRad, Richmond, CA).

When IPA was used, the nitrocellulose replicas were reacted with a 1:200 dilution of goat antibovine immunoglobulin following the primary immunoglobulin. After three 10 minute TTBS washes, 1 μCi of ^{125}IPA in TBS-BSA was placed on each nitrocellulose replica and incubated for 1 hour at room temperature on a rocking platform. Following three 10 minute washes with TTBS, the replicas were exposed to Kodak XAR-5 at -70° C for 4 to 48 hours (Lum and Reed 1983).

RESULTS

Purification of BVDV with Ricinus communis Agglutinin Lectin

Purification parameters obtained with the RCA lectin method using 6 isolates of BVDV were inconsistent (Table 1). Low percentages of infectivity were recovered for 3 isolates, 8% for TGAC, and 1% for NADL and NY-1. For the remaining isolates, the percentage of recovered infectivity was higher, 80% for Singer, 160% for Tifton, and 200% for 7443. With the latter virus preparations, some concentration was achieved. In the best case (isolate 7443), 80-fold (1.9 $\log_{10}$) purification was recorded.

High Performance Gel Permeation Chromatography

The isolates examined by HPgPC were NADL, NY-1, Singer, and 7443, along with the BT cell control. In the nondenaturing system, the major portion of the viral and BT cellular proteins came off the column in the void volume having a molecular weight of greater than 440 kd. A secondary peak in the molecular weight range of 66 kd to 96 kd was seen for NADL, NY-1, and 7443 (Figure 1).

In the denaturing system, an increase in numbers and a decrease in molecular weight of the polypeptides were seen for the four virus preparations and the BT cell control (Figure 2). The major peak occurred in the molecular weight range of 45 kd to 66 kd, with a secondary peak smaller than the 12.5 kd marker. Several small peaks were observed within the primary peak, but distinct differences could not be seen between the individual virus isolates and/or the BT cell control.

Table 1: Infectivity of BVDV during concentration and purification by hydroextraction and affinity chromatography

Preparative Steps	Total Infectivity (ID_{50})	Recovery (%)	Total Protein (mg)	Infectivity (ID_{50}/mg protein)
Singer				
Stock	$10^{9.2}$	100	75	$10^{7.3}$
Concentrate	$10^{9.5}$	200	115	$10^{7.4}$
Eluate	$10^{9.1}$	80	5	$10^{7.3}$
NADL				
Stock	$10^{9.3}$	100	80	$10^{7.4}$
Concentrate	$10^{8.0}$	5	107	$10^{6.0}$
Eluate	$10^{7.1}$	1	19	$10^{5.8}$
TGAC				
Stock	$10^{8.0}$	100	47	$10^{6.3}$
Concentrate	$10^{7.3}$	20	65	$10^{5.5}$
Eluate	$10^{6.9}$	8	7	$10^{6.1}$
7443				
Stock	$10^{9.0}$	100	44	$10^{6.9}$
Concentrate	$10^{9.2}$	160	88	$10^{7.3}$
Eluate	$10^{9.3}$	200	3	$10^{8.8}$
NY-1				
Stock	$10^{7.0}$	100	56	$10^{4.8}$
Concentrate	$10^{6.2}$	16	88	$10^{4.3}$
Eluate	$10^{5.1}$	1	8	$10^{4.2}$
Tifton				
Stock	$10^{7.6}$	100	50	$10^{5.9}$
Concentrate	$10^{7.7}$	126	115	$10^{5.6}$
Eluate	$10^{7.8}$	160	18	$10^{6.6}$
BT Cell Control				
Stock	---	---	50	---
Concentrate	---	---	98	---
Eluate	---	---	17	---

Figure 1 HPgPC tracings of the native polypeptides obtained from nondenatured BVD virus preparations. Arbitrary values have been assigned to the ordinate scale

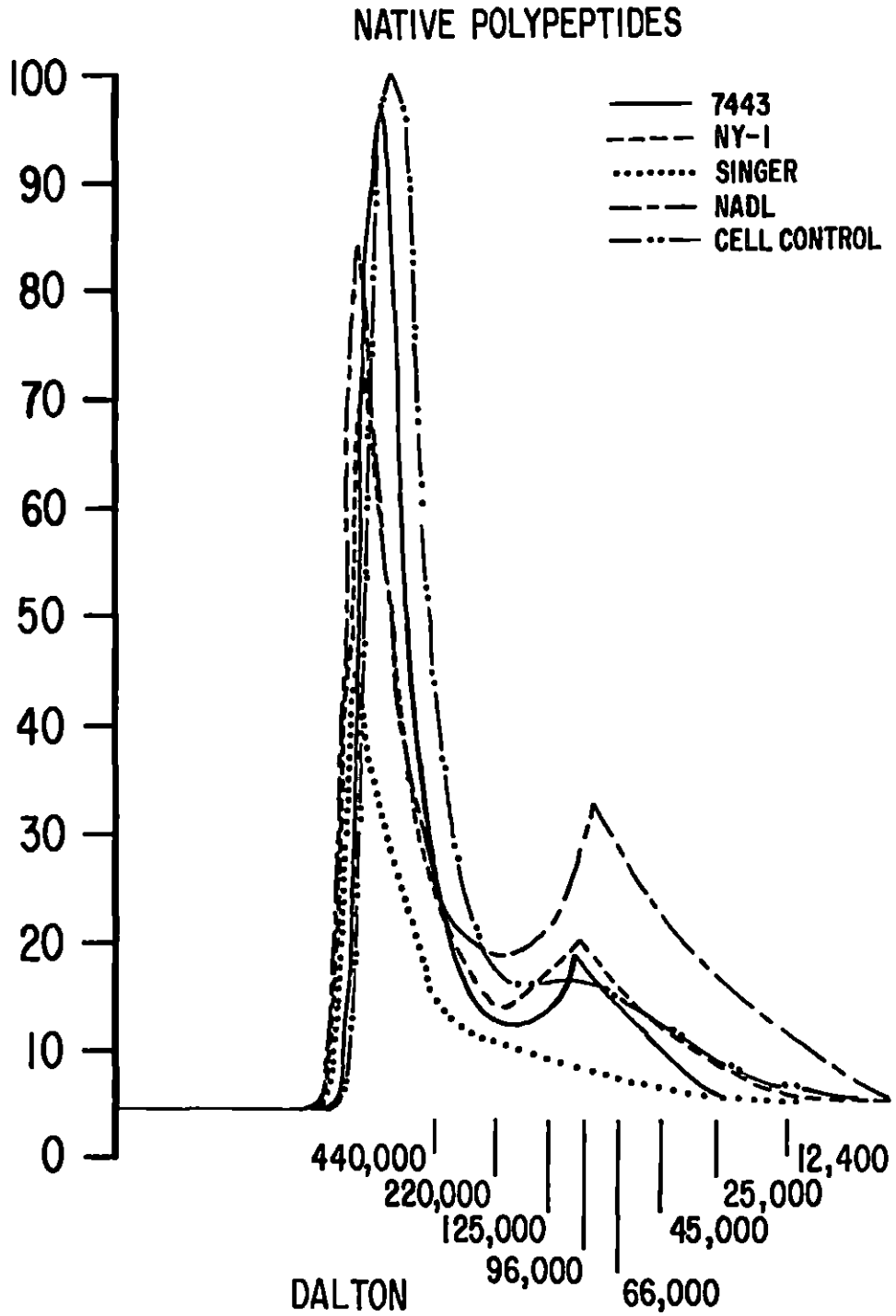
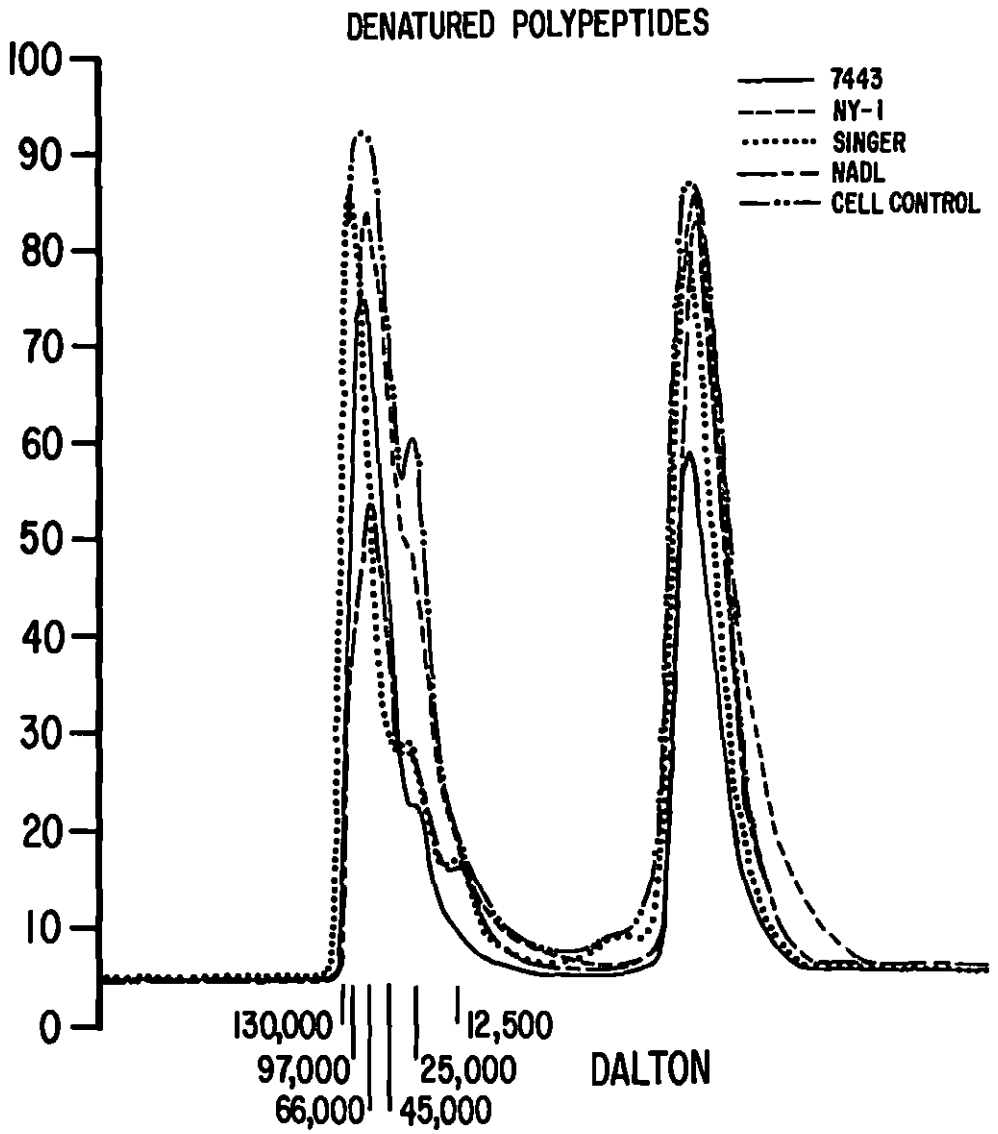

NATIVE POLYPEPTIDES
7443
NY-1
SINGER
NADL
CELL CONTROL
100
90
80
70
60
50
40
30
20
10
0
440,000
220,000
125,000
96,000
66,000
45,000
25,000
12,400
DALTON

Figure 2 HPgPC tracings of the polypeptides obtained from denatured BVD virus preparations. Arbitrary values have been assigned to the ordinate scale


DENATURED POLYPEPTIDES
100
90
80
70
60
50
40
30
20
10
0
7443
NY-1
SINGER
NADL
CELL CONTROL
130,000
97,000
66,000
45,000
25,000
12,500
DALTON

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Continuous SDS-PAGE of NADL, NY-1, Singer, 7443, and BT cell control resulted in the separation of several polypeptides (Figure 3). The estimated molecular weights of the separated polypeptides of the four isolates were similar to those previously reported by Pritchett and Zee (1975), Felmingham and Brown (1977), Matthaeus (1979), Akkina (1982), Coria et al. (1983b), and Purchio et al. (1984a). The virus preparations and the BT cell control each had 5 polypeptides which migrated to similar distances from the origin and ranged in molecular weights from 24 kd to >92.5 kd. Variations between the virus isolates and BT cell control could be noted. The variations were contained within 4 molecular weight ranges. Isolates NADL, 7443, and NY-1 had additional bands above the 92.5 kd marker, while NADL and Singer had several bands in the molecular weight ranges of 30 kd to 50 kd and <20 kd. The fourth area of variation is in the molecular weight range of 80 kd to 90 kd, with NADL having the only band.

Isolates Singer, NADL, TGAC, 7443, NY-1, and Tifton, along with the BT cell control were also examined with discontinuous SDS-PAGE (Figure 4). Banding patterns for all of the virus isolates and BT cell control were relatively similar with variations occurring in 3 molecular weight areas. All of the virus preparations and the BT cell control had a band within the molecular weight range of 66 kd to 95 kd; however, individual locations varied. Isolates NADL, TGAC, Tifton and the BT cell control each had a band within molecular weight ranges of 45 kd to 50 kd and 35 kd to 40 kd. Isolate 7443 also has a band within the molecular weight range of 35 kd to 40 kd.

Figure 3 Continuous SDS-PAGE of BVD isolates 7443, NY-1, Singer, and NADL, and the BT cell control. Molecular weight standards are included

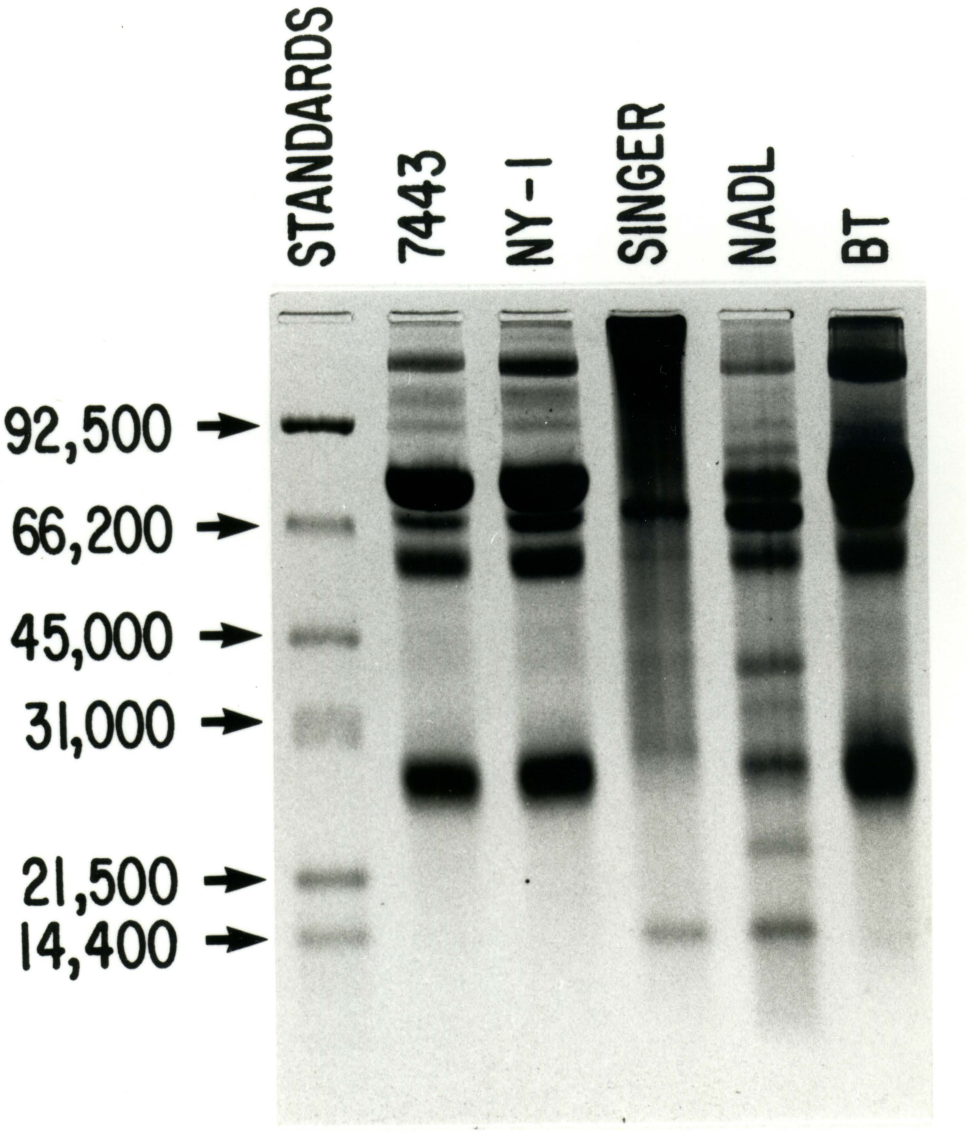
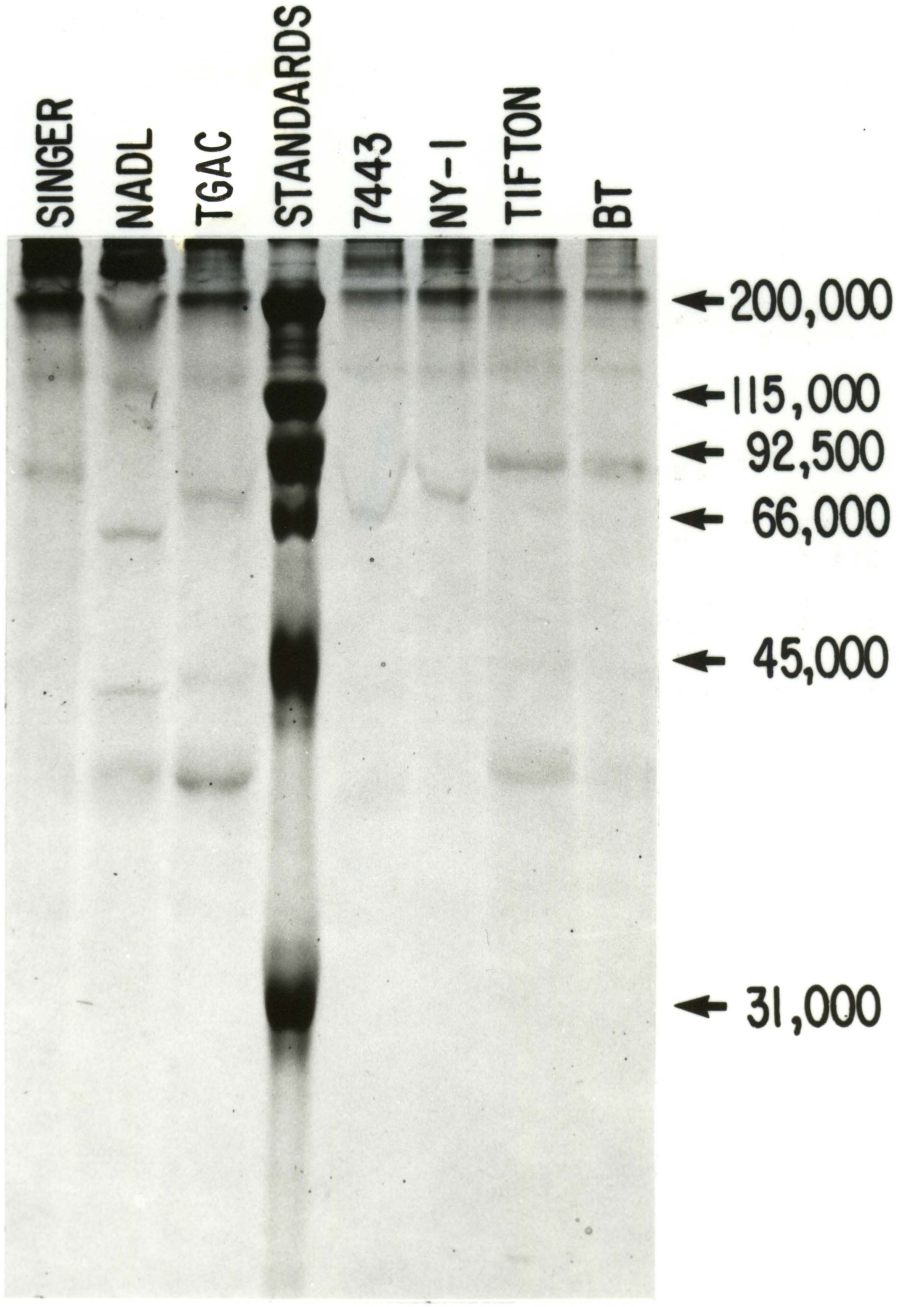

STANDARDS
7443
NY-1
SINGER
NADL
BT
92,500
66,200
45,000
31,000
21,500
14,400

Figure 4 Discontinuous SDS-PAGE of BVD isolates Singer, NADL, TGAC, 7443, NY-1, and Tifton, and the BT cell control. Molecular weight standards are included


SINGER
NADL
TGAC
STANDARDS
7443
NY-1
TIFTON
BT
200,000
115,000
92,500
66,000
45,000
31,000

Identification of Viral Specific Protein

Antigenic reactivity could not be observed on the nitrocellulose replicas when the standard method, as described by Leammli (1970) was followed; or if this method was modified by the elimination of 2-mercaptoethanol from the electrophoretic denaturing buffer. Only after soaking in the renaturing buffer prior to trans-blotting was antigenic reactivity restored.

Reactions of specific antibody-antigen complexes on the renatured nitrocellulose replicas with BPA and SA-HRP were weak. In order to increase sensitivity, goat anti-bovine immunoglobulin and ^{125}IPA were used.

Two polypeptides in the virus preparations and one in the BT cell control were labeled on the nitrocellulose replicas by specific BVDV immunoglobulin. The larger molecular weight polypeptide was seen in both isolates and BT cell control while the smaller polypeptide was present only in the virus isolates. Molecular weights of these polypeptides exceeded 200 kd.

Anti-BT immunoglobulin also labeled a polypeptide with a molecular weight of greater than 200 kd in the six isolates and the BT cell control. This polypeptide corresponds to the larger polypeptide labeled by specific BVDV immunoglobulin.

Immunoglobulin obtained from a normal calf failed to label any polypeptides on the nitrocellulose replicas of the six virus preparations and the BT cell control.

DISCUSSION

Purification methods which result in recovery of relatively high levels of infectivity for most viruses are not effective with BVDV. These physical methods are believed to disrupt the BVD viral envelope, reducing infectivity. In order to recover more infectivity by avoiding mechanical stress, Moreno-Lopez et al. (1981) proposed a gentler purification method. This method involved the use of affinity chromatography with Crotalaria juncea (Cj) lectin which binds the viral particles via terminal galactosyl residues. Since the Cj lectin has a greater affinity for galactose when a glucosyl residue is adjacent, the virus is easily desorbed from the lectin with lactose. Using this method, Moreno-Lopez et al. (1981) were able to recover 65% of the BVD viral infectivity, but could not demonstrate the presence of any intact viral envelopes in the purified preparations when these were examined by negative stain electron microscopy. Coria et al. (1983b) successfully showed in negative stain electron micrographs that the viral envelope is left intact during this purification method.

Another lectin with the same affinity as the Cj lectin, Ricinus communis agglutinin (RCA), was used by Neukirch et al. (1981) to purify hog cholera virus. Using this lectin in affinity chromatography, Neukirch et al. recovered 40% to 60% of the hog cholera virus infectivity.

Purification of BVDV with the RCA lectin was attempted, since hog cholera virus and BVDV belong to the same virus group. The inconsis-

tency seen in the purification of six BVD isolates using this method does not fully support the reported effectiveness of viral purification by affinity chromatography with RCA lectin. The difference in the rates of concentration and purification between the various isolates can not be easily explained (Table 1). Infectivities recovered as a result of RCA lectin affinity chromatography for 7443 and Tifton were 200% and 160% respectively. This high yield of infectivity is in contrast to that seen for isolates NADL, TGAC, and NY-1, which had infectivity reduced to less than 10%. Eighty percent of the infectivity was recovered for Singer. This rate is in closer agreement with rates reported earlier for affinity chromatography.

The step which appears to be most critical in the purification procedure is concentration by hydroextraction. When total infectivity in the concentrate was reduced by > 0.7 $\log_{10}$ of virus, as for isolates NADL, TGAC, and NY-1, low levels of total infectivity were recovered from the RCA column (eluate). In contrast, when total infectivity was increased in the concentrate, as for Singer, 7443, and Tifton, higher levels of total infectivity were obtained in the eluate.

Explanations for the increase in infectivity at this step are conjectural. The concentrates were derived from hydroextraction of the cell culture lysates (stocks) with PEG-20 at 4° C for up to 36 hours. During this time period, cellular hydrolases released as a result of freezing and thawing of the stocks had the opportunity to degrade membrane-bound vesicles in which viral maturation occurred, liberating more viral particles (Ohmann and Bloch 1982, Mahnel 1984). In contrast,

aliquots of the stocks reserved for titration were held at -90° C, preventing the degradation of the vesicles. Low molecular weight polyethyleneglycol, which may have crossed the dialyzing membrane, could also be responsible for additional degradation of the infectious vesicles. This may be especially true for the noncytopathic isolates since their replication does not destroy the cell, and hydrolases are not present in significant amount in the cell culture supernatant during the replication cycle.

Pritchett and Zee (1975) used a series of sucrose gradients to purify ^{3}H-labeled BVDV from ^{14}C-labeled cell proteins. They recovered 45% of the viral infectivity after purification, but were unsuccessful in separating cellular proteins from the viral proteins. Felmingham and Brown (1977) recovered 79% of the viral infectivity using ammonium sulphate precipitation, Tween-80, and sucrose gradient purification procedures. They found labeled cellular proteins banding in similar locations as the labeled BVD viral proteins after isopycnic-zonal centrifugation. Moreno-Lopez et al. (1981) reported that purification methods utilizing the Cj lectin also resulted in the co-purification of cellular proteins with BVD viral proteins. Like many other methods of purification, use of the RCA lectin did not effectively remove the cellular proteins. As can be seen in Table 1, a large portion of protein from the BT cell control was bound by the RCA lectin. As a result, RCA lectin affinity chromatography does not effectively remove cellular proteins during the purification process.

Although the RCA lectin was inefficient in removing cellular proteins from the viral preparations, some purification was achieved. With isolate 7443, an 80-fold increase in the amount of infectivity relative to the quantity of protein present in the eluate was seen over that of the stock. This rate of purification compares favorably to that reported by Pritchett and Zee (1975). By using sucrose gradient and isopycnic centrifugation, they obtained a 100-fold purification rate.

