Monoclonal Antibodies to the p80/125 and gp53 Proteins of Bovine Viral Diarrhea Virus: Their Potential Use as Diagnostic Reagents

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ABSTRACT

Monoclonal antibodies reactive to the bovine viral diarrhea virus (BVDV) protein gp53 were produced and characterized. These antibodies and our panel of anti-p80/125 monoclonal antibodies were tested for their cross-reactivity with 11 different North American and European (Danish) BVDV strains and isolates including viruses of both cytopathic and noncytopathic biotypes. The four anti-gp53 monoclonal antibodies were neutralizing for the homologous Danish cytopathic isolate and cross-reacted with all BVDV strains examined except for the Draper strain. Further, anti-gp53 monoclonal antibodies neutralized the majority of BVDV strains examined. The anti-p80/125 monoclonal antibodies cross-reacted with all eleven strains and isolates tested. This indicated that various strains of BVDV have common epitopes. The broad cross-reactivities demonstrated by these monoclonal antibodies suggest that a pool of these antibodies may be used for detection of BVDV cellular contamination or for virus isolation, in place of polyclonal antiserum.

RÉSUMÉ

Des anticorps monoclonaux reconnaissant la protéine gp53 du virus de la diarrhée à virus des bovins (BVDV) ont été caractérisés. Les réactions croisées entre ces anticorps et les anticorps monoclonaux anti-p80/125 ont été comparées avec 11 souches différentes (américaines et européennes (danoise)) du BVDV incluant les biotypes cytopathogène et noncytopathogène. Les quatre anticorps monoclonaux anti-gp53 étaient neutralisants contre la souche homologue cytopathogène danoise et réagissaient aussi contre toutes les souches examinées à l'exception de la souche Draper. Les anticorps monoclonaux anti-gp53 ont aussi neutralisé la majorité des souches examinées. Les anticorps monoclonaux anti-p80/125 ont aussi réagi avec les 11 souches et prélèvements étudiés. Les résultats de cette étude indiquent que plusieurs souches de BVDV ont des épitopes communs et que les réactions croisées observées entre ces anticorps monoclonaux démontrent qu'un mélange de ces anticorps au lieu d'un antisérum polyclonal pourrait être utilisé afin de démontrer la présence du virus. ***(Traduit par Dr Pascal Dubreuil).***

INTRODUCTION

Bovine viral diarrhea virus (BVDV), is currently classified as a pestivirus within the family Togaviridae (1,2). It is the causal agent of a multifarious syndrome comprising subclinical infections, reproductive disorders, congenital defects, enteritis and respiratory signs and the inevitably fatal syndrome, mucosal disease (MD) (3). Results obtained in recent years indicate that two biotypes of BVDV are required to induce MD: a noncytopathic (i.e. not showing overt cytopathic effect in tissue culture) and a cytopathic virus strain. Following establishment of a persistent fetal infection with noncytopathic virus in the first trimester of gestation, the calf is born and remains viremic and antibody-negative. After postnatal encounter with a second, cytopathic virus strain the animal may develop clinical MD (4-6). The origin of the cytopathic virus, i.e. whether the cytopathic virus arises by mutation of the noncytopathic virus or represents a later infection, remains to be fully established (7-9).

Due to fetal infections resulting in virus persistence, contamination of fetal bovine and calf serum as well as primary fetal bovine cell lines is a common phenomenon (10,11). This is a problem of great concern both in diagnostic work and in vaccine production where requirements for the complete exclusion of extraneous agents is paramount. Thus other alternatives to fetal bovine serum such as lamb or horse serum for supplementation are often used, when feasible, in order to mitigate against BVDV contamination. Even with these precautions, however, BVDV contamination of tissue cultures can still occur, and because of the noncytopathic nature of these viruses their presence in tissue culture can only be detected by immunological methods.

Hyperimmune polyclonal antisera for detection of BVDV in tissue culture are commonly used. However, the production and use of antisera can be problematic. The main reason for this is that purification of the virus is very difficult because of its low level of production, density, instability and cell-associated nature. This leads

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inevitably to production of antibodies to cellular antigens in hyperimmune sera and can cause high background reactions to occur in immunological assays. These cellular reactions can be reduced but not totally eliminated by growth of BVDV in cells derived from the species used for the production of BVDV antiserum. Another problem is one of consistency in the quality of antiserum obtained, which may depend on the virus strain(s) and the immunization schedule used. Monoclonal antibodies have the advantage of high specificity which virtually eliminates background reactions in many immunological assays. However, a potential disadvantage of monoclonal antibodies for diagnostic reagents is their inherent monospecificity to single epitopes, as virus strains lacking a particular epitope may escape detection. This problem, however, can be solved by using a pool of monoclonal antibodies which together cross-react with all known virus strains and isolates.

In an effort to produce good reagents for BVDV diagnosis, particularly for the identification of noncytopathic BVDV in tissue culture, as contaminants or for diagnostic virus isolation, we produced four monoclonal antibodies reactive to the BVDV glycoprotein gp53. These monoclonal antibodies, together with our panel of anti-p80/125 monoclonal antibodies (Deregt *et al*, unpublished observations) were examined for their cross-reactivity with 11 different BVDV strains and isolates, including four noncytopathic viruses, by virus neutralization and an indirect immunoperoxidase assay.

MATERIALS AND METHODS

VIRUS AND CELLS

A Danish cytopathic BVDV isolate (12,13), designated DCP, was the virus used for immunization of mice in hybridoma production. The cytopathic strains, Singer, Oregon, and NADL and the noncytopathic Draper strain were obtained from Dr. A. Bouffard at the Animal Diseases Research Institute, Nepean, Ontario. The Singer strain was subsequently plaque-purified. The noncytopathic virus NY-1 (New York-1) was obtained from the American Type Culture Collection, Rockville, Maryland. All Danish viruses (cytopathic and noncytopathic) were isolated by one of us (HBO) and purified by the limiting dilution method. These viruses were designated as DCP,-2,-3,-4 for (Danish) cytopathic viruses and DNCP,-2 for noncytopathic viruses. All viruses were propagated in BVDV-free Madin Darby bovine kidney (MDBK) cells using minimal essential medium (MEM-Gibco Canada Inc., Burlington, Ontario) containing 5% horse serum (Gibco) as the growth medium.

PRODUCTION OF MONOCLONAL ANTIBODIES

Balb/C mice were immunized with partially purified cytopathic (DCP) BVDV by a modification of the procedure of Cianfriglia *et al* (14). Danish cytopathic BVDV was concentrated from the clarified media of 50 flasks (175 cm^2, Falcon, Canlab, Edmonton, Alberta) of infected cells by ultracentrifugation (18,000 rpm for 4.5 h in a Beckman R21 rotor) and resuspended in 1 mL of saline. Mice were immunized by intraperitoneal (IP) injection of 100 μL of concentrated virus in complete Freund's adjuvant (CFA) on days 15 and 8 before cell fusion, followed by IP injection of 500 μL and 200 μL of virus in saline on days 3 and 2, respectively. These procedures were completed before the new Canadian Council on Animal Care guidelines on the use of CFA were issued. Splenocytes were fused with P3/NSI/1-Ag4-1 (NS-1) cells (15) using polyethylene glycol essentially as described previously (16). An immunoperoxidase test (see below) against virus-infected and noninfected cells was used to screen hybridoma spent media for BVDV-specific monoclonal antibodies. Hybridoma cell lines were subsequently subcloned by the limiting dilution method to ensure monoclonality. Monoclonal isotypes were determined by an enzyme immunoassay of hybridoma supernatants (Hyclone Laboratories, Logan, Utah).

INDIRECT IMMUNOPEROXIDASE ASSAYS

For screening hybridoma spent media and for monoclonal antibody cross-reactivity studies with different BVDV isolates, virus infection of MDBK cells in 96 well microtiter plates (Microtest III, Falcon, Canlab) was allowed to proceed for two to five days. Cell monolayers were then washed with phosphate-buffered saline (PBS) and fixed with 20% acetone (in PBS) containing 0.2 g/L of bovine albumin, fraction V (Calbiochem, San Diego, California) for 30 min at room temperature (RT). After fixation the plates were allowed to dry for 3 h at 37°C and then used immediately or stored in plastic bags at -63°C. Plates were incubated with 50 μL monoclonal antibody as undiluted tissue culture supernatants or as ascites fluid, diluted 1/100 in ST buffer (0.5 M NaCl, 0.05% Tween-20 [Bio-Rad Lab., Richmond, California]), for 1.5 h at 37°C. Plates were then rinsed in wash buffer (PBS, 0.05% Tween-20) and further incubated with 50 μL of goat antimouse IgG and IgM conjugated to horseradish peroxidase (Cappel, Organon Teknika Inc., Scarborough, Ontario) diluted 1/1000 in ST buffer containing 2% normal rabbit serum (Gibco) for 45 min at 37°C. Following rinsing of plates in wash buffer, the immunolabeling was visualized by incubation with substrate containing 1 mL of a 0.8% solution of 3-amino-9-ethylcarbazole (Sigma Chemical, St. Louis, Missouri) in N,N-dimethylformamide (Sigma) per 19 mL 0.1 M acetic acid/acetate buffer, pH 5, and 0.01% hydrogen peroxide for 30 min at RT. Bovine polyclonal anti-BVDV serum was used as a positive control serum. Negative controls consisted of noninfected MDBK cells and the use of monoclonal antibodies to bovine coronavirus (16,17).

Alternatively, BVDV antigens were detected by indirect immunoperoxidase staining of cytospin preparations as described in detail elsewhere (18). Briefly, cytospin preparations, following blocking of endogenous peroxide activity and nonspecific antibody binding, were incubated with monoclonal antibodies (as mouse ascites fluids) at a 1/100 dilution. After incubation with goat antimouse IgG and IgM conjugated to horseradish peroxidase (Boehringer-Mannheim Canada, Dorval, Quebec), at a 1/750 dilution, the specific binding was

visualized with 3-amino-9-ethylcarbazole substrate.

NEUTRALIZATION ASSAY

To test the virus-neutralizing ability of monoclonal antibodies to various BVDV strains and isolates, twofold dilutions of monoclonal antibodies (mouse ascites fluids) in MEM from an initial 1/100 dilution were incubated with 100 median tissue culture infectious doses ($TCID_{50}$) of virus in 96-well microtiter plates (Microtest III, Falcon, Canlab) for 1 h at 37°C. Madin Darby bovine kidney cells were then added to the mixture and further incubated for four or five days until 100% of the cells in the virus control wells were infected. The endpoint of neutralization was calculated by determining the maximum dilution in which an estimated 50% of the cells or less showed either cytopathic effect or evidence of BVDV infection as determined by an indirect immunoperoxidase assay.

RADIOIMMUNOPRECIPITATION

To determine the protein specificity of monoclonal antibodies specific for BVDV, virus was allowed to adsorb to cells (approx. 0.5 $TCID_{50}$/cell) for 2 h. Mock-infected and virus-infected cells were labeled at 20-24 h postinfection for 3-4 h with 50 μCi/mL of L-^{35}S-methionine (Amersham Canada Ltd., Oakville, Ontario) after a 1 h incubation of cells in methionine-free medium. Cells were then harvested and lysates were immunoprecipitated as previously described (16).

POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) AND FLUOROGRAPHY

Immunoprecipitates were resuspended in electrophoresis sample buffer (19) and boiled for 2 min. Electrophoresis and fluorography were performed as previously described (16).

WESTERN IMMUNOBLOTTING

Western immunoblotting of mock-infected and BVDV-infected cell lysates were performed as previously described (12).

RESULTS

PROTEIN SPECIFICITY

Following the initial screening of hybridoma supernatants for BVDV reactivity, positive samples were tested for virus protein specificity by immunoblotting. By this method a protein of 52-55K was prominently labeled by several of the supernatants. Other protein bands appeared far less prominently and were considered to result from nonspecific labeling (data not shown).

To further establish the protein specificity of BVDV positive monoclonal antibodies, cells were infected with BVDV and labeled with ^{35}S-methionine. Cell lysates were prepared and immunoprecipitated with these antibodies and the immunoprecipitated proteins were fractionated by polyacrylamide gel electrophoresis (PAGE) and visualized by fluorography. Four monoclonal antibodies, 157-77, 348-14, 450-19 and 477-77 (Table I) immunoprecipitated the same proteins from Singer-, DCP-, DNCP- and NY-1-infected cells, although virus-dependent variation in protein mobility was observed (Fig. 1, lanes 3-6). These four monoclonal antibodies immunoprecipitated a prominent protein, known to be a glycosylated protein (gp) (2, data not shown) of an approximate molecular size of 53K (gp53). Unexpectedly, several other BVDV glycoproteins, gp75 (a relatively minor protein), gp62 and gp25 and two higher molecular weight protein species (of approx. 175 K and 125-135K) were also observed in immunoprecipitations with these monoclonal antibodies. An initial experiment suggests that the latter protein is also glycosylated (data not shown). For comparison, the profile of BVDV-specific proteins as observed from immunoprecipitations of Singer-infected cell lysates with bovine anti-BVDV serum is shown in lane 1. In lane 8, for further comparison, an immunoprecipitation result with a representative anti-p80/125 monoclonal antibody is shown. There also appeared to be a reaction to a higher molecular weight protein, which may be p175, in immunoprecipitation of infected cell lysates with anti-p80/125 monoclonal antibodies, as previously noted (Deregt *et al*, unpublished observations). Its significance remains to be determined.

VIRUS NEUTRALIZATION

To test the virus-neutralizing abilities of anti-gp53 monoclonal antibodies, dilutions of antibody were incubated with virus before cells were added to the mixture. Cells were subsequently examined for cytopathic effects or evidence of BVDV infection as determined by an indirect immunoperoxidase assay. Monoclonal antibodies to p80/125 were previously found to be nonneutralizing to the homologous (DCP) virus (Table I).

TABLE I. Neutralizing activity of monoclonal antibodies to Danish cytopathic BVDV

Designation	Protein specificity	Isotype	Neutralization[a]
157-77	gp53	G2a	6,400
348-14	gp53	G2b	12,800
450-19	gp53	G2a	100
477-77	gp53	M	200
115-21	p80/125	G1	< 100
183-10	p80/125	G1	< 100
184-14	p80/125	G2a	< 100

[a]Neutralizing ability of monoclonal antibodies (mouse ascites fluids) to DCP virus expressed as the reciprocal of the maximum dilution that gave 50% virus neutralization

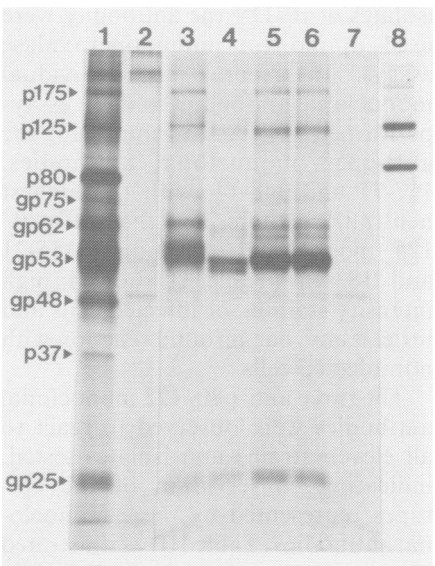

Fig. 1. Protein specificities of BVDV-specific monoclonal antibodies. Cells were infected with various strains of BVDV and labeled with ^{35}S-methionine and cell lysates were immunoprecipitated with bovine BVDV antiserum or monoclonal antibodies. Immunoprecipitated proteins were solubilized and electrophoresed in 10% polyacrylamide gels. BVDV strain (or isolate) and antibody used were: Singer, antiserum (lane 1); mock-infected, antiserum (lane 2); Singer, 348-14, a representative anti-gp53 monoclonal antibody (lane 3); DCP, 348-14 (lane 4); DNCP, 348-14 (lane 5); NY-1, 348-14 (lane 6); mock-infected, 348-14 (lane 7); DCP, 115-21, a representative anti-p80/125 monoclonal antibody (lane 8).

The p80/125 monoclonal antibodies were also tested against other BVDV strains and isolates (Singer, NADL, Oregon, DCP-2, DCP-3, DCP-4, DNCP, DNCP-2, NY-1 and Draper) and were likewise found to be nonneutralizing to these viruses. All four monoclonal antibodies reactive to gp53, however, neutralized the homologous (DCP) virus. The monoclonal antibodies 157-77 and 348-14 demonstrated high virus-neutralizing ability to DCP virus (Table I) and were neutralizing for all viruses tested with the exception of the Draper strain (Table II). In contrast, monoclonal antibodies 450-19 and 477-77 had low neutralizing titers to the homologous DCP virus (Table I) and showed more variability in neutralizing ability against other viruses (Table II).

TABLE II. Neutralization[a] of BVDV strains and isolates with anti-gp53 monoclonal antibodies

	Monoclonal antibodies			
Virus	157-77	348-14	450-19	477-71
DCP, Singer, Oregon, NADL, DCP-4, DNCP, NY-1	+	+	+	+
DCP-2, DCP-3	+	+	+	–[b]
DNCP-2	+	+	–	–
Draper	–	–	–	–

[a]As determined by the microtiter method using 100 $TCID_{50}$ of virus: +, antibodies (mouse ascites fluids) which gave 50% virus neutralization at ≥ 1/100 dilution; –, not neutralizing at 1/100 dilution
[b]Partial neutralization (< 50%) was observed at 1/100 dilution for DCP-2 in some assays

CROSS-REACTIVITY WITH BVDV STRAINS

To test the cross-reactivity of anti-gp53 and anti-p80/125 monoclonal antibodies to various strains and isolates of BVDV the antibodies were used in indirect immunoperoxidase assays. In general, the individual monoclonal antibodies in these assays performed well. Assays employing the anti-gp53 monoclonal antibodies, 157-77 and 348-14, with the highest neutralizing titers, and the anti-p80/125 monoclonal antibodies, 115-21 and 183-10 (Table I) showed high intensity staining of infected cells and little, if any, background reaction with noninfected cells.

The three anti-p80/125 monoclonal antibodies were observed to react to all eleven strains and isolates tested, indicating conservation of the epitopes represented by these monoclonal antibodies (Table III) as illustrated using monoclonal antibody 183-10 in Fig. 2a,d,g. The four anti-gp53 monoclonal antibodies reacted with all cytopathic viruses and with three of the four noncytopathic viruses (Table III) as illustrated in Fig. 2b,e using monoclonal antibody 157-77. In contrast, the anti-gp53 monoclonal antibodies reacted with only a small subpopulation of cells infected with our Draper stock virus (Fig. 2h, compare with Fig. 2g). This seemed to indicate that our Draper stock virus either contained antigenic variants of the Draper virus or possibly, was contaminated with a second, unknown BVDV NCP virus. An initial attempt to purify the Draper stock by the limiting dilution method was unsuccessful in eliminating this population of virus. Although the reason for this is unknown, it may suggest that antigenic variants of Draper virus arise at a high frequency.

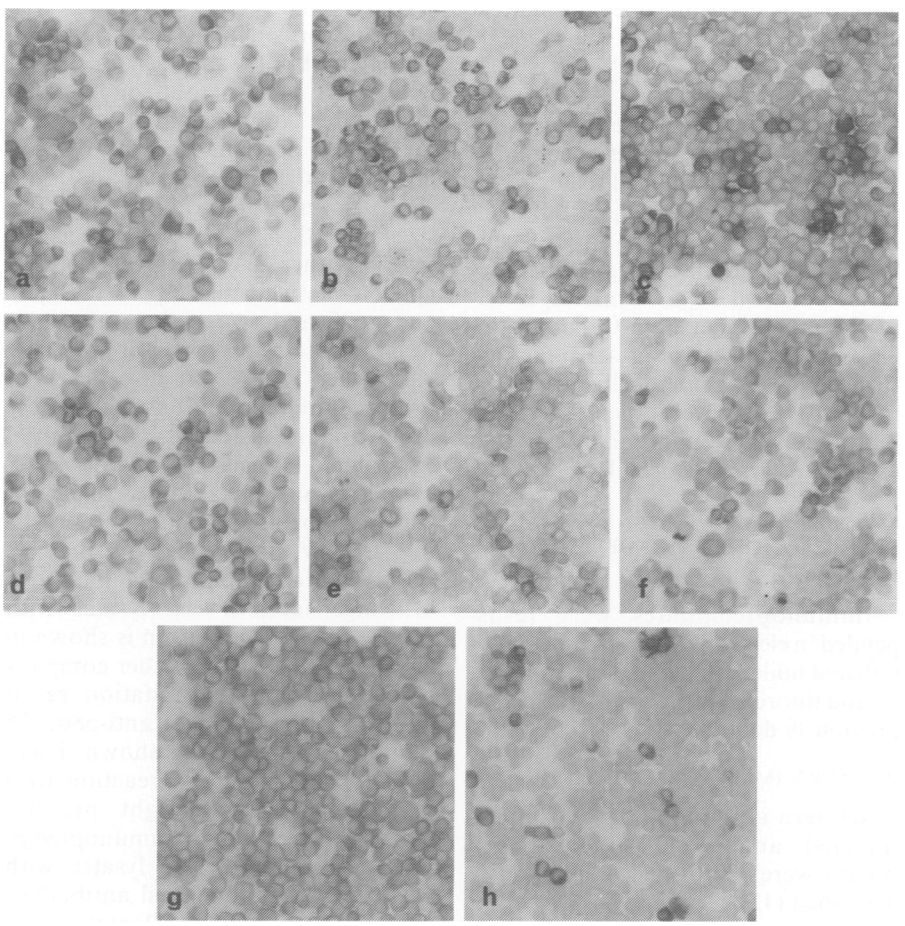

Fig. 2. Immunoperoxidase labeling of cytopathic and noncytopathic BVDV-infected cells by representative monoclonal antibodies and polyclonal antiserum (18). Cells were inoculated with BVDV and the infection allowed to proceed until approximately 50% (a-f) or 100% (g,h) of the cells were infected. Following gentle trypsinization and washing, cells were cytocentrifuged onto slides and labeled. Virus and antibody used were: (a) DCP, 183-10, a representative anti-p80/125 monoclonal antibody; (b) DCP, 157-77, a representative anti-gp53 monoclonal antibody; (c) DCP, antiserum; (d) DNCP, 183-10; (e) DNCP, 157-77; (f) DNCP, antiserum; (g) Draper, 183-10; (h) Draper, 157-77.

TABLE III. Cross-reactivity[a] of monoclonal antibodies with BVDV strains and isolates

Virus	Monoclonal antibodies anti-gp53 157-77	348-14	450-19	477-71	anti-p80/125 115-21	183-10	184-14
All[b] except Draper	+	+	+	+	+	+	+
Draper	–[c]	–	–	–	+	+	+

[a]As determined by an indirect immunoperoxidase test on virus-infected cells
[b]Viruses listed in Table II
[c]The anti-gp53 monoclonal antibodies reacted with only a small subpopulation of virus-infected cells. See text for details

