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Characterization of Antigenic Sites on the Rinderpest Virus N Protein Uusing Monoclonal Antibodies

Kang-seuk Choi*, Jin-ju Nah, Young-joon Ko, Cheong-up Choi, Jae-hong Kim, Shien-young Kang1 and Yi-seok Joo

National Veterinary Research and Quarantine service, Ministry of Agriculture and Forestry, 480 Anyang, Kyounggi 430-824, Korea
1Research Institute of Veterinary Medicine, College of Veterinary Medicine, Chungbuk National University, 48 Gaeshin-dong, Heungduk-gu, Cheongju, Chungbuk 361-763, Korea

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Abstract

The N protein of the rinderpest virus (RPV) was analyzed topologically and antigenically by using anti-N monoclonal antibodies (Mabs). Ten Mabs were raised against the N protein of the RPV. At least six non-overlapping antigenic sites (sites A-F) were delineated by competitive binding assays using biotinylated Mabs. Of them 5 sites (A, C, D, E and F) on the N protein were recognized by RPV-specific Mabs in ELISA and IFA while site B was recognized by Mabs reacting with both RPV and PPRV. Non-reciprocal competition was found among sites C, D and E. Recombinant RPV N protein after exposure to 0.2% SDS exhibited higher ELISA titers in all Mabs recognizing 6 sites. Four sites (A, B, E and F) on 2% SDS-treated N protein lost completely reactivity with Mabs while the remaining sites (C and D) on the protein retained their antigenicity to some degree. It indicates that two sites (C and D) were sequential. Six representative Mabs bound to each site exhibited competition with rinderpest antibodies in a blocking ELISA, indicating that the sites were actively involved in antigenicity in cattle.

Key words: monoclonal antibody, N protein, rinderpest virus, antigenicity

Introduction

Rinderpest virus (RPV) has caused an acute, febrile and highly contagious disease in cattle and wild bovids in Africa, the Middle East and South Asia for several decades. Recently, rinderpest has been eradicated but enzootic foci are still present in East Africa and Asia, particularly Pakistan [19, 30]. Korean peninsular has been maintained as a rinderpest free status since the last outbreak in the Northern part in 1931 [20]. Between 1945 and 1985, Korea has carried out ring-vaccination annually in cattle population along the demilitarized zone in order to prevent transmission of the disease from the Northern region [3, 21]. Since then, a vaccine stock policy for emergency without vaccination has been established in place of the restricted vaccination.

The RPV belongs to the genus *Morbillivirus* in the family *Paramyxoviridae*. The other members of the genus include peste-des-petits-ruminants virus, measles virus, canine distemper virus, phocine distemper virus and dolphin morbillivirus [1, 6, 7].

The genome of RPV contains genes encoding structural proteins of fusion (F), haemagglutinin (H), nucleocapsid (N), matrix (M), polymerase (L), phosphoprotein (P) and two nonstructural proteins C and V [6, 11, 12]. The N protein gene, which is highly conserved among morbilliviruses, is located at the 3'end of the genome and contains an open reading frame (ORF) of 1,575 bp encoding encoding 525 amino acids [11,12].

The N protein is one of the most abundant viral proteins so the majority of antibodies produced during infection are specific for the N protein of RPV [8, 23, 25]. The gene is, therefore, an attractive target for diagnostic applications in morbilliviruses, including ELISA [10, 11, 15, 16, 17, 27, 28].

Monoclonal antibodies (Mabs) have been used as tools in studies of epitope mapping on the viral protein, diagnostic applications, and pathological/immunological mechanisms. Especially epitope mapping studies have been successfully carried out on the basis of competitive binding assay, serological specificity and biological activity of the Mabs [22, 24, 25].

With regard to the N protein of RPV, the antigenic sites on the N protein of RPV-L [25] and RPV-RBOK strains [18] have been mapped by the competitive ELISA using anti-N

*Corresponding author: Kang-seuk Choi
National Veterinary Research and Quarantine Service, Ministry of Agriculture and Forestry, 480 Anyang, Kyounggi 430-824, Korea
Tel: +82-31-467-1860; Fax: +82-31-449-5882
E-mail: choiks@nvrqs.go.kr

Mabs. The antigenic sites of the RPV-L strain were inconsistent with those of RPV-RBOK since subtle antigenic differences between virus strains and the preparation of the Mabs depend on the delineation of the sites. Therefore, additional information about the antigenic sites on the N protein of the RPV-LATC strain remains to be elucidated.

In this study, we prepared 10 anti-N Mabs against the RPV-LATC strain, a vaccine strain of RPV in Korea and characterized antigenic sites on the N protein of the RPV-LATC strain using these Mabs.

Materials and Methods

Viruses and sera

RPV-LATC strain [3] was grown in monolayers of Vero cells (American Type Culture Collection, USA) in alpha minimum essential medium supplemented with 5% fetal bovine serum, antimycotics and antibiotics (GibcoBRL, USA) using roller culture apparatus (Bellco, USA).

The viral antigens from RPV-LATC strain were partially purified from infected Vero cell cultures by centrifugating thawed cell lysates through a 25% (w/v) sucrose cushion as described previously [29]. The purified viral antigens were adjusted to the concentration of 0.1 mg/ml and stored at -20 oC until use.

Eighteen serum samples were obtained from 9 cattle by bleeding before and 3 weeks after experimental rinderpest vaccination (RPV-LATC). All sera from vaccinated cattle were determined having virus-neutralizing antibody titers of 1:11-1:16. All sera from pre-vaccinated cattle gave negative results to the VN test.

Preparation of hybridoma cell lines and ascitic fluids

BALB/c mice were immunized with purified viral antigen (50 μg per dose in Freund's incomplete adjuvant) via foot-pad route [5]. Ten to fifteen days after immunization, the lymphocytes derived from popliteal lymph nodes of immunized mice were harvested and fused with the SP2/0 myeloma cells using polyethylene glycol 1500 (Boeringer Manheim, Germany) by the conventional method. Hybridoma cells secreting anti-N Mabs were screened by immunofluorescence assay (IFA) and then selected by indirect ELISA using recombinant N protein of RPV-LATC strain [4]. The positive hybridoma cells were subjected to cloning by the limiting dilution method and finally inoculated intraperitoneally into BALB/c mice, which were primed by Freund's incomplete adjuvant. Ascitic fluid was collected 1 to 2 weeks later.

The isotype of an antibody was determined by commercial ELISA kit (Boeringer Manheim) according to the manufacturer's instruction. Ascitic fluids were purified using a Immunopure (A/G) IgG Purification Kit (PIERCE, USA) and then biotinylated additionally by a Biotin Labeling Kit (Boeringer Manheim).

The concentration of immunoglobulins in ascitic fluid was measured using a commercial BCA Protein Assay Kit (PIERCE) according to the manufacturer's instructions.

IFA

Vero cells were cultured on coverslips for 3 days after infection with RPV-LATC strain, washed with 0.01 M phosphate buffered saline (PBS) once, air-dried and fixed with cold acetone for 20 min at -20 oC. The fixed cells were reacted with the Mabs (1:1,000 and 1:10,000 dilutions of ascitic fluid) and then stained with fluorescein isothiocyanate (FITC)-labelled anti-mouse IgG (Kirkegaard -Perry Laboratories, Inc., USA). The cells were mounted in buffered glycerol and examined by fluorescence microscopy (Olympus).

N proteins

Four baculovirus-expressed N proteins from strains of RPV-LATC [4], RPV-RBOK, RPV-RGK, and PPRV-Nig75/1 [17] were used. Recombinant N proteins from strains of RPV-RBOK, RPV-RGK and PPRV-Nig75/1 were kindly supplied by Dr. G. Libeau, CIRAD-EMVT, Montpellier, France.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting

Whole viral proteins were fractionated on the vertical 12% SDS-PAGE under denaturing conditions [14]. The proteins were subsequently transferred to nitrocellulose membranes [26]. Immunodetection was performed by standard techniques using Mabs (1:1,000 dilution), optimally diluted alkaline phosphatase-conjugated second Abs (Kirkegaard-Perry Laboratories, Inc.), and BCIP/NPT solution (Kirkegaard-Perry Laboratories, Inc.) as a substrate.

Mab affinity analysis by indirect ELISA

The wells of MaxisorpTM ELISA plate (Nunc, USA) were coated with 50 μl of purified viral antigen (1.0 μg/ml) in 0.01 M PBS for 1 h at 37℃. The antigen-coated plates were incubated with 50 μl of serial dilutions of purified Mabs in blocking buffer (0.01 M PBS containing 3% skimmed milk and 0.05% Tween 20) for 1 h at 37℃. The plates were then incubated with 50 μl of optimally diluted peroxidase -labelled mouse IgG (Kirkegaard-Perry Laboratories, Inc.) in blocking buffer for 1 h at 37℃. The plates after each incubation step were washed with PBST (0.002 M PBS containing 0.05% Tween 20) three times. Color development of the reaction was carried out by 10 min incubation with a chromogen solution (ortho-phenylenediamine) and stopