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The Characterization of the Neutralizing Bovine Viral Diarrhea Virus Monoclonal Antibodies and Antigenic Diversity of E2 Glycoprotein

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ABSTRACT. Bovine viral diarrhea virus (BVDV) is associated with a range of economically important diseases of cattle including reproductive disorders and an acute fatal hemorrhagic disease. Neutralizing antibodies that bind to the E2 glycoprotein are important predictors of vaccinal immunity. Neutralization tests using the NADL strain of BVDV and five anti-E2 monoclonal antibodies showed one, Wb163, neutralized the NADL strain of BVDV in an unexpected manner. Its titer was 10,000 compared to <35 as reported previously. The present stock of NADL differed from that of the earlier study in that the amino acid at position 79 of E2 was Valine instead of Glutamic acid. MAb Wb163 may, however, recognize a less important neutralizing epitope than another mAb Wb166, because it was less cross reactive than mAb Wb166, had a neutralizing titer 50-fold lower than Wb166 and was of lower relative affinity than Wb166. Variations in the amino terminus of E2 will be discussed in the context of vaccinal immunity.

KEY WORDS: Bovine Viral Diarrhea Virus, neutralization, relative affinity, viral diversity.

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Bovine Viral Diarrhea Virus (BVDV) is a member of the genus *Pestivirus,* a group of small-enveloped RNA viruses in the family *Flaviviridae* [1]. Two genotypes of BVDV have been recognized (BVDV-1 and BVDV-2) [2, 7, 16]. The major envelope glycoprotein, E2, is the most variable and immunodominant glycoprotein of BVDV [5, 6, 9]. The neutralizing antibody induced by E2 after natural infection or vaccination is considered the main protective mediator against BVDV infection [12, 13, 21]. It is known that BVDV isolates vary greatly. This variability of E2 can compromise the protective efficacy of traditional vaccines and the development of new generation marker vaccines [16].

Paton *et al*. [19] mapped neutralizing epitopes of BVDV using a panel of E2-specific mAbs to 2 strains of BVDV-1a (BVDV NADL and BVDV Oregon C24V). The major domain shared by both viruses was recognized by mAbs that were broadly cross-reactive to BVDV strains. A cluster of 3 amino acids, (71–74), within the amino terminus of E2 was involved in this domain. Another site was defined by a strain specific mAb that bound NADL but not Oregon. A more recent epitope mapping study using different E2-specific mAbs also identified the cluster of amino acids within the amino terminal half of the E2, and a mutation of BVDV-2 was immunoselected at position 77 by a mAb raised to BVDV-1, Deregt *et al.* [5, 6]. Both studies, however, considered the neutralizing epitopes of E2 to be discontinuous and conformational, because other amino acids defining the above epitopes were more distal to the amino terminal than the cluster.

Affinity is the strength of binding between an antibody and its epitope. High affinity antibodies are more effective than low affinity antibodies for a number of biological functions and *in vitro* reactivities [22]. The present study sought to link the varying anti-NADL neutralizing titers of Paton's panel of 5 mAb [19] to their relative affinities. When one mAb, Wb 163, had a high instead of a low titer, see later, the NADL E2 sequence data for the present virus [14] were compared to those of Paton *et al*. [19] and Collett *et al*. [4]. The sequence of BVDV Oregon [19] was included in this comparison.

MATERIALS AND METHODS

Propagation of BVDV NADL: The NADL strain of BVDV-1a was donated by the American Tissue Culture Collection (ATCC), U.S.A. It was plaque-purified and expanded by Iqbal [14]. Iqbal's two plaque purified virus was plaque-purified once more and then amplified to make working stocks.

Preparation of antigens: BVDV cell lysate and mock cell lysate were prepared from infected and mock-infected Madin Darby bovine kidney cells [13]; both contained 40 μg protein/m*l* using a Micro BCA protein assay reagent kit (Pierce).

Neutralizing titers of the BVDV E2 monoclonal antibodies: BVDV E2 mAbs (Wb166, Wb214, Wb158, Wb163 and Wb215) were those used by Paton *et al.* [19]. A microtiter

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ABBREVIATIONS: Bovine viral diarrhea virus (BVDV); Enzyme-linked immunosorbent assay (ELISA); Monoclonal antibody (ies) (mAbs); Optical density (OD).

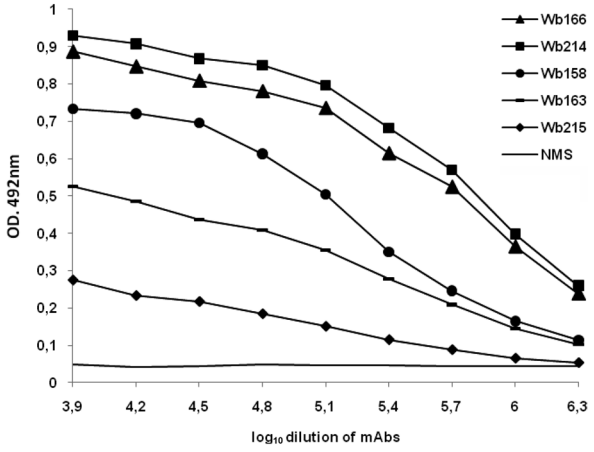

Fig. 1. Estimation of the dilutions of mAbs to be used in ELISA for testing their relative affinity.

Reactivity of E2 specific BVDV mAbs was tested against BVDV cell lysate. 96 well plates were coated with the BVDV cell lysate and mock cell lysate at a dilution of 1:100 (0.4 μg/m*l* total protein concentration). Serial two fold dilutions of mAbs (Wb166, Wb214, Wb163, Wb158 and Wb215) were titrated across the plate and reactivity was tested by the addition of secondary antibody followed by addition of the substrate. A normal mouse serum was used as a negative control.

neutralization test was performed essentially as described by Howard *et al.* [11]. A bovine antibody (2359) to BVDV [18] was a positive control. Neutralization was the complete loss of viral cytopathic effect. Duplicate wells were used, and the 50% endpoint titer was determined by the Kärber formula.

Estimation of the working dilutions of antigen and of mAbs for testing relative affinities: Microtiter ELISA plates were coated with a 2-fold dilution series of the BVDV-cell lysate and the mock-infected cell lysate from 1:25 to 1:3,200. MAb Wb166, normal mouse sera and an irrelevant mAb (to the E2 of classical swine fever virus, Wh303, [20]), were all diluted 1:16,000 and added to different wells. A peroxidase-conjugated rabbit anti-mouse IgG (Dako) was added [8]. The absorbency was measured at 492 nm. The binding of mAb Wb166 was related to the concentration of BVDV cell lysate such that OD values dropped from 1.6 to 0.2. The negative controls showed no reactivity. The 1:100 dilution of BVDV cell lysate, which gave OD of 1.0, was used in future assays.

To determine the dilution of each mAb for future relative affinity assays, microtiter ELISA plates were coated with BVDV cell lysate. MAbs Wb166, Wb214, Wb158, Wb163, Wb215 and normal mouse serum were diluted from $10^{3.9}$ to $10^{6.3}$ in 2-fold steps. The working dilution of each mAb that gave an OD in the range of 0.5 to 0.7 was selected. This dilution was $10^{5.1}$ for Wb166, $10^{5.4}$ for Wb214, $10^{4.5}$ for Wb158, and $10^{3.9}$ for Wb163 (Fig. 1). MAb Wb215 did not achieve an OD of 0.5.

The estimation of relative affinity of neutralizing BVDV E2 mAbs by ELISA: The relative affinities of the E2- specific mAbs were assessed by an ELISA based affinity elution assay [10]. In preliminary assays, a pre-treatment of BVDV-cell lysate with 8M urea did not reduce the binding of mAb Wb166 (result not shown). The selected dilution of each mAb that gave an OD of 0.5–0.7 was incubated with BVDV-cell lysate coated plates. Serial 2 fold dilutions of urea (8 M to 0.06 M) were then added to the bound mAb [9]. The % affinity index was (OD with urea/ OD without urea) × 100 [9].