DISCUSSION

In this study four anti-gp53 monoclonal antibodies are described. In immunoprecipitations of BVDV-infected cell lysates with these monoclonal antibodies several BVDV-specific proteins in addition to gp53 were observed. It is unlikely that all of these proteins share common epitopes. Rather, co-immunoprecipitation of several of these proteins may be due to either strong noncovalent protein associations or nonspecific protein-antibody reactions (20). Multiple proteins observed in immunoprecipitations with monoclonal antibodies, however, may be expected if proteins arise from proteolytic processing of precursor proteins and/or other posttranslational modifications to proteins (i.e. glycosylation with complex sugars) occur (16,17,21). For BVDV it has been suggested that viral proteins arise from proteolytic processing of a large polyprotein (20). Similar to our study, Donis *et al* (22) observed several additional proteins in immunoprecipitations with anti-gp53 monoclonal antibodies. However, their presence was regarded as due to nonspecific interactions (22). Others have not observed these proteins in their immunoprecipitations with anti-gp53 monoclonal antibodies (23-25). It is possible, however to overlook less prominent proteins if autoradiographic exposure times are of short duration. Collett *et al* (20) observed two glycoproteins, gp53 and gp116, immunoprecipitated from BVDV (NADL strain)-infected cell lysates with antiserum prepared by immunization with a BVDV fusion protein. Antiserum prepared by immunization with two other BVDV fusion proteins immunoprecipated gp116, gp62 and gp48, and gp116, gp62 and gp25, respectively. This suggests that gp116 is a common precursor to gp53 and gp62 and that the latter protein is cleaved to gp48 and gp25 (20). Pulse-chase labeling experiments and peptide mapping studies (26) are needed to further establish these protein relationships. These experiments are currently underway in our laboratory.

All four monoclonal antibodies reactive to gp53 neutralized the homologous (DCP) virus. Of the eleven BDV viruses examined only the Draper strain could not be neutralized by any of the monoclonal antibodies. All neutralizing monoclonal antibodies so far produced to BVDV have been gp53-specific (22-25). Our anti-p80/125 monoclonal antibodies and a single gp48 monoclonal antibody (22) were found to be nonneutralizing. The monoclonal antibodies of Peters *et al* (27) which were later reported to be specific to p80/125 apparently are also nonneutralizing (2). Therefore, it remains to be determined whether gp53 is the only neutralizing antigen of BVDV. Magar *et al* (24) produced a gp53-specific monoclonal antibody to the NADL strain which could neutralize NADL, Singer and Draper strains efficiently but did not neutralize the Oregon strain. In contrast, our monoclonal antibodies do neutralize the Oregon strain but not the Draper strain of BVDV, suggesting that these monoclonal antibodies recognize different neutralizing epitopes. Bolin *et al* (23) examined the neutralizing ability of nine gp53-specific monoclonal antibodies to various strains of BVDV and concluded that multiple epitopes are involved in virus neutralization.

The monoclonal antibodies examined in this study appear to meet several requirements for good diagnostic reagents. First, the monoclonal antibodies in this study showed strong avidities for viral antigens as indicated by their good performances in an indirect immunoperoxidase assay. Second, and as expected, the monoclonal antibodies showed high specificity for viral antigen as background staining of uninfected cells was minimal. Third, and most importantly, these monoclonal antibodies demonstrated broad cross-reactivities with the eleven BVD viruses examined. These viruses included North American and European (Danish) BVDV strains and isolates. Thus, BVD viruses isolated from different geographical locations have conserved epitopes.

Other monoclonal antibodies with broad cross-reactivities to BVDV isolates have been produced (2,7,28). It has been reported that one monoclonal antibody of Peters *et al* (27) reacted with all of 50 pestivirus (BVDV, border disease virus of sheep, and hog cholera virus) isolates tested (2). This supports the notion that serotypes to BVDV do not exist (2). In our study, monoclonal antibodies reactive to p80/125 reacted with all viruses while anti-gp53 monoclonal antibodies failed to react with only one of the eleven viruses. Testing of BVDV field isolates from across Canada has begun in our laboratory to determine further, in parallel with BVDV antiserum, the ability of these monoclonal antibodies to detect different BVD viruses. Our results to date indicate that a pool of these monoclonal antibodies could be used for detection of BVDV in place of polyclonal antiserum.

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