Rotavirus, a member of the Reoviridae family, is an impor-
tant cause of gastroenteritis in young children, calves, mon-
keys, chickens, pigs, sheep, and horses (1, 14). It is nonen-
veloped and has double-shelled capsids surrounding a genome of
11 double-stranded RNA segments. Seven serological groups
of rotavirus, A to G, have been identified, but only groups A,
B, C, D, and G have been characterized well (15). Each group
can be differentiated by polyacrylamide gel electrophoretic
mobilities (2, 23).

Among the seven serogroups, group A rotavirus has been
studied in greatest detail, and it is the serogroup most com-
monly found in cattle worldwide. The virus is composed of a
core surrounded by VP6, the major inner capsid protein. The
outer capsid layers of infectious bovine rotavirus (BRV)
particles contain two proteins, VP4 and VP7. The VP4 (P)
types are spike protein encoded by RNA segment 4 (19, 21).
They constitute important outer capsid proteins with various func-
tions such as hemagglutinating activity (22) and neutralization
activity (10, 25, 37), and when cleaved by trypsin into VP3
and VP8, they enhance the infectivity of the virus. There is evi-
dence that rotavirus VP4 sequences are diverse (32). Using
monoclonal antibodies (MAbs) against VP4, diversity has been
shown in the amino acid sequences of epitopes that are critical
for cross-reaction and neutralization of rotaviruses (18, 19, 22,
33). Both VP4 and VP7 are associated with stimulation of
serotype-specific antibodies and in vivo protection. Serotypes 1
to 4 of VP7 are glycosylated (6). Proteins other than VP4 and
VP7, such as VP6, associated with stimulation of serotype-
specific antibodies, may participate in protection against BRV
infection; however, neutralizing antibodies in vitro have been
shown to be specific against VP4 and VP7. Protection against
rotavirus infection appears to rely mainly on stimulation of
neutralizing antibodies against the outer capsid proteins, VP4
and VP7 (27).

Many established protocols and commercial kits are avail-
able to detect rotavirus infection for human diagnostic medical
applications including electron microscopy and enzyme-linked
immunosorbent assay (ELISA). The objective of this study was
to develop MAbs against bovine rotavirus that can detect
group A rotavirus antigen in bovine fecal samples by ELISA
and indirect fluorescent-antibody assay (IFA) for diagnostic
and research use.

MATERIALS AND METHODS

Virus propagation and purification. The Nebraska calf diarrhea strain of BRV
(serogroup A, serotype G6), obtained from the National Veterinary Service
Laboratory at Ames, Iowa, was passaged six times in Madin-Darby bovine kidney
(MDBK) cells in Dulbecco’s modified Eagle medium containing trypsin (5 µg/
ml) and pancreatic (5 µg/ml) (16). Virus was harvested when 75% of the infected
monolayer showed typical cytopathic effects such as rounding and detachment
of cells. A previously described procedure for virus purification was followed (17).
After three cycles of freezing and thawing, the cells were scraped, pooled, and
centrifuged at 35,000 g for 20 min at 4°C in a Sorvall TH641 rotor. The
supernatant was passed through a 0.45-µm-pore-size filter, and then polyethyl-
en glycol 8000 was added at a final concentration of 8% (wt/vol). After incu-
bation overnight at 4°C, the precipitated virus was centrifuged at 10,800 g for
20 min at 4°C in a Sorvall TH641 rotor. Pelleted virus was resuspended in a
minimal volume of TNE buffer (100 mM NaCl, 50 mM Tris-HCl [pH 7.5], 1 mM
EDTA). Virus was purified on a discontinuous sucrose gradient (10 to 60%
wt/wt) and then centrifuged at 90,000 × g for 2 h at 4°C in a Sorvall TH641
rotor. The interphase band was collected, diluted in 1× TNE buffer (pH 7.5), and
layered on a 20 to 60% (wt/wt) sucrose gradient for centrifugation at 90,000 ×
g for 2 h. The purified virus pellet was resuspended in 1× TNE buffer (pH 7.5) for
storage at −20°C, and the protein content was quantitated by the
bicinchoninic acid method (Pierce Chemical Company, Rockford, Ill.).

Production of MAbs. Four-week-old BALB/c mice were injected subcutane-
ously with 60 µg of purified BRV viral proteins mixed with an equal volume of
adjuvant containing TDM plus MPL plus pokeweed mitogen (Ribi Immuno-
Chem Research, Inc., Hamilton, Mont.). After three injections were adminis-
tered at 2-week intervals, the mice were sacrificed and their spleen cells were
fused with mouse Ag8 myeloma cells by a standard protocol (7). ELISA, IFA,
immunodot assay, Western blot assay, immunoprecipitation, and immunohisto-
chemistry (IHC) were used to screen hybridoma supernatants for reactivity to
BRV. The BRV-positive hybridomas were selected and cloned by limiting dilu-
tion. Isotyping of the MAbs was performed using a commercial kit (Bio-Rad
Labs, Richmond, Calif.) according to the manufacturer’s instructions.

Indirect ELISA for hybridoma screening. Immunolun 1 flat-bottom microtiter
plates (Dynex Technologies, Chantilly, Va.) were coated with 50 ng of purified
BRV protein per well and incubated overnight at 4°C. A blocking solution of 2%
casein enzymatic hydrolysate (Sigma, St. Louis, Mo.) in 0.01 M phosphate-
buffered saline (PBS) (pH 7.0) was added to each well (50 µl), and the plate was
incubated at 37°C for 25 min. Wells were washed five times with PBS containing 0.05% Tween 20 (PBS-T). Fifty microliters of hybridoma supernatant was added to each well, and the plate was incubated at 37°C for 25 min. The plate was washed five times with PBS-T, followed by the addition of 50 μl of secondary goat anti-mouse immunoglobulin G (IgG) horseradish peroxidase (HRPO)-conjugated antibody (dilution, 1:10,000) to each well. The plate was incubated at 37°C for 25 min and then washed five times with 5 min each time with PBS-T. For an color development, 50 μl of 2.2'-azino-bis(3-ethylbenzthiazoline sulfonic acid) (ABTS) substrate was added to each well, the plate was incubated for 15 to 45 min at 37°C. The absorbance values were read at 405 nm.

IFA test. BRV-infected (Nebraska calf diarrhea strain) cell culture slides were fixed in acetone for 10 min at 4°C, air dried for 10 min, and then incubated with 50 to 70 μl of hybridoma supernatant for 30 min at 37°C in a humidified chamber. The cultures were incubated with 0.01 M PBS (pH 7.4) for 10 min, incubated with 1:50 dilution of secondary goat anti-mouse IgG fluorescein isothiocyanate-labeled antibody at 37°C for 30 to 60 min. After the slides were washed in PBS for 10 min, they were mounted with buffered glycerol (pH 7.2), coverslapped, and then examined with a fluorescence microscope for the presence of apple-green fluorescence indicating BRV-infected cells.

Immunodot assay. A biodot apparatus (Bio-Rad Labs) was used to stabilize the nitrocellulose membrane for application of BRV-positive and -negative controls. One microliter of purified BRV was applied per well for each MAb to be tested. Uninfected MDBK cell lysate (1 μg) was used for the negative control. After air drying for 10 min, the membrane was cut into strips that were placed in multiresevoir trays and blocked with 10% horse serum for 1 h at 4°C with gentle agitation. Each MAb was tested individually against the BRV-positive and -negative samples by incubating with the nitrocellulose strips for 1 h on a shaker at 4°C. The membrane strips were washed with Tris-buffered saline (TBS) five times for 5 min per wash. A 1:9,000 dilution of horse anti-mouse IgG HRPO-conjugated antibody was applied to the strips at 4°C for 1 h. After the membrane strips were washed with TBS five times for 5 min, secondary horse anti-mouse IgG HRPO-conjugated antibody was applied, and the strips were incubated for 1 h at 4°C. Prior to adding of 4-chloro-1-naphthol peroxidase (4CN) substrate for color development, the membranes were washed five times in TBS for 5 min per wash.

