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Monoclonal Antibodies to Bovine Coronavirus: Characteristics and Topographical Mapping of Neutralizing Epitopes on the E2 and E3 Glycoproteins[1]

DIRK DEREGT* AND LORNE A. BABIUK*,†,[2]

*Department of Veterinary Microbiology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 0W0, and the †Veterinary Infectious Disease Organization, 124 Veterinary Road, Saskatoon, Saskatchewan, Canada S7N 0W0

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Monoclonal antibodies to the Quebec isolate of bovine coronavirus were produced and characterized. Monoclonal antibodies to both the E2 and the E3 glycoproteins were found to efficiently neutralize virus *in vitro*. None of the monoclonal antibodies directed against the E1 glycoprotein neutralized virus infectivity. Neutralizing monoclonal antibodies to the E2 glycoprotein were all found to immunoprecipitate gp190, gp100, and their intracellular precursor protein gp170. Neutralizing monoclonal antibodies to the E3 glycoprotein immunoprecipitated gp124 and showed differential reactivity to its precursor proteins gp59 and gp118. These monoclonal antibodies also showed differential reactivity to an apparent degradation product of E3. Neutralizing monoclonal antibodies to E2 bound to two distinct nonoverlappig antigenic domains as defined by competitive binding assays. Neutralizing monoclonal antibodies to the E3 glycoprotein also bound to two distinct antigenic sites as defined by competitive binding assays plus a third site which overlapped these regions. Other results indicated that one domain on the E3 glycoprotein could be further subdivided into two epitopes. Thus four epitopes could be defined by E3-specific monoclonal antibodies. © 1987 Academic Press, Inc.

INTRODUCTION

Bovine coronavirus (BCV), an enteric virus, is composed of four structural proteins. Three of these proteins are analogous to the mouse hepatitis virus (MHV) A59 strain; E1, a transmembrane protein of 23K–26K (molecular weight, MW); E2 the large peplomeric protein of coronaviruses, which exists in both uncleaved and cleaved forms of approximately 180K–190K and 90K–120K, respectively; and N, the nucleocapsid protein (50K–54K) and its trimer (160K) (Sturman and Holmes, 1977; Sturman *et al.*, 1985; Storz *et al.*, 1981; King and Brian, 1982; Hogue *et al.*, 1984; Robbins *et al.*, 1986; Deregt *et al.*, in press). The fourth protein of BCV, E3, is a disulfide-linked dimer with a molecular weight of approximately 124K–140K, reducible to two apparently identical subunits of 62K–65K (King and Brian, 1982; Deregt *et al.*, in press). This protein has been identified as the BCV hemagglutinin and does not appear on MHV-A59 (Sturman and Holmes, 1977; Hogue *et al.*, 1984; King *et al.*, 1985).

BCV (Quebec isolate) intracellular precursor proteins have been recently identified by us (Deregt *et al.*, in press). The precursor protein to the BCV E2 protein (gp190/gp100) is a glycoprotein of 170K (MW), gp170. This protein is apparently further glycosylated to yield gp190/E2 before subsequent proteolytic cleavage to yield gp100. Two presumptive comigrating gp100/E2 species are believed to be the result of cleavage.

The precursor proteins to the BCV E3 glycoprotein (gp124/gp62) are gp59 (monomer) and gp118 (disulfide-linked dimer). The primary precursor was found to be gp59 which apparently undergoes rapid dimerization to produce gp118 before further glycosylation to gp124/E3.

In this report, monoclonal antibodies to BCV were characterized as to their specificities and their abilities to neutralize virus infectivity. Monoclonal antibodies to both BCV E2 and E3 glycoproteins were found to efficiently neutralize virus infectivity *in vitro* in the absence of complement. Also, in this report, neutralizing epitopes of the E2 and the E3 proteins were topographically mapped and further characterized.

MATERIALS AND METHODS

Virus and cells

The Quebec isolate of BCV (obtained from S. Dea, Department of Pathology and Microbiology, Faculty of Veterinary Medicine, University of Montreal, St. Hyacinthe, Quebec) was used in these studies (Dea *et al.*, 1980). Virus was propagated in Madin–Darby bovine kidney (MDBK) cells and purified as described previously (Deregt *et al.*, in press).

[1] A portion of this work was presented at the Third International Coronavirus Symposium at Asilomar (1986).

[2] To whom requests for reprints should be addressed.

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Plaque assay and virus neutralization

BCV infectivity was assayed in MDBK cells and plaques were allowed to develop under 0.8% agarose as previously described (Deregt *et al.*, in press). To quantify the virus-neutralizing ability of monoclonal antibodies 100 PFU of virus in 250 μl MEM was incubated with an equal volume of various dilutions of heat-inactivated (56° for 30 min) mouse ascites fluid for 1 hr at 37°. The virus–ascites fluid mixtures were then allowed to adsorb to confluent MDBK monolayers for 1 hr. Controls included ascites fluid containing monoclonal antibodies to bovine herpes virus-1. Neutralization endpoint titers were expressed as the reciprocal of the highest dilution of monoclonal antibody which gave a 50% reduction in plaque numbers.

Production and conjugation of monoclonal antibodies

BALB/c mice were immunized with either purified whole virus or SDS-denatured virus preparations (Cianfriglia *et al.*, 1983). The production of monoclonal antibodies is described elsewhere (Deregt *et al.*, in press). Monoclonal isotypes were determined by an enzyme immunoassay of hybridoma supernatants (Hyclone Laboratories, Logan, UT). Total IgG concentration of ascites fluids was determined by radial immunodiffusion (ICN Immuno-biologicals, Lisle, IL).

Monoclonal antibodies were conjugated to horseradish peroxidase (HRP) by the method of Nakane and Kawaoi (1974) as modified by van den Hurk and Kurstak (1980).

Antibody-binding and competitive binding assays

An enzyme-linked immunosorbent assay (ELISA) for antibody-binding curves and titration of ascites fluids was performed. Ninety microliters of purified BCV (7.5 μ/ml) in coating buffer (0.5 *M* $NaHCO_3/Na_2CO_3$, pH 9.6) was applied to each well of polystyrene microtiter plates (Immulon 2; Dyntech Laboratories, Inc., Fisher Scientific, Edmonton, Alberta) and incubated overnight at 4°. The plates were washed five times in washing buffer [0.01 *M* PBS, pH 7.2, containing 0.05% Tween 20, (PBST)] and then 150 μl of blocking buffer, PBST containing 10% heat-inactivated horse serum (GIBCO), was added to each well and incubated for 1–2 hr at room temperature to block unreacted sites. The plates were then washed as before with PBST and 75 μl of monoclonal antibody serially diluted in PBST containing 1% horse serum (PBSTS) was added and incubated for 2 hr at room temperature. After washing the plates to remove unbound antibody, 75 μl of a 1:1600 dilution of HRP-conjugated goat anti-mouse IgG and IgM (Boehringer-Mannheim, Dorval, Quebec) diluted in PBSTS was then added and incubated for 1 hr at room temperature. After final washing of the plates in PBST, 75 μl of substrate solution containing 1 mg/ml of recrystallized 5-aminosalicylic acid and 0.005% hydrogen peroxide in 0.01 *M* phosphate buffer, pH 6.0, was added to each well. The enzyme reaction was terminated after 30 min of incubation at room temperature by the addition of 75 μl of 1.0% sodium azide (Saunders *et al.*, 1977). The optical density at 490 nm (OD_{490}) was determined immediately after the addition of sodium azide using a microELISA reader (MR580, Dynatech). Endpoint titers were determined from the point at which the antibody-binding curve crossed the average absorbance attributed to nonspecific binding of the conjugated antibody for each plate.

For experiments using saturating amounts of HRP-conjugated goat anti-mouse IgG and IgM, dilutions as low as 1:50 were used and incubated for 3–4 hr. The enzyme reaction with substrate was terminated after 4 or 6 min before substrate became limiting.

For competitive binding assays, the procedure was the same as first described above except that HRP-conjugated monoclonal antibodies at concentrations of 1:400–1:6400 (equivalent to approximately 60–90% saturation levels for different HRP-conjugated monoclonal antibodies) in PBSTS were used in place of HRP-conjugated goat anti-mouse IgG and IgM. The competition curves were calculated as described by Kimura-Kurado and Yasui (1983) using the formula $[100(A - n)]/(A - B)]$ where A is the OD in the absence of competing antibody, B is the OD in the presence of homologous antibody at 1:10 (or 1:100) dilution, and n is the OD in the presence of competitor at $1:10^1$–$1:10^7$ dilutions. Thus, by definition, self-competition at low dilution of homologous unlabeled antibody was equal to 100% and the measurement of competition by heterologous unlabeled antibody was a relative measure. Competition at the plateau was regarded as strong (+) if greater than 70%, significant (±) if between 30 and 50% and negative (–) if less than 30%. Actual self-competitions were calculated using the formula $[100(A - B)]/A$, where A and B in this instance were corrected for nonspecific binding as measured by the binding of anti-mouse-conjugated antibody to wells incubated without unlabeled competitor antibody.

Labeling of intracellular proteins and radioimmunoprecipitation

Confluent monolayers of MDBK cells were washed once with MEM. Virus, at a multiplicity of infection of 5–10 PFU/cell, was then adsorbed for 1 hr at 37°.

After adsorption the inoculum was removed and replaced with MEM + 2% FBS. This medium was removed and replaced with 50 μCi/ml of [^{35}S]methionine in methionine-deficient medium 8–14 hr postadsorption. At 24–36 hr postadsorption the cells were washed and harvested in ice-cold PBS and after pelleting were prepared for radioimmunoprecipitation or electrophoresis.

For pulse–chase experiments the cells were starved for methionine for 2 hr and then labeled with 200 μCi/ml of [^{35}S]methionine in methionine-deficient medium beginning at 18–23 hr for different experiments. After a 15-min pulse, the cells were washed with MEM or MEM containing 10 times the normal concentration of methionine and then further incubated with this same medium for 60 min before harvesting.

The procedure used for radioimmunoprecipitation was essentially as that described previously (Deregt *et al.*, in press).

Western immunoblotting

Purified BCV was solublized with various sample buffers (see PAGE, below) and proteins were fractionated in a 10% polyacrylamide gel. Proteins were then transferred to nitrocellulose paper (Bio-Rad) by electroblotting at 120 V (constant) for 8–10 hr at 4° in Tris–glycine methanol buffer (20 m*M* Tris–hydrochloride, pH 8.3, 190 m*M* glycine, 20% methanol).

After transfer, blots were blocked with 3% skim milk powder (SMP) in 0.01 *M* PBS overnight at 4°. Blots were cut into strips and monoclonal antibody [1:2 dilution of hybridoma supernatants or ascites fluids (1:200 dilution)] or rabbit anti-BCV polyclonal serum (1:100 dilution) in PBS containing 0.05% Tween 20 (PBST) and 1% SMP (PBSTS) was incubated with individual strips in an incubation tray (Bio-Rad) and rocked for 2 hr at room temperature. Strips were then washed with PBST for 30–60 min at room temperature and then incubated with a 1:2000 dilution of HRP-conjugated goat anti-mouse IgG and IgM or a 1:5000 dilution of HRP-conjugated goat anti-rabbit IgG (Boehringer-Mannheim) in PBSTS for 90 min at room temperature. Strips were again washed and developed by reaction with 0.05% 4-chloro-1-naphthol (Bio-Rad) substrate for 15 min–1 hr. Alternatively, the substrate 0.02% dianisidine dihydrochloride (Sigma) was used in a slightly modified procedure (Dergt *et al.*, in press).

Polyacrylamide gel electrophoresis (PAGE)

Samples were resuspended in sample buffer (Laemmli, 1970) with or without 2-mercaptoethanol, boiled for 2 min, and stored at −20° or analysed immediately on various percentages of polyacrylamide gels. Alternatively, samples were resuspended in a sample buffer containing urea, without 2-mercaptoethanol, pH 6.8 (Sturman, 1980), and incubated at 37° for 30 min. Electrophoresis and fluorography were performed as previously described (Deregt *et al.*, in press).

RESULTS

General characteristics of BCV-specific monoclonal antibodies

From a total of 105 hybridoma cell lines, 15 monoclonal antibodies were chosen for detailed characterization, 7 were specific for the E2 protein, and 4, 3, and 1 for the E3, E1, and N proteins, respectively (Fig. 1). These antibodies were chosen based on their overall superior reactivity in assays as well as our particular interest in analyzing BCV glycoproteins. All monoclonal antibodies were of the IgG immunoglobulin class. The predominant isotypes were IgG_{2a} and IgG_1 (six of each). ELISA titers of ascites fluids ranged from $10^{4.5}$ to $10^{7.5}$. Total ascites IgG, as determined by ra-

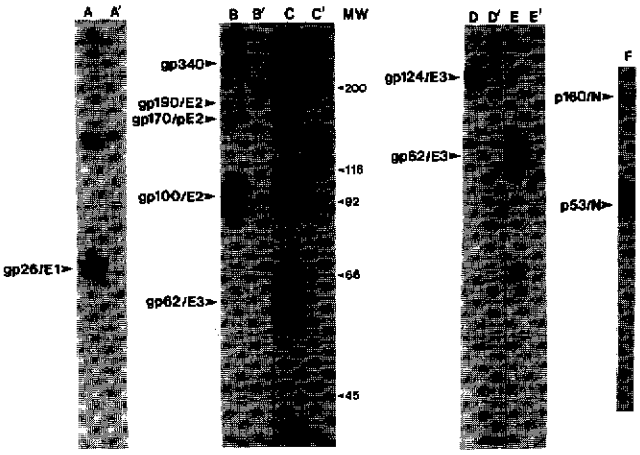

FIG. 1. Protein specificity of representative BCV monoclonal antibodies. Radioimmunoprecipitation of cell lysates, lanes A–E'. Western immunoblotting of purified BCV, lane F. Immunoreactivity of mABs to the E1 protein (AE12–11, lanes A, A'), E2 protein (BB7-14, lanes B, B'), E3 protein (BD9-8C, lanes D–E'), or N protein (MC5, lane F) or rabbit anti-BCV serum (lanes C, C'). BCV-infected cells (A–E) or mock-infected cells (A'–E') were labeled wth [^{35}S]-methionine (A, A', D–E) or [^{3}H]glucosamine (B–C'). After immunoprecipitation proteins were solubilized in a urea buffer (final concentration, 3 *M*) without 2-mercaptoethanol (A, A', D, D', F) or in Laemmli buffer containing 2-mercaptoethanol (B–C', E, E'). Analysis was done in polyacrylamide gels ranging from 7–12.5%. For lane F, reactivity was detected using HRP-conjugated rabbit anti-mouse IgG and IgM antibody and the substrate used was dianisidine dihydrochloride. MW, positions of molecular weight standards. Note that the protein gp340, a probable aggregate of gp170/pE2, was more prominant in this analysis then was usually observed. Its MW was calculated from other gels by use of thyroglobulin (MW 330K, Pharmacia) as a MW standard (see also Fig. 2).

dial immunodiffusion, ranged from less than 1 to 32 mg/ml.

Neutralization with monoclonal antibodies

To determine which monoclonal antibodies could neutralize virus infectivity *in vitro* without the aid of complement, heat-inactivated ascites fluids and virus were mixed and incubated in a plaque reduction assay (Table 1). Monoclonal antibodies to both the E2 and the E3 proteins were found to neutralize virus infectivity with plaque reduction (50%) titers of up to 130,000 and 50,000, respectively. None of the monoclonal antibodies to the E1 or the N protein neutralized virus infectivity *in vitro*.

Patterns of reactivity of E2- and E3-specific monoclonal antibodies

(a) Monoclonal antibodies to the E2 protein. Upon initial screening, by radioimmunoprecipitation, most hybridoma supernatants reactive to E2 were observed to bind to both gp100/E2 and gp170 (designated as pE2, for precursor to E2) as well as to higher molecular weight species, which were apparently aggregated forms of E2 or pE2. Reactivity to gp190/E2 was sometimes difficult to assess when regularly labeled lysates were used, because this protein often occurred in small amounts (Fig. 1). However, in pulse–chase experiments, gp190/E2 occurs quite prominently prior to cleavage to gp100/E2 (Deregt *et al.*, in press). Thus to assess the binding of selected E2 monoclonal antibodies to these related proteins, lysates were prepared from [^{35}S]methionine pulse–chased cells and used for radioimmunoprecipitation. All of these monoclonal antibodies immunoprecipitated gp100/E2, gp190/E2, gp170/pE2, and gp340, an apparent aggregate of pE2 (Fig. 2B).

FIG. 2. Comparison of virion glycoproteins and intracellular BCV E2 and E2-related proteins. BCV (lane A) was labeled with [^{3}H]-glucosamine in MDBK cells and purified. Lanes B and C, BCV-infected and mock-infected cells, respectively, were pulsed for 15 min and chased with MEM for 60 min before immunoprecipitation with a representative E2-specific mAB (BB7-14). Proteins were solubilized in Laemmli sample buffer without 2-mercaptoethanol before analysis in a 6% polyacrylamide gel. MW, positions of molecular weight standards. Note that lane A represents virus that had undergone two freeze–thaw cycles after solubilization because a portion of this virus was used for a previous analysis. Aggregation of gp100/E2 was apparent as reflected in the relative prominance of the gp190/E2 band compared with the previous analysis (not shown) and the appearance of band a. Band a has a MW of approximately 300K when gp340 was used as a molecular weight standard and is a presumptive aggregate (trimer) of gp100/E2.

TABLE 1

CHARACTERISTICS OF BCV MONOCLONAL ANTIBODIES

Protein specificity	Clone designation	Isotype	ELISA titer ($\log_{10}$)[a]	Ascites IgG (mg/ml)[b]	Neutralization titer[c]
E3	HC10-6	G_{2a}	7	10	50,000
	KC4-3	G_{2a}	7	10	1,100
	KD9-40	G_{2a}	7	15	50,000
	BD9-8C	G_{2a}	6	3	1,250
E2	HE7-3	G_1	6.5	15	130,000
	JB9-3	G_1	7.5	32	2,500
	HB10-4	G_1	6	7	12,500
	JB5-6	G_{2a}	7	15	125,000
	HF8-8	G_1	6.5	10	130,000
	BB7-14	G_{2b}	6.5	5	125,000
	BB10-27	G_1	6	14	125
N	MD8-3	G_1	6	1	<50
E1	AE12-11	G_3	5.5	<1	<50
	CC7-3	G_{2b}	4.5	1	<50
	GB10-5	G_{2a}	5	14	<50

[a] Ascites fluid.
[b] Total IgG as determined by radial immunodiffusion.
[c] Reciprocal of the highest dilution of antibody (ascites fluid) which gave a 50% reduction in plaque number.

(b) Monoclonal antibodies to the E3 protein. (i) Differential reactivity of E3-specific monoclonal antibodies to an apparent degradation fragment of gp124/E3. Upon initial characterization by radioimmunoprecipitation it was noted that monoclonal antibodies to the E3 protein reacted differentially to gp124/E3 and a 64K (MW) polypeptide (Fig. 3A, lanes A–C). This 64K polypeptide (gp64) was found to be glycosylated (data not shown) and virus specific by its absence in mock-infected cell lysates (Fig. 3B, lanes A, G) and its occasional appearance in samples of purified virus (data not shown).

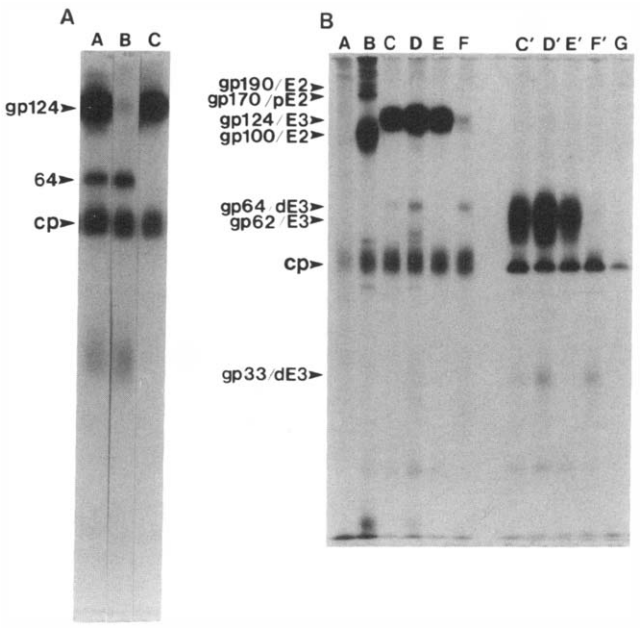

FIG. 3. Differential reactivity of E3-specific mABs to the apparent degradation product gp64/dE3. (A) Initial characterization of E3 mABs by immunoprecipitation of lysates from [^{35}S]methionine-labeled BCV-infected cells (BD9, KC4, HC10; lanes A–C, respectively). Immunoprecipitates were solubilized in a urea buffer (final concentration, 3 *M*). Analysis was in a 11% polyacrylamide gel. Protease inhibitor used in lysate: Aprotinin, 2%. (B) Immunoprecipitates of E3-specific mABs: KD9-40 (lanes C, C'), BD9-8C (lanes D, D'), HC10-5 (lanes E, E'), and KC4-3 (lanes F, F') solubilized under nonreducing (C-F) and reducing (C'–F') conditions. Lane B, E2-specific mAB BB7-14. Lanes B–F, C'–F', represent immunoprecipitates of BCV-infected cells. Lanes A and G represent mock-infected cells immunoprecipitated by mABs BD9-8C and HC10-5, respectively. Cells were labeled with [^{35}S]methionine and solubilized with Laemmli buffer without 2-mercaptoethanol (A–F) or with 2-mercaptoethanol (C'–G). Analysis was in a 10% polyacrylamide gel. Protease inhibitors used in lysate: aprotinin, 2%; PMSF, 0.2 m*M*. Note that BD9-8C, HC10-5, and KC4-3 are subclones of BD9, HC10, and KC4 (A), respectively. (A and B) Nonspecific binding of a cellular protein, cp.

Monoclonal antibodies BD9-8C, KD9-40, and KC4-3 reacted with gp64 (KC4-3 with apparent higher avidity then with gp124/E3 itself) while monoclonal antibody (mAB) HC10-5 did not. This differential reactivity did not change by increasing (10-fold) or decreasing (5-fold) the amount of mAB HC10-5 used, nor did the exclusion of SDS in the incubation mixture have any effect on HC10-5 immunoprecipitation. Further, it was possible to immunoprecipitate gp64 from supernatants of HC10-5 incubation mixtures with mAB BD9-8C, indicating that the differential reactivity was, indeed, due to binding (i.e., mABs, BD9-8C, KD9-40, and KC4-3) or lack of binding of this polypeptide (i.e., mAB HC10-5; data not shown).

It had further been noted that when immunoprecipitates of mAB BD9-8C and purified virus preparations containing gp64 were reduced with 2-mercaptoethanol, a polypeptide of approximately 33K (MW) appeared (Fig. 3B, lane D'). This polypeptide was also glycosylated (data not shown). It was suspected that the 33K polypeptide (gp33) was derived by reduction of gp64 and that the latter protein was a disulfide-linked dimer (similar to gp124/E3). If this were true, gp64 should be absent under reducing conditions. Since gp124/E3 is itself reduced to gp62/E3, the presence or absence of gp64 would normally be difficult to assess under reducing conditions due to a lack of resolution between these proteins. However, when immunoprecipitates of mAB KC4-3 (which demonstrated greater reactivity to gp64 than to gp124/E3) were examined, an assessment could be made. Under reducing conditions gp64 was absent and apparently replaced by gp33 (compare Fig. 3B, lanes F and F'). This indicated that gp64 was a disulfide-linked dimer of probably identical gp33 subunits. Since these polypeptides have no apparent role in the genesis of gp124/E3, as observed in pulse–chase experiments (Deregt *et al.*, in press), they were concluded to be degradation products of gp124/E3 and designated gp64/dE3 and gp33/dE3).

(ii) Differential reactivity to gp59/pE3 and gp118/pE3 by monoclonal antibodies to E3. The differential reactivity of E3-specific monoclonal antibodies to the E3 degradation product, gp64, suggested that these monoclonal antibodies might also bind differentially to the E3 precursor monomer gp59 and precursor dimer gp118 (designated as pE3). Thus to determine the binding ability of E3 monoclonal antibodies to these proteins, BCV-infected and mock-infected cells were pulsed with [^{35}S]methionine and chased with MEM, and lysates were prepared and used for immunoprecipitation. The results show that although all E3 monoclonal antibodies could bind to gp118/pE3 (Fig. 4A, lanes A, C, E, G), only mAB BD9-8C was able to immunoprecipitate the monomer gp59/pE3 (Fig. 4A, lane A). Monoclonal antibody KC4-3 revealed the same weak binding for gp118/-E3 as it did for gp124/E3. This reaction was better observed after a 30-min pulse with [^{35}S]methionine (Fig. 4B, lane C). The same results were obtained when the concentration of monoclonal antibody was increased (10-fold) or decreased (5-fold). When SDS was excluded from the lysates of pulsed cells a minor amount of what was apparently gp59 in mABs HC10-5 and KD9-40 immunoprecipitations was detected along with apparent nonspecific binding to p53, the nucleocapsid protein. This was observed in lanes A–D when the gel shown in Fig. 4B was exposed 10 times longer (data not shown). We could not distinguish whether this was due to a very

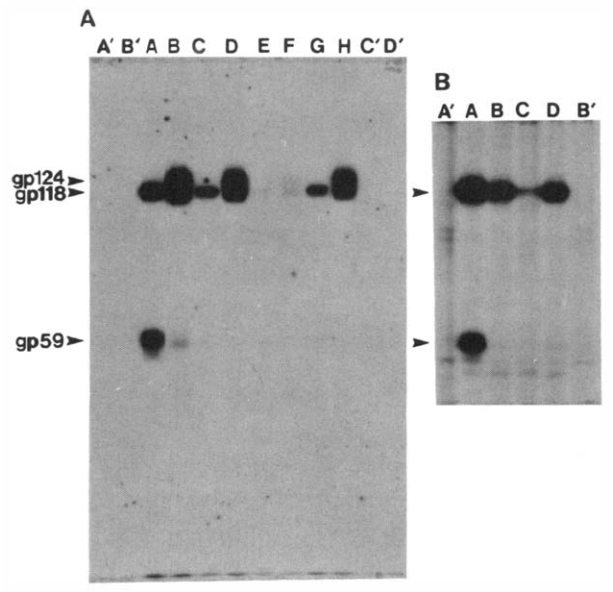

FIG. 4. Differential reactivity of E3-specific monoclonal antibodies to the E3 precursor proteins gp59 and gp118. (A) BCV-infected (A–H) and mock-infected cells (A'–D') were pulsed with [^{35}S]-methionine for 15 min (A', A, C, E, G, C') or pulsed and then chased with MEM for 60 min (B', B, D, F, H, D'). Cell lysates were then immunoprecipitated with mAB BD9-8C (A'–B), HC10-5 (C, D, C', D'), KC4-3 (E, F), or KD9-40 (G, H). (B) BCV-infected (A–D) and mock-infected cells (A', B') were pulsed with [^{35}S]methionine for 30 min and cell lysates were immunoprecipitated with mAB BD9-8C (A', A), HC10-5 (B, B'), KC4-3 (C), or KD9-40 (D). SDS was excluded from these lysates. Only the upper middle part of the gel is shown. (A and B) Immunoprecipitates were solubilized in Laemmli sample buffer without 2-mercaptoethanol and analysis was done in a 10% polyacrylamide gel.

weak reaction with gp59, nonspecific binding, or a slight amount of reduction of gp118, after immunoprecipitation, during sample preparation. Nevertheless, we considered it as a negative result. Based on their differential reactivities with gp124/E3, gp64/dE3, gp59/pE3, and gp118/pE3, summarized in Table 2, the E3 monoclonal antibodies defined four epitopes on the E3 protein.

Nature of epitopes on BCV proteins

To determine the nature of BCV epitopes, the binding of monoclonal antibodies in Western immunoblotting assays was studied. These results are shown in Fig. 5 and summarized in Table 3. All monoclonal antibodies to the E2 protein bound to SDS-denatured antigen in Western immunoblots (Figs. 5E and 5F). In contrast, only one E2-specific monoclonal antibody (BB10-27) bound to antigen denatured by SDS plus 2-mercaptoethanol although a weaker reaction (than with antigen denatured with SDS alone) was observed (Fig. 5E'). All monoclonal antibodies directed against

TABLE 2

REACTIVITY OF E3-SPECIFIC MONOCLONAL ANTIBODIES TO E3-RELATED PROTEINS[a]

Clone designation	Antigen			
	gp124/E3	gp118/pE3[b]	gp59/pE3	gp64/dE3[c]
HC10-5	+	+	–	–
KC4-3	±[d]	±	–	+
KD9-40	+	+	-	+
BD9-8C	+	+	+	+

[a] Determined by studying the reactivity of the antibody in radioimmunoprecipitation assays.
[b] pE3, precursor to E3.
[c] dE3, degradation product of E3.
[d] ±, weak positive reaction.

E3 also bound to SDS-denatured antigen (Fig. 5G). However, mABs HC10-5 and KC4-3 showed relatively weak reactions compared to mABs BD9-8C and KD9-40 (data not shown). None of the E3 monoclonal antibodies could react with antigen denatured by SDS plus-2-mercaptoethanol (Fig. 5G'). Thus all E2 and E3 epitopes represented by this panel of monoclonal antibodies, with the exception of that represented by mAB

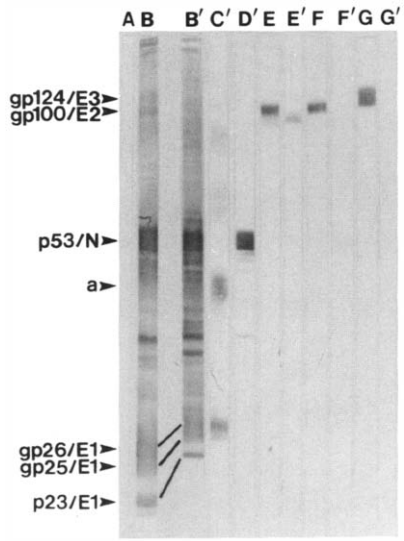

FIG. 5. Western immunoblotting of representative mAbs to purified BCV. BCV was solubilized with Laemmli sample buffer without 2-mercaptoethanol (A, B, E–G) or with 2-mercaptoethanol (B'–G') fractionated in a 10% gel and transferred to nitrocellulose. Individual nitrocellulose strips were incubated with rabbit nonimmune serum (A), rabbit anti-BCV serum (B, B'), mAB CC7-3 (C'), MD8-3 (D'), BB10-27 (E, E'), BB7-14 (F, F'), or KD9-40 (G, G'). Reaction was detected using HRP-conjugated goat anti-rabbit IgG antibody (lanes A–B') or conjugated rabbit anti-mouse IgG and IgM antibody (other lanes). Substrate used was 4-chloro-1-naphthol and color development was allowed to proceed for 15 min (lanes A–B') or 1 hr (other lanes).

TABLE 3

REACTIVITY OF BCV MONOCLONAL ANTIBODIES TO DENATURED ANTIGEN[a]

Protein specificity	Clone designation	*In vitro* neutralization[b]	Treatment[c] SDS	Treatment[c] SDS + 2-ME
E3	HC10-5, KC4-3, KD9-40, BD9-8C	+	+	−
E2	BB10-27	−	+	+
	HE7-3, JB9-3, HB10-4, JB5-6, HF8-8, BB7-14	+	+	−
E1	AE12-11, CC7-3	−	+	+
	GB10-5	−	−	−
N	MD8-3	−	+	+

[a] Determined by studying the reactivity of the antibody in immunoblotting assays.

[b] +, 50% plaque reduction at ascites dilution of more than 1:1000 (see Table 2 for actual titers).

[c] Antigen boiled in the presence of SDS or SDS plus 2-mercaptoethanol (2-ME) prior to PAGE.

BB10-27 (E2), were apparently dependent upon disulfide linkages for their antigenic integrity. Rabbit polyclonal anti-BCV serum also showed an apparent lack of reaction to reduced species of E2 and E3. However, weak reactions may have been obscured by background reactions in these experiments (Fig. 5B′). Transfer of these reduced proteins was confirmed when [^{35}S]methionine-labeled virus was immunoblotted (data not shown).

Two of three mABs specific to E1 proteins, mABs AE12-11 (to gp26/E1) and CC7-3 (to gp26/E1 and gp25/E1), reacted with antigen denatured under both nonreducing and reducing conditions (Fig. 5C′) as did mAB MD8-3 (N specific) (Fig. 5D′). The E1 monoclonal antibodies also reacted with apparent aggregates of E1 (most notably, species a) (Fig. 5C′). There was some distortion in the gel front for reduced proteins which caused the E1 proteins to migrate with apparent lower mobilities (Figs. 5B′ and 5C′). The extraneous bands observed were apparently the result of some protein degradation or aggregation (Fig. 5, particularly lanes B and B′).

Binding characteristics of E2 and E3 monoclonal antibodies used in competitive binding assays (CBA)

Binding characteristics of E2 and E3 monoclonal antibodies to BCV were determined by parallel ELISA titrations using a single antigen preparation. Binding curves of monoclonal antibodies used in CBA were constructed and all monoclonal antibodies apparently saturated BCV at a dilution of 1:10^3 (data not shown). This was confirmed by CBA which typically demonstrated maximum levels of competition over three $\log_{10}$ dilutions. Thus, the same amount of input antibody was represented on the plateaus of the binding curves.

Absorbances reached at the plateau, reflecting the amount of monoclonal antibody bound to BCV, were used as an approximate measure of relative avidity of monoclonal antibodies for their respective proteins (Frankel and Gerhard, 1979; Stone and Nowinski, 1980; Bruck *et al.*, 1982). However, since anti-IgG_1 antibodies were significantly underrepresented in the HRP-conjugated probe, as determined by later titration, comparisons were not made between E2 monoclonal antibodies across isotypes at limiting dilutions of probe. The results suggested that mABs JB9-3 and HF8-8 had significantly lower relative avidities for the E2 protein than did HE7-3 and HB10-4. When binding was measured using saturating or near saturating levels of the probe, similar adsorbances were reached at the plateau for E2 monoclonal antibodies HE7-3 (IgG_1) and BB7-14 (IgG_{2b}), suggesting that their avidities were similar.

Avidities of the anti-E3 monoclonal antibodies (all of IgG_{2a} isotype) appeared similar in this assay. However, since the E3 protein is a disulfide-linked dimer of apparently identical subunits, variation in the number of epitopes (one or two) or type of antibody binding (monovalent versus bivalent) were possibilities that would make binding comparisons meaningless at limiting dilutions of the probe. Thus binding was also measured at saturating levels of the probe. Again very similar absorbances were reached at the plateau for E3 monoclonal antibodies indicating that approximately the same amount of antibody binding occurred for the same amount of input antibody.

Characterization of antigenic domains on the BCV E2 and E3 proteins by competitive binding assays

To determine the topography of epitopes on the E2 and E3 glycoproteins involved in neutralization, monoclonal antibodies described in Table 1 (with the exception of JB9-3 which induced an insufficient amount of ascites and BB10-27 which was inefficient at neutralization and whose epitope could be distinguished by other means) were conjugated to HRP and used as probes in CBAs. Conjugation had no apparent adverse effect on the binding properties of these monoclonal antibodies and endpoint titers ranged from approximately $10^{4.4}$–10^5. Binding of HRP-conjugated monoclonal antibody was inhibited from approximately 70 to greater than 99% (≥85% for all monoclonal antibodies,

except HF8-8, 73%) in the presence of saturating unlabeled homologous antibody. As a measure of avidity these results were in general agreement with those derived from the binding curves.

The results of typical competition binding experiments are shown in Figs. 6 and 7 and summarized in Tables 4 and 5. For each experiment a monoclonal antibody to another BCV protein was used as a "competitor" control. Two nonoverlapping antigenic regions were defined by E2-specific neutralizing monoclonal antibodies (Fig. 6). Monoclonal antibodies HF8-8, HB10-4, HE7-3, and JB5-6 demonstrated reciprocal competition and defined one region which was designated A(E2) (Figs. 6A–6D). The monoclonal antibody BB7-14 defined a second region which was designated B(E2) (Fig. 6E). The mAB JB9-3, an antibody of apparent low avidity, showed intermediate levels of competition with the BB7-14 probe. However, since a reciprocal competition experiment could not be done because of lack of conjugated (JB9-3) probe this monoclonal antibody was not assigned to a particular antigenic group.