Table 1. Neutralizing titers of BVDV E2 mAbs and their estimated relative affinities (AI)

Monoclonal antibodies	Neutralizing titers tested by BVDV NADL in present study	% Affinity Index
Wb 166 anti-NADL	$10^{5.7}$	66
Wb 214 anti-NADL	$10^{5.3}$	17
Wb 158 anti-NADL	$10^{4.9}$	33
Wb 163 anti-Oregon	10^{4}	18
Wb 215 anti-Oregon	$<10^{2}$	ND

Neutralization assays were performed by using BVDV NADL plaque purified and propagated in our laboratory. BVDV strains against which monoclonal antibodies directed are indicated. Neutralization was quantal and neutralizing titers of mAbs were determined as reciprocal dilution of mAb neutralising 50% of replicates of 100TCID50 of BVDV NADL. The affinity index, (AI) expressed as percentage, was (OD with urea/ OD without urea) × 100.
ND: Not determined

RESULTS

Confirmation of neutralizing activities of BVDV E2 mAbs: MAbs Wb166, Wb214, Wb158 and Wb163 displayed high neutralizing titers of $10^{5.7}$ to 10^{4}, whereas mAb Wb215 showed no neutralizing activity at 10^{2} (Table 1). The bovine antiserum (2359) to BVDV had a neutralizing titer of 10^{3} as expected. The mAb Wb163 that was raised against BVDV Oregon had a neutralizing titer of 10,000 to NADL compared to that of 35 in the earlier study [19].

The present stock of NADL, as sequenced by Iqbal [14], was compared not only to the NADL of Paton *et al.* [19] but also to that of Collett *et al*. [4], see Table 2. At position 53 Iqbal's and Paton's were the same but Collett's was not. Conversely, at position 79 Iqbal's and Collett's were the same but Paton's was not. At position 266 Collett's and Paton's were the same but Iqbal's was not. Collett's NADL was the same as Paton's Oregon in all 3 positions (Table 2).

The estimation of relative affinity of neutralizing BVDV E2 mAbs by ELISA: Urea had little effect on the elution of bound mAb until its molarity reached 4 M (Fig. 2). The estimated affinity indexes of the mAbs Wb166, Wb214, Wb158 and Wb163 with 8 M urea were 66%, 17%, 33% and 18%, respectively (Fig. 2).

DISCUSSION

A comparison of the neutralizing titers of mAbs tested in present and previous studies: The assessment of the neutralizing activity of 5 E2 specific BVDV mAbs (Wb166, Wb214, Wb158, Wb163 and Wb215) to BVDV NADL grown in our laboratory revealed that four had neutralizing activity, (Table 1). MAb Wb166 showed the highest neutralizing titer

Table 2. Neutralization titers of the mAb Wb163 against 2 different BVDV's (NADL and Oregon) and the amino acid at positions 53, 79 and 266 of different stocks of BVDV E2

Virus strains used by researchers	Amino acid in positions			History of NADL in laboratories	Titer
	53	79	266		
Oregon C24V Paton *et al*. (1992)	L	V	V	Cloned three cycles and a total of 67 passages	420
NADL Collett *et al*. (1988)	L	V	V		ND
NADL Paton *et al*. (1992)	F	E	V	Two plaque purifications and a total of 12 passages	35
NADL Iqbal (2001)	F	V	A	Three plaque purifications and <7 passages	ND
NADL used in present study	ND	ND	ND	Two plaque purifications by Iqbal and another plaque purification and <7 passages in this study	10.000

ND: Not determined.

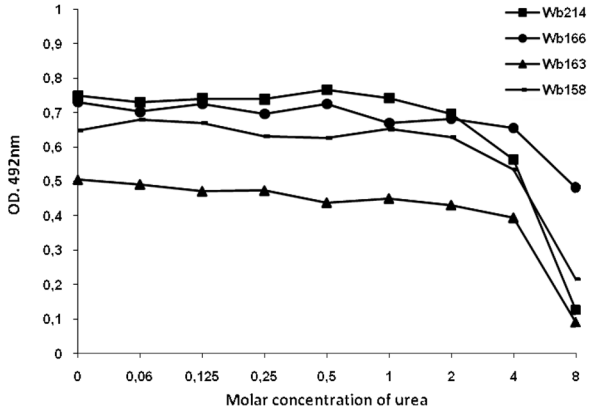

Fig. 2. The estimation of relative affinity of neutralizing BVDV E2 mAbs by ELISA.

ELISA plates were coated with the BVDV cell lysate at a dilution of 1:100. The dilution of mAbs that gave 0.5–0.7 OD492 nm value with BVDV cell lysate was allowed to bind. Urea was added to duplicate wells in concentrations ranging from 0 to 8 molar and incubated for 30 min. The% affinity index was calculated as: (OD with 8 M / OD without urea) × 100. The binding of mAbs Wb166, Wb214, Wb163, and Wb158 to BVDV cell lysate in the absence of urea were OD492 0.749, 0.731, 0.506, and 0.649. The percentage affinity index was 66% for Wb166, 17% for Wb214, 33% for Wb158 and 18% for Wb163.

of 500,000. The anti-BVDV Oregon (C24V) mAb Wb163 neutralized our virus to a titer of 10,000 compared to titers of <35 to NADL and 420 to Oregon [19]. Such large differences between the neutralizing titer of mAb Wb163 reported by Paton *et al.* [19] and that presented in this study could be related to the variability of the E2 glycoprotein sequences between BVDV NADL grown in different laboratories [3]. The present virus used in our antibody study was plaque-purified from Iqbal's twice-plaqued stock [14]. Picking one plaque in 3rd round of plaque purification and then expanding it under non-selective conditions was unlikely to have resulted in mutation (s) compared to the NADL stock Iqbal sequenced. Iqbal's NADL shared a V at position 79 with the NADL of Collett *et al*. [4] and the Oregon of Paton *et al.* [19], whereas Paton's NADL had E at position 79 (Table 2). It was likely that V instead of E at position 79 was critical for the strong neutralization by mAb Wb163.

Iqbal found two other unexpected amino acid changes of NADL compared to Collett *et al.* [14]. These changes were L to F at position 53 and V to A at position 266 (Table 2). The amino acid change of L to F at position 53 was also in Paton's NADL (Table 2). Although Iqbal suggested that these variations were likely to have been induced by *Taq* polymerase during PCR and/or sequencing, their presence in other BVDV isolates remain to be investigated. It should, however, be noted that changes at different regions of E2 might be continuing sources of antigenic variation of between BVDV strains and vaccine stocks produced in different laboratories.

The estimating relative affinities of neutralizing mAbs: The use of urea for estimating relative antibody affinity is based on the elution of antibodies bound to the antigen by urea in a solid phase ELISA [10]. This method has been applied to test the antibody avidity for evaluating measles vaccine efficacy in ELISAs [9], measuring antibody affinity in patients with respiratory syncytial virus infection [17] and serodiagnosis of Hepatitis C virus infection [15]. The highest affinity index of 66% was observed with the mAb Wb166 that had the highest neutralizing titer (Fig. 2). There was not such a great difference between the relative affinities of the mAbs Wb214, Wb163 and Wb158 (17% − 33%). This indicates that a urea-based dissociation assay for testing relative affinities of the high titer neutralizing mAbs might not be discriminatory enough.

In conclusion, the preliminary results of the present study highlighted the importance of the variability in amino terminal part of BVDV E2. In particular, the replacement of Valine by Glutamic acid at position 79 may weaken the neutralizing ability of mAb Wb163. To confirm this, the present virus would need to be sequenced, and Iqbal's NADL would need to be used in neutralization test with the mAb Wb163.

An extension to this work would be to compare strains of BVDV and vaccines held in different laboratories not only by neutralization using a panel of mAb but also by sequencing of their E2 genes. Sequence variations might be related to those of mAb escape mutants. This would enhance our knowledge of the antigenic diversity of BVDV and its vaccines.

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