Separation and molecular weight determination of the polypeptides which compose BVDV were attempted by means of HPgPC in both a nondenaturing and a denaturing system. Neither system resulted in the separation or identification of polypeptides which could distinguish between isolates and/or the BT cell control. Preparations of isolates Singer, NADL, NY-1, and 7443, and the BT cell control did not separate into smaller peaks under nondenaturing HPgPC (Figure 2). Peaks with similar molecular weights, greater than 440 kd, were seen for the 4 isolates and BT cell control. Significance of the secondary peak seen for isolates NADL, 7443, and NY-1 can not be determined since the BT cell control may also possess this peak. Upon denaturing, several smaller polypeptides were separated for each BVD isolate and the BT cell control (Figure 3). Peaks seen for each isolate and the BT cell control co-migrated, again making resolution between the isolates and the BT cell control impossible. A large secondary peak is present for the 4 isolates and BT cell control, emerging considerably later than the 12.5 kd marker. This peak most likely results from use of 2-mercaptoethanol in the denaturing process (Montelaro et al. 1981).

Failure to demonstrate distinct differences within these samples in both the native and denatured modes was due in part to the quantity of cellular proteins which co-purified with the BVD isolates on the RCA lectin. Incomplete resolution of the separated peaks in the denatured chromatographs may be attributed to the fact that molecular weight ratios of the separated peaks were smaller than the minimum molecular weight ratio (Rm) of 2 required for complete resolution by the TSK 3000 SW column (Pfannkoch et al. 1980). Another factor to be considered in the failure to obtain distinctions within the isolates is incomplete denaturation of the viral polypeptides prior to chromatographing.

Continuous SDS-PAGE of the isolates Singer, NADL, 7443, and NY-1 resulted in the separation of polypeptides which demonstrated distinct differences between the isolates, especially between the cytopathic and noncytopathic isolates. However, SDS-PAGE of the BT cell control resulted in several polypeptides with estimated molecular weights similar to those of the other isolates, making identification of the polypeptides of viral origin impossible (Figure 3).

In an effort to make this identification, discontinuous SDS-PAGE and electrophoretic transfer of separated polypeptides to nitrocellulose (Western blotting) with subsequent labeling staining of the discontinuous SDS electrophoretic gel prior to blotting revealed slight variations in the molecular weights and numbers of polypeptides composing each isolate. The BT cell control contained polypeptides of similar molecular weights to those of isolates Singer, NADL, TGAC, 7443, NY-1, and Tifton (Figure 4).

To maintain antigenic integrity of the viral polypeptides in the Western blots, modifications to the standard methods of Laemmli (1970) for SDS-PAGE and Towbin et al. (1979) for Western blotting were required. In SDS-PAGE, 2-mercaptoethanol was omitted from the sample buffer. Prior to electrophoretic transfer of the separated polypeptides from the electrophoretic gel to nitrocellulose, renaturation of the polypeptides was necessary. Soaking the gel in the renaturation buffer containing 4M urea removes SDS, allowing the polypeptides to assume a configuration closer to that of the native polypeptide.

Detection of the bound specific immunoglobulin was done with Protein A. Two Protein A systems were used, BPA followed by HRP-SA and ^{125}IPA. Low levels of reactivity were detected with the BPA/HRP-SA system. The use of autoradiography with ^{125}IPA increased sensitivity.

Anti-BVDV immunoglobulin labeled two protein bands on each of the viral nitrocellulose replicas, while a single protein band was labeled on the BT cell control. These labeled protein bands had molecular weights of greater than 200 kd, with the molecular weight of the viral specific protein band being smaller than the nonspecific band. When the nitrocellulose replicas were labeled with anti-BT immunoglobulin, a protein band of greater than 200 kd was identified in the viral and the BT cell control replicas. Immunoglobulin from a normal calf did not identify any protein band.

Anti-BVDV immunoglobulin labeled a protein band on the BT cell control which corresponds to one of the protein bands seen on the viral

replicas. A protein band of greater than 200 kd also was labeled by anti-BT immunoglobulin on the viral and BT cell control replicas. These proteins are most likely of cell origin. Antibodies to these cellular antigens in the anti-BVDV and anti-BT immunoglobulins are due to the presences of cellular protein complexes in the immunizing preparations due to viral induced and mechanically induced lysis of BT cells. The total lack of reactivity seen with the normal calf immunoglobulin indicates that this is an induced immune response following exposure to BT cell debris.