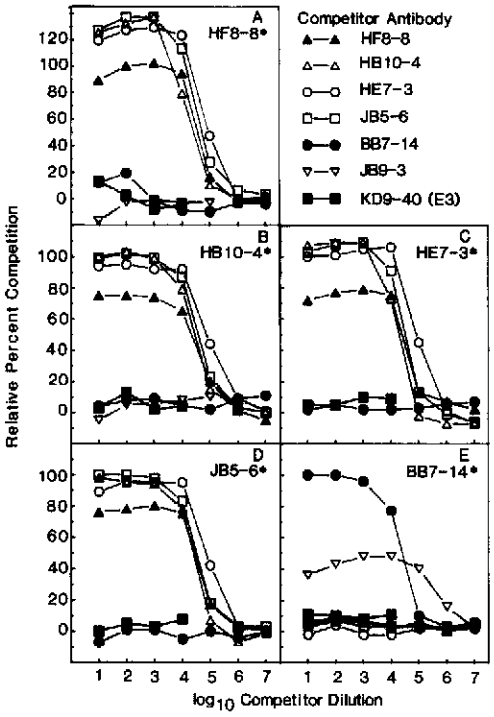

FIG. 6. Competition binding assay with HRP-conjugated monoclonal antibodies directed against the E2 protein. Serial dilutions of unconjugated antibodies (competitor) were allowed to react with coated antigen (BCV). After removal of unbound antibodies the binding of HRP-conjugated monoclonal antibody (probe) was observed. The extent of blocking by competitor antibody of conjugated probe was calculated and expressed as relative percentage competition as described in the text. HRP-conjugated (*) antibodies were mABs HF8-8 (A), HB10-4 (B), HE7-3 (C), JB5-6 (D), and BB7-14 (E).

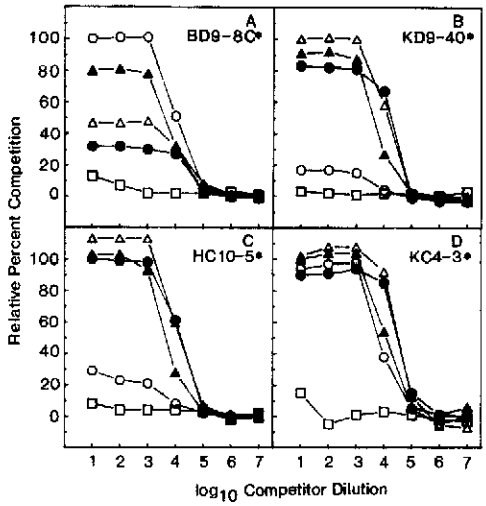

FIG. 7. Competition binding assay with HRP-conjugated monoclonal antibodies directed against the E3 protein. Assays were done as described in the legend to Fig. 7. HRP-conjugated (*) antibodies were ○, BD9-8C (A); △, KD9-40 (B); ●, HC10-5 (C), ▲, KC4-3 (D). Control antibody, □, BB7-14 (E2 specific).

The competitive binding experiments with E3-specific monoclonal antibodies suggest the presence of closely adjacent and overlapping antigenic domains (Figs. 7A–7D and Table 5). The mABs KD9-40, HC10-5, and KC4-3 reciprocally competed with each other (Figs. 7B–7D) while KC4-3 and BD9-8C also engaged in reciprocal competition (Figs. 7D and 7A). The mABs KD9-40 and HC10-5 revealed only intermediate levels of blocking the binding of BD9-8C probe (Fig. 7A) while BD9-8C showed little, if any, blocking of KD9-40 and HC10-5 probes (Figs. 7B and 7C). Thus,

TABLE 4

SUMMARY OF COMPETITION BINDING ASSAYS WITH E2 MONOCLONAL ANTIBODIES[a]

Competitor antibody	Probe antibody: HE7-3	HB10-4	JB5-6	HF8-8	BB7-14	Antigenic group
HE7-3	+	+	+	+	–	A
HB10-4	+	+	+	+	–	
JB5-6	+	+	+	+	–	
HF8-8	+	+	+	+	–	
BB7-14	–	–	–	–	+	B
JB9-3		–		–	±	

Note. Summary of data is presented in Fig. 6.

[a] Percentage competition at the plateau: +, >70%; ±, 30–50%; –, <30%.

TABLE 5

SUMMARY OF COMPETITION BINDING ASSAYS WITH E3 MONOCLONAL ANTIBODIES[a]

Competitor antibody	Probe antibody				Antigenic group
	HC10-5	KD9-40	KC4-3	BD9-8C	
HC10-5	+	+	+	±	A
KD9-40	+	+	+	±	
KC4-3	+	+	+	+	B
BD9-8C	−	−	+	+	C

Note. Summary of data is presented in Fig. 7.

[a] Percentage competition at the plateau: +, >70%; ±, 30–50%, −, <30%.

the competitive binding experiments delineated one distinct antigenic region defined by HC10-5 and KD9-40 monoclonal antibodies and another by BD9-8C and these were designated A(E3) and C(E3), respectively. The antigenic site defined by KC4-3, which overlapped these two regions, was designated B(E3). Since other data presented in this report indicate that mAB HC10-5 and KD9-40 bind to different epitopes, the antigenic region defined by these monoclonal antibodies was further subdivided and designated as A1(E3) and A2(E3) epitopes, specified by HC10-5 and KD9-40, respectively.

DISCUSSION

The results presented show that both BCV E2 and E3 glycoproteins can induce the production of antibodies that can efficiently neutralize virus infectivity in the absence of complement. Previous studies have shown the E2 peplomeric protein, the protein responsible for cell membrane fusion and cellular attachment, to be the major neutralizing antigen of coronaviruses, while antibodies to the E1 glycoprotein had little if any neutralizing ability in the absence of complement (Collins *et al.*, 1982; Vautherot and Laporte, 1983; Talbot *et al.*, 1984; Wege *et al.*, 1984; Laude *et al.*, 1986; Jimenez *et al.*, 1986). The coronaviruses MHV-A59, infectious bronchitis virus, and transmissible gastroenteritis virus (TGEV), some of the best-studied coronaviruses, all apparently lack an analogous protein to the BCV and human coronavirus (HCV-043) E3 glycoprotein (Sturman and Holmes, 1977; Rottier *et al.*, 1981; Cavanagh, 1981; Stern *et al.*, 1982; Garwes and Pocock, 1975; Hogue *et al.*, 1984; Hogue and Brian, 1986; Deregt *et al.*, in press). There may be an analogous protein on MHV-JHM; however, the identity of this protein remains unclear (Wege *et al.*, 1979; Siddell *et al.*, 1981; Siddell, 1982; Taguchi *et al.*, 1986). Since the E3 protein can induce efficient neutralizing antibodies, this suggests that this protein has an important function in bovine coronavirus infectivity. However, the exact nature of this function remains to be determined.

Monoclonal antibodies directed against E2 defined three antigenic regions on the BCV protein. Two of these regions can induce the production of highly efficient neutralizing antibodies and their antigenic integrity was dependent upon disulfide linkages. At least six major antigenic sites on the E2 protein of MHV-JHM were identified by competitive binding assays in previous studies. Three of these sites were involved in neutralizing virus (Talbot *et al.*, 1984; Wege *et al.*, 1984). Four major antigenic sites were found on TGEV E2 protein by CBA. Two of these sites were responsible for most of the neutralization mediating determinants and up to six neutralizing epitopes could be distinguished by various other criteria in another study (Delmas *et al.*, 1986; Jimenez *et al.*, 1986). These studies employed, in general, a larger panel of monoclonal antibodies to the E2 protein than reported here. Thus whether more neutralizing epitopes on the BCV E2 protein sites could have been identified had a larger panel of monoclonal antibodies been used remains to be determined.

Wege *et al.* (1984) could distinguish three classes of monoclonal antibodies by their pattern of immunoprecipitation of E2 (gp170 and gp98) and pE2 (gp150) proteins. Only monoclonal antibodies that could bind all three proteins in the immunoprecipitation assay had high neutralizing titers. Similarly, all neutralizing monoclonal antibodies to the BCV E2 protein reported here immunoprecipitated the analogous proteins gp190/E2, gp100/E2, and gp170/pE2.

Three antigenic domains designated A, B, and C representing four distinct epitopes could be defined on the E3 protein by the present panel of E3-specific monoclonal antibodies. Differential reactivity of E3 monoclonal antibodies to gp124/E3 and gp64/dE3, in radioimmunoprecipitation assays (RIPA), distinguished three epitopes: epitope A1 and epitope B (defined by mABs HC10-5 and KC4-3, respectively) from each other and from the epitopes A2 and C (defined by mABs KD9-40 and BD9-8C, respectively). The differential avidity of mAB KC4-3 for gp124/E3 and gp64/dE3 in this assay was considered significant and suggested that an apparent change in epitope conformation occurs when gp124/E3 is proteolytically cleaved to gp64/dE3, one which allows better binding of KC4-3 under the conditions of the assay. Assay conditions were likely important for mAB KC4-3 binding since although KC4-3 bound poorly to gp124/E3 in RIPA, it

appeared to bind as well as other monoclonal antibodies to E3 in antibody binding assays.

Two patterns of reactivity to gp59/pE3 and gp118/pE3 were observed by E3 monoclonal antibodies. These patterns distinguished epitope C defined by mAB BD9-8C from epitope A2 defined by mAB KD9-40 (and from epitopes A and B) because only mAB BD9-8C bound the precursor monomer gp59.

Epitope C defined by mAB BD9-8C exists apparently complete on gp59/E3 which indicates the existance of two epitopes on each dimer (gp118/pE3 and gp124/E3). Other E3 mABs bound only the dimeric proteins indicating that epitope (A1, A2, and B) conformation was dependent on protein dimerization. However, there may be two A1, A2, and B epitopes on each E3 dimer since the behavior of these monoclonal antibodies (HC10-5, KD9-40, and KC4-3, respectively) was similar to that shown by mAB BD9-8C in antibody binding assays.

Results obtained by Western immunoblotting suggested that epitopes A1 and B defined by mAB HC10-5 and KC4-3, respectively, might be partially altered by SDS denaturation as indicated by a weak response in this assay while epitopes A2 and C, defined by mAB KD9-40 and BD9-8C, respectively, were apparently fully resistant. Alternatively, this may indicate slight avidity differences between the E3 monoclonal antibodies. All E3 epitopes were, however, apparently destroyed when SDS and 2-mercaptoethanol were used, suggesting that disulfide linkages are essential for their antigenicity. E3 occurs as gp62 (monomer) under reducing conditions, thus the loss of antigenicity under these conditions would be expected for epitopes A1, A2, and B, as the defining monoclonal antibodies do not bind the precursor monomer gp59 in radioimmunoprecipitation assays. Conversely, since mAB BD9-8C (epitope C) can bind the monomer gp59/pE3 it might have been expected to react with gp62/E3 in Western immunoblots. However, since this monoclonal antibody did not react with gp62/E3 and since intermolecular disulfide bonds are apparently not required for this epitope's antigenicity (as indicated by the reactivity of mAB BD9-8C for gp59/pE3) this suggests that an intramolecular disulfide bond may be involved in preservation of this epitope's conformation.

Competitive binding assays indicate that the E3 epitopes are in close spatial proximity to each other (with possible overlap) as antigenic region A (containing epitopes A1 and A2) overlaps with B and B with C. Results presented indicate that epitopes A2, B, and C are located on gp64/dE3 which remains ill-defined, but apparently contains at least one of the intermolecular disulfide bond(s) bridging gp62/E3 monomers. The location of epitope A1 is speculative with regard to gp64/dE3, although its apparent proximity to A2 suggests that this epitope may be destroyed in some manner upon cleavage of gp124/E3.

The important antigenic region on the E3 protein involved in neutralization is apparently region A which probably contains or is closely adjacent to a biologically important domain on this protein. We have not yet examined whether these monoclonal antibodies can inhibit BCV hemagglutination. Thus, whether this biologically important domain is the same domain for erythrocyte binding (King *et al.*, 1985) remains to be determined. Of greater interest, however, is the determination of the actual function of this protein in bovine coronavirus–cell interactions. Such studies are presently in progress.

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