Western blot assay. Proteins from purified BRV were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane by electrophotblotting. The membranes were cut into strips that were placed in a multichannel reservoir, blocked with 10% horse serum in TBS (0.025 M Tris [pH 7.4], 0.8% NaCl, 0.02% KCl) at 4°C for 1 h, and then incubated with 1 ml of hybridoma supernatant at 4°C for 1 h. After the membrane strips were washed with TBS five times for 5 min, secondary horse anti-mouse IgG HRPO-conjugated antibody was applied, and the strips were incubated for 1 h at 4°C and then washed five times for a total of 25 min. Antigen-antibody complexes were detected colorimetrically by adding 4CN substrate. The BRV protein with which each MAb reacted was identified by molecular weight compared to a protein molecular mass marker.

Immunoprecipitation test. The BRV proteins were immunoprecipitated overnight at 4°C with gentle rocking by addition of 1 ml of clarified infected cell lysate to 50 μl of hybridoma supernatant or a positive, polyclonal control serum from BRV-immunized mice. The immune complexes were incubated on ice for 2 h with 10 μl of formalin-fixed Staphylococcus aureus (Cowan 1) which bound to the MAbs. Cells were centrifuged at 4,000 × g for 10 min and washed three times for 5 min, first with trypsin soy agar (TSA) containing 1% Triton X-100 and 1% sodium deoxycholic acid, then with TSA alone, and finally with 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA. After centrifugation at 4,000 × g for 10 min, the pellet was resuspended in 20 μl of sample loading buffer and electrophoresed on a 12% SDS-polyacrylamide gel electrophoresis gel. The gel was blotted onto a nitrocellulose membrane, and specificity was tested using bovine anti-rotavirus, polyvalent, hyperimmune serum (National Veterinary Service Laboratory) as an antibody probe. The blot was incubated with secondary goat anti-bovine IgG HRPO-conjugated antibody. For chromagen development, 4CN was chosen as the substrate.

IHC. Formalin-fixed, paraffin-embedded intestinal tissues in 4-μm sections were used. The tissue sections were heat fixed to glass slides at 55°C for 30 min and then deparaffinized in three changes of Hemo-De (Fisher Scientific, St. Louis, Mo.) xylene substitute for a total of 15 min, followed by rehydration through graded alcohols (100% > 95% > 80%) to distilled water. Sections were treated with 0.01% trypsin for 10 min at room temperature, followed by a 5-min distilled water wash. To quench endogenous peroxidase, 3% H2O2 was applied to the tissues for 5 min at room temperature. The slides were rinsed in distilled water for 5 min and then soaked in PBS-T for 10 min. A protein blocker (PBA; Lipshaw, Pittsburgh, Pa.) was applied to the sections for 10 min at room temperature. Excess blocker was removed, and anti-BRV hybridoma supernatant (dilution, 1:50) was applied to the tissues for a 2-h incubation at 37°C. After a 10-min distilled water wash in PBS-T, the slides were incubated with a secondary anti-mouse IgG biotinylated antibody (Vector Labs, Burlingame, Calif.) for 30 min at room temperature and then washed with PBS-T for 10 min. Avidin-biotin complex (Vector Labs) was applied to the tissues, which were then incubated for 30 min at room temperature, washed in PBS-T, and soaked in distilled water for 5 min. As a substrate, DAB was applied to the tissues for 10 min. Following a 5-min distilled water wash, the sections were counterstained with Gill’s hematoxylin 1 (Fisher Scientific) for 30 s and placed under lukewarm running tap water for 5 min. After the tissues were dehydrated through graded alcohols (80% > 75% > 100%) to xylene, they were coverslapped with Permount (Fisher Scientific) and examined by light microscopy for BRV-positive stained cells.

Antigen capture ELISA for BRV antigen detection. Immunoloon 1 flat-bottom microtiter plates (Dynes Technologies) were coated with 12.5 μg of semipurified or concentrated MAb (SB4 or SB11) per well and incubated overnight at 4°C. The plates were washed five times with PBS-T, and residual moisture was tapped onto a paper towel. A 50-μl aliquot of blocking solution (0.5% glycin in PBS) was added to each well, and the plate was incubated at 37°C for 25 min and washed five times with PBS-T. Fifty microliters of a 10% (+/+) fecal suspension in PBS was added to each well, and the plate was incubated at 37°C for 25 min. The plate was washed five times with PBS-T, and then 50 μl of rabbit anti-bovine IgG HRPO-conjugated antibody was added to the wells. The plate was incubated at 37°C for 30 min and then washed five times with PBS-T. For development, 50 μl of ABTS substrate was added to each well, the plate was incubated for 15 to 45 min at 37°C, and the absorbance value was read at 405 nm.

Calculation of specificity and sensitivity. The following formulas were used to calculate the specificity and sensitivity of the antigen capture ELISA compared to the Rotavirus II kit (Abbott Laboratories, Abbott Park, Ill.). Bovine fecal samples were tested using the Rotavirus II kit following the manufacturer’s instructions for human fecal samples.

[Tables and figures are not included in the text.]
Most of the MAbs had a kappa light chain, and two (10B1 and 1B12) had a lambda light chain.

The size of viral protein immunoprecipitated by each of the seven MAbs was calculated by comparing the relative mobility of the bound protein to that of the protein molecular mass marker. A calibration curve of the protein standard was used, and the value of relative mobility of the protein to which the MAb bound was applied to the curve to find the anti-log corresponding to the molecular mass of that protein. All seven MAbs were shown to precipitate with or bind to a 37.5-kDa protein (Fig. 1), which corresponds in molecular mass to rotavirus VP7.

MAbs were used in antigen capture ELISA, and the results were compared with those obtained with the Rotazyme II kit (Abbott Laboratories). Clones 2B11 and 8B4 had the ability to detect rotavirus antigen in fecal samples. Of 20 samples tested, 18 that were positive by the Rotazyme II kit were also found positive by MAbs 8B4 and 2B11, with 85 and 39% sensitivity, respectively (Table 2). Sensitivity and specificity of the antigen capture ELISA using each of the two MAbs were evaluated based on the Rotazyme II kit being the reference method (specificity of both MAbs was 100%).

Cross-reactivity between BRV antibody and bovine coronavirus, another virus commonly encountered in fecal specimen of neonatal calves, was not observed. Cross-reactivity was not observed with canine rotavirus or with uninfected MDBK cells.

**TABLE 2. Agreement of Rotazyme II kit results with antigen capture ELISA results**

| Result of test | ELISA | Frequency |
|----------------|-------|-----------|
| Rotazyme II kit | SB4 | 2B11 |
| + | + | 4 | 0.20 |
| - | - | 2 | 0.10 |
| + | + | 11 | 0.55 |
| + | - | 3 | 0.15 |

*Agreement was determined with reference to the total number of specimens tested (n = 20). Either MAb SB4 or MAb 2B11 was used in the ELISA for detecting BRV in calf feces. The total frequency was 20, and ri = frequency/n, where n is the number of samples having a particular outcome (in this case, positive).

DISCUSSION

The BRV serotypes are defined according to the reactivity of VP7 to specific MAbs (2, 34). Different segments of BRV encode the VP7 gene (segment 7, 8, or 9, depending on the strain of the virus) (6). VP7 is the main antigen for neutralizing antibodies and determining serotypic differences.

Because binding of an antibody to an antigen is dependent on the recognition of specific amino acid epitopes by the antibody, MAb technology has facilitated the development of sensitive and specific tests for the detection of many microbial and viral antigens in clinical specimens. Several immunological techniques that incorporate the use of MAbs have been described, including ELISA (3, 5, 7, 23, 24, 26, 29, 34), IFA, and fluorescent-antibody assay (8, 28, 30, 31, 33), immunohistochemical staining of fixed tissues (35), and immunoblot assays (9). The advantages of using dot blot immunoassays to detect viral antigens directly from tissues, swabs, washings, and body secretions are as follows: (i) large numbers of specimens can be handled simultaneously; (ii) culture of virus in cells, eggs, or laboratory animals is not required; and (iii) immunoassays are sensitive, specific, and rapid to perform. Coating an ELISA plate with an antigen of interest can result in a change of the conformational epitope to which the MAb may bind. To solve this problem, a sandwich ELISA, in which antibodies are coated in a microtiter plate to anchor the protein of interest and so retain its native conformation, is an alternative.

Accurate and effective diagnosis of BRV infection is important for disease prevention. Current methods for diagnosing infection with BRV are electron microscopic examination of fecal samples, ELISA, and direct fluorescent-antibody inspection of frozen sections of the small intestine. Transmission electron microscopy can detect virus only if large numbers of particles are present (100,000 particles/g of feces), and it is expensive, requiring an operator, special equipment, and skilled personnel. Direct immunofluorescence testing of tissues is rapid, but it is less sensitive than IFA. The latter generally is considered to be more sensitive than direct immunofluorescence because of signal amplification of secondary, labeled antibody. ELISA is sensitive for detecting rotavirus in feces of humans and calves. Commercial ELISA kits, such as the Rotazyme II kit, are useful for detecting rotavirus of several species because all have a common group-specific antigen. Most available kits are designed primarily for human diagnostic testing and are not approved for veterinary application. Therefore, the use of IFA to detect viral antigen in acetone-
fixed tissues is a good alternative to the ELISA, because it is a specific and rapid aid for the study of viral pathogenesis.

In this study, we have produced and characterized MAbs specific for bovine rotavirus. Several screening methods were used to identify BRV-reactive MAbs: immunodot blotting, Western blotting, immunoprecipitation, and ELISA. Differences noted in the reactivity of each MAb by the various tests could be due to the sensitivity of the test or to the conformation of the proteins. For example, both immunoprecipitation and the immunodot assay identify conformational epitopes, whereas Western blotting identifies linear epitopes. Differences in protein conformation can affect reactivity of the antibodies by sequestering or revealing epitopes or weak affinity to MAbs. Differences in the immunodot assay and the indirect screening ELISA could be due in part to differences in test sensitivity. The indirect ELISA is less sensitive than the antigen capture ELISA. Our results show that IFA had a higher sensitivity than indirect ELISA. Immunoprecipitation allows better detection of native conformational epitopes by antibodies than does IFA or dot blot assay. None of the seven MAbs were positive by the IHC test. We believe that the MAbs were nonreactive by IHC because BRV epitopes were distorted by cross-linking induced during formalin fixation of the tissues (3).

This is the first report of MAbs produced against VP7 of Nebraska calf diarrhea strain BRV by using purified viral particles as immunogen. MAbs produced by using synthetic peptide from VP7 as the immunogen for MAb production were unable to detect antiviral antibodies as tested by ELISA (12). In another study that used a VP7 peptide-VP6 conjugate as the immunogen, the MAbs produced provided less protection than those obtained by using BRV as the immunogen (13).

The MAbs described in this study should aid in serotyping for selection of vaccine candidates and in development of better diagnostic methods for detection of BRV. Because the outer capsid shell, VP7, is associated with stimulation of serotype-specific antibodies, these MAbs may work well for G-typing (G6 serotype) of group A BRV (11, 19). Both MAb SB4 and MAb 2B11 were G6 subtype specific, and they may be good tools for typing of American and European strains of BRV. These MAbs serve well for diagnosing BRV infection when acetone-fixed slides are submitted for diagnostic investigation. Further study is needed to define and compare the major epitopes and to decide which serotypes (of serotypes G1, G3, and G6) are bound, because each subunit of VP7 induces a different degree of serum neutralization response (4), and then to develop a multivalent vaccine against BRV isolates common in the United States.

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