The virus-specific protein bands retained their antigenicity, but were incompletely dissociated. Specific polypeptides were not identified. Failure to identify individual polypeptides may be the result of the methodology. The successful electrophoretic transfer of the smaller polypeptides took place since all of the molecular weight markers could be identified on the nitrocellulose replica. Antigenicity of the smaller denatured polypeptides was not restored by the renaturing buffer. Akkina (1982) successfully immunoprecipitated 1% NP40, 0.5% deoxycholate, and 1% trasylol dissociated BVD viral polypeptides from cell lysates; however, this step occurred prior to the solubilizing step required in SDS-PAGE. In contrast, in this study dissociation, denaturation, and solubilization of the viral polypeptides for SDS-PAGE occurred in the same step. Consequently, failure to restore antigenicity to the separated polypeptides could be due to incomplete removal of the SDS in the renaturation step concealing the haptens, inability of the polypeptides

to regain their original antigenicity, or complete denaturation resulting in polypeptides which have lost the specificity of the whole virion.

Earlier studies of the molecular weights of the structural polypeptides of BVDV made use of radiolabeling. Pritchett and Zee (1975) labeled cellular proteins with ^{14}C and viral proteins with ^{3}H leucine. After electrophoresis, location of the radiolabeled products was determined, and origins of the polypeptides could be distinguished by the type of label present. A large amount of ^{14}C radiolabeled cellular protein co-migrated during electrophoresis with ^{3}H radiolabeled virus. Matthaeus (1979), Akkina (1982), and Purchio et al. (1984a) immunoprecipitated ^{35}S methionine labeled BVDV from cell cultures thus eliminating most of the cellular polypeptides. Although, BVDV is not easily radiolabeled, these methods may prove to be the most beneficial in future studies of BVDV.

CONCLUSION

Affinity chromatography with the RCA lectin was able to achieve a purification rate for BVDV which is comparable to other purification methods. The lectin purified virus retained its infectivity, although in some instances at moderately reduced levels. Only one of the 6 isolates purified with this method demonstrated a purification rate similar to the 100-fold purification rate reported by Pritchett and Zee (1975).

Factors contributing to the low purification rates of the other viral isolates can only be surmised. These factors may include cytopathogenicity of the isolate, level of infectivity present in the initial virus stock, and length of time the viral concentrates were exposed to PEG-20 during hydroextraction.

Identification of the structural polypeptides composing the BVD virion and their respective molecular weights could not be determined by HPgPC, continuous SDS-PAGE or discontinuous SDS-PAGE. A common difficulty in using these methods which could not be overcome was the failure to identify whether the separated polypeptides were of viral or cellular origin. This inability to identify the source of the polypeptides is associated with the affinity of the RCA lectin for galactosyl residues present on both the viral polypeptides and cellular polypeptides.

It had been hoped that subsequent use of Western blots and labeling of the electrophoretically separated polypeptides with specific immunoglobulin would overcome this problem. Akkina (1982) had successfully immunoprecipitated the dissociated polypeptides of BVDV prior to SDS-

PAGE. Since these dissociated polypeptides maintained their antigenicity, it was presumed that polypeptides resulting from dissociation, denaturation and solubilization of whole BVDV prior to SDS-PAGE would also be antigenic. During electrophoresis and Western blotting, these polypeptides had lost their antigenicity. In order to restore antigenicity to these polypeptides on the nitrocellulose replicas, the electrophoretic gels were soaked in a renaturing buffer prior to Western blotting. This renaturing step restored antigenicity to the large protein aggregates but not the small polypeptides.

Other renaturing buffers have been formulated (Bers and Garfin 1985). Use of one of these could result in renaturation of the solubilized and denatured viral polypeptides, allowing for the identification of these polypeptides on the nitrocellulose replicas as of viral origin. This in turn would allow similar studies to be conducted in which radiolabeling would not be required for determining molecular weights of the structural polypeptides of BVDV.

A large proportion of the viral and BT cell control proteins deposited on the nitrocellulose replicas were not completely denatured prior to electrophoresis. Both anti-BVDV and anti-BT immunoglobulins identified a large band on the nitrocellulose replica with the same electrophoretic mobility. This protein aggregate may be cellular proteins which co-purified with the isolates.

Due to the inability of the RCA lectin to adequately remove the cellular proteins from the viral preparations, separation techniques

such as HPgPC, continuous SDS-PAGE, and discontinuous SDS-PAGE could not be used to successfully identify the viral polypeptides. These techniques may be useful in the identification of viral polypeptides only if a viral preparation can be obtained free of host cell protein.

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