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Khalid A. Hussain
School of Veterinary Medicine

Johannes Storz
School of Veterinary Medicine

Konstantin G. Kousoulas
School of Veterinary Medicine

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Comparison of Bovine Coronavirus (BCV) Antigens: Monoclonal Antibodies to the Spike Glycoprotein Distinguish between Vaccine and Wild-Type Strains

KHALID A. HUSSAIN, JOHANNES STORZ,¹ AND KONSTANTIN G. KOUSOULAS

*Department of Veterinary Microbiology and Parasitology, School of Veterinary Medicine,
Louisiana State University, Baton Rouge, Louisiana 70803*

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Monoclonal antibodies (MAbs) against two major structural proteins of the cell-adapted Mebus strain of bovine coronavirus (BCV-L9) were produced and characterized. Seven MAbs reacted with the peplomeric glycoprotein, gp100/S, while three MAbs reacted with the nucleoprotein p53/N in Western blot analysis of BCV polypeptides. MAbs to gp100/S reacted with discontinuous epitopes of gp100/S in Westerns under mild but not under standard denaturing conditions. In contrast, MAbs to p53/N reacted in both types of Westerns, and those epitopes were thus continuous. MAbs to p53/N failed to neutralize BCV infectivity, while 4 MAbs to gp100/S neutralized BCV effectively. Cross reactivity of MAbs to gp100/S specified by five virulent wild-type strains and two high passage, cell-culture-adapted strains in mildly denaturing Westerns and neutralization assays indicated that two epitopes were conserved in all seven strains, while two epitopes of the avirulent strains were not detected in the wild-type strains. Non-neutralizing MAbs of gp100/S reacted with all seven strains in Westerns with the exception of one MAb that was specific for the highly cell-adapted strain BCV-L9. © 1991 Academic Press, Inc.

Bovine coronavirus (BCV) is a member of the coronavirus family that causes neonatal diarrhea, a disease with substantial economic impact in the dairy and beef cattle industries. The viral particle is spherical, enveloped with a diameter of around 100 nm, possessing a single-stranded nonsegmented RNA genome of positive polarity. The viral particle is composed of sets of four major structural proteins (1–7). These consist of the peplomeric glycoprotein (gp190/S, gp100/S), the nucleocapsid protein (p53/N) and its apparent trimer (p160/N), a family of small matrix glycoproteins (gp25/M, gp26/M, p23/M), and the putative hemagglutinin (gp124/HE). Protein processing studies in infected cells indicated that the glycosylated precursor gp190 is proteolytically cleaved to yield gp100/S (4). GP100/S was shown to be the predominant form of S for BCV as two presumptive comigrating gp100/S species or a doublet band was reported (4, 5). A number of other coronaviruses possess similar structural proteins that are targets of the humoral antibody response. MAbs to S of mouse hepatitis virus type 4 (MHV-4), transmissible gastroenteritis virus (TGEV), feline infectious peritonitis virus (FIPV), and feline enteric coronavirus (FECV) neutralized respective virus infectivity (8–12). Immunization of mice with purified S from MHV-4 protected them against a challenge infection with the virus (8). Neutralizing MAbs to S and HE glycoproteins of BCV (Quebec strain) defined two distinct antigenic domains

on the S and three domains on the HE glycoprotein (13).

Antigenic diversity among isolates of several groups of coronaviruses was documented. MAbs to MHV-4 gp180/90 revealed little conservation among isolates (14). Comparison of TGEV strains with a panel of MAbs evidences differential retention of epitopes (10, 11, 15). Monoclonal antibodies to the S glycoprotein of FIPV were used to distinguish virulent and avirulent FIPV strains, and to demonstrate antigenic differences between FIPV and FECV strains (9, 16). Serological variation among 5 BCV strains was observed (17). Oral vaccination of newborn calves with attenuated BCV protected against infection by the virulent virus within 2–3 days postimmunization of colostrum-deprived calves (18). However, the vaccine has not been completely effective in field trials (19). We analyzed with a panel of MAbs the cross-reactivity of gp100/S and p53/N specified by five virulent wild-type strains and two high passage, cell-adapted strains, which includes the currently used vaccine strain.

The cell culture-adapted prototype BCV-L9 was originally isolated in bovine fetal kidney (BFK) cells from diarrhea fluid of a calf (20). This virus was serially passaged 42 times in BFK cells, 16 times in bovine fetal brain cells, 15 passages in bovine fetal spleen (BFS) cells, and for 5–10 passages in human rectal tumor (HRT-18) cells. Five other wild-type BCV isolates, initially maintained by calf inoculation, were adapted to HRT-18 cells from diarrhea fluid or intestinal mucosal scrapings of calves with clinical diarrhea and electron micro-

¹ To whom reprint requests should be addressed.

scopic evidence of coronavirus infection (21, 22). These strains are: LY-138 (Utah, 1965), BCV C-50 (Colorado, 1972), BCV-Miller (Colorado, 1974), BCV-Meeker (Colorado, 1975), and BCV-Fisher (Colorado, 1980). The vaccine strain of BCV was cultured in HRT-18 cells from the vaccine of Norden Laboratories (Omaha, NE) (20, 23). Specific markers of this strain are currently not available. Importantly, cultivation of the BCV wild-type field strains remained difficult until it was demonstrated that the cytopathic expression of BCV-L9 in cultured bovine cells was enhanced by trypsin (24) and that HRT-18 cells were susceptible to BCV (25).

Plaques of BCV were selected by a method similar to the one described by Takayama and Kim (26) and modified by Jimenez *et al.* (10) in a plaque assay described earlier (24). The infected cultures were overlaid with 3 ml of 1.2% (w/v) agarose in DMEM (Dulbecco's modified Eagle medium) containing 4 μ g/ml final trypsin concentration. A stock of virus was prepared after two consecutive plaque purification passages. The BCV was purified as described (27). MAbs were produced against purified virus preparations from the prototype BCV-L9 as described previously (28).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in 12% slab gels by the Laemmli procedure (29). Detailed procedures for the analysis of BCV proteins by SDS–PAGE and Western blot were described (5, 28).

Western blots were performed under mildly denaturing conditions according to Cohen *et al.* (30). Briefly, this technique involved the use of low concentration of SDS (0.1%) and no heat or reducing agent which allows retention of much native conformation and detection of discontinuous epitopes. The presence of antibodies neutralizing BCV infectivity in sera from naturally or experimentally infected calves, or ascitic fluid was detected by the plaque neutralization test as described previously (13, 24).

Three independent cell fusions produced 200 different hybridoma lines which yielded in 10 cell lines selected on the basis of their reactivity with BCV-specific proteins in Western blots performed under mildly denaturing and denaturing conditions. The most frequent isotype was immunoglobulin G1 (IgG1) (seven MAbs), followed by IgM and IgG2a. The MAb titer, determined by ELISA of ascitic fluids ranged from $10^{3.6}$ to $10^{4.5}$. Seven MAbs reacted specifically with gp100/S in mildly denatured Westerns (Fig. 1), but failed to react in denatured Westerns (not shown). Three MAbs reacted with nucleoprotein p53/N in mildly denatured westerns (Fig. 1) and denatured Westerns (not shown). Four MAbs to gp100/S neutralized BCV effectively by exhibiting neutralization titers that were 160, 320, or 640.

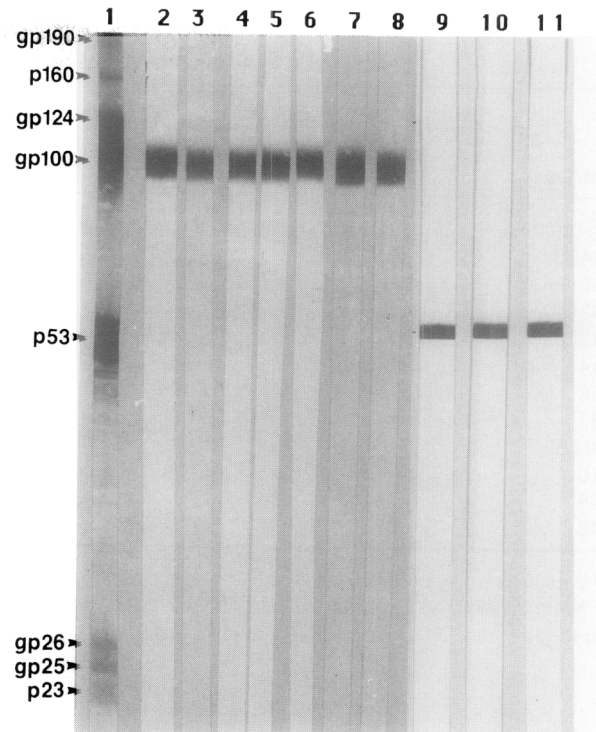


FIG. 1. Native Western blot analysis under mild denaturing conditions and polypeptide specificity of 10 anti-BCV monoclonal antibodies. Proteins of density gradient-purified BCV-L9 preparations were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The strips were incubated with MAbs 43F6, 34B8, 43C2, 44, 38, 16, 31, 10, 36, and 46 in lanes 2 to 11, respectively. A blot of reference serum showing reactivity with BCV structural proteins (lane 1) is included for reference.

In contrast, all three MAbs to nucleoprotein p53/N failed to neutralize the virus infectivity (Table 1).

MAbs were reacted with proteins specified by five wild-type strains originating from different geographical locations and isolated at different times, and with two related cell-adapted strains in Westerns. The latter group included the currently used vaccine strain and its progenitor laboratory strain BCV-L9 (Table 1). MAbs to p53/N reacted similarly against p53/N specified by all strains in both Westerns. Three MAbs to BCV-L9 gp100/S failed to react with gp100/S specified by other viral strains with the exception of antibodies 44 and 38 which reacted with the vaccine strain (Table 1). Specifically, three antigenic types of virus were distinguished. One type was the cell-adapted Mebus strain BCV-L9 used as the immunogen for the isolation of the MAbs. The second type was represented by the modified live virus vaccine strain, which differed from the prototype BCV-L9 through the loss of reactivity to MAb 31. The third antigenic type included all five virulent wild-type isolates, distinguished by their failure to react with MAbs 31, 38, and 44.

TABLE 1
REACTIVITY OF MONOCLONAL ANTIBODIES WITH BCV STRAINS IN WESTERNS AND NEUTRALIZATION TESTS

| MAbs | BCV strains | | | | | | |
|------------|---------------------------------|---------|--------|--------|-------|--------|--------|
| | L9 | Vaccine | LY-138 | Meeker | C-50 | Miller | Fisher |
| Anti-gp100 | | | | | | | |
| 34B8 | + ^a <20 ^b | + ND | + ND | + ND | + ND | + ND | + ND |
| 16 | + <20 | + ND | + ND | + ND | + ND | + ND | + ND |
| 43C2 | + 640 | + 320 | + 160 | + 640 | + 320 | + 320 | + 320 |
| 43F6 | + 640 | + 320 | + 320 | + 640 | + 320 | + 320 | + 320 |
| 44 | + 640 | + 320 | - <20 | - <20 | - <20 | - <20 | - <20 |
| 38 | + 640 | + 320 | - <20 | - <20 | - <20 | - <20 | - <20 |
| 31 | + <20 | - ND | - ND | - ND | - ND | - ND | - ND |
| Anti-p53 | | | | | | | |
| 10 | + <20 | + ND | + ND | + ND | + ND | + ND | + ND |
| 36 | + <20 | + ND | + ND | + ND | + ND | + ND | + ND |
| 46 | + <20 | + ND | + ND | + ND | + ND | + ND | + ND |

^a Westerns were run under mildly denaturing conditions; + and - indicate positive and negative reaction in Westerns, respectively.

^b Neutralization titers are expressed as the reciprocal of the dilution of ascites fluid that produced 50% reduction in plaque forming units (PFU); 100 PFU per test. ND, neutralization test not done.

The infectivity neutralizing ability of MAbs against heterologous BCV strains was tested. MAbs 43F6 and 43C2 neutralized all strains, exhibiting small variations in titers. In contrast, MAbs 38 and 44 neutralized the avirulent BCV-L9 and vaccine strains but failed to neutralize the five wild-type strains (Table 1).

All MAbs to gp100/S reacted in mildly denatured Westerns but failed to react with gp100/S in denatured Westerns which reveals that the major antigenic domains of gp100/S were composed of discontinuous amino acids brought together by virtue of the folding of the molecule in three dimensions. Furthermore, the addition of a reducing agent abolished the antigenic reactivity indicating that disulfide bonds were important in stabilizing the tertiary structure of gp100/S. Similar results were reported for gp100/S specified by the BCV Quebec strain (13).

MAbs to gp100/S reacted exclusively with gp100/S and failed to detect its precursors gp170/S and gp190/S in Westerns. It is not clear whether gp100/S represents the amino-terminal (S₁) or carboxyl-terminal (S₂) component of the proteolytic cleavage products of S. In contrast, MAbs to BCV (Quebec-strain) were reported to react with gp100/S precursors and higher molecular weight aggregates (13). The observed differences in reactivity may be due to the different conditions used to solubilize BCV viral proteins or may reflect viral strain-host cell differences in the relative amounts of processed gp100/S used to produce the immunogens for the generation of the hybridomas. Comparative reactivity of these sets of MAbs would resolve these differences.

MAb cross-reactivity tests with different viral strains revealed strong antigenic variation in gp100/S among virulent and avirulent strains. In contrast, MAbs to nucleoprotein p53/N cross-reacted with all strains. Cross-reactivity of six out of seven MAbs with gp100/S specified by the related avirulent vaccine and BCV-L9 strains infers that these glycoproteins folded into nearly identical conformations. The failure of MAb binding to gp100/S specified by the wild-type strains implies that their three-dimensional configurations differed significantly. Interestingly, one MAb failed to react with the vaccine strain although it reacted with its progenitor strain BCV-L9. A limited number of replication cycles of the virus in cell culture is evidently sufficient for the emergence of an antigenic variance.

MAbs to the nucleoprotein p53/N reacted in both Westerns illustrating that their target epitopes were composed of continuous amino acids located proximal to each other, thus rendering these epitopes insensitive to denaturation.

Neutralization tests with MAbs to gp100/S revealed the presence of epitopes associated with virus neutralization. This is in agreement with findings for coronaviruses from other animals (4, 11, 31, 32), and the Quebec strain of BCV (13). Cross-neutralization studies with MAbs to gp100/S revealed that wild-type strains were not neutralized by two MAbs. Conformational differences detected by the reactivity in Westerns are thus within antigenic areas that are crucial for virus neutralization. Similar observations were reported for mouse hepatitis virus 4 (31, 33), and the transmissible gastroenteritis virus (10, 11). It is conceivable that con-

formational differences in gp100/S of wild-type strains, as compared to the vaccine strain, result in loss of neutralizing sites with subsequent loss of protection following vaccination. None of the five wild-type BCV strains were cultivatable in bovine fetal spleen cells even in the presence of trypsin (22). A unique plaque morphology characterized each BCV strain grown in HRT-18 cells (22). The epitopes to MAb 31, 38, and 44 appear to reflect the wider host cell range of the highly cell-cultured adapted L9 and vaccine strains.

Glycoprotein S of coronaviruses is generally implicated in virus adsorption onto eukaryotic cells and in cell fusion (5, 35). The biological differences in BCV strains reported previously and the differences in the reactivity of MAbs with BCV strains apparently reflect changes in gp100/S related to virulence and adaptation to cultured cells. Our findings highlight again that gp100/S is a major target of the humoral response, and it may contain the majority of antigenic domains that are targets for neutralizing antibodies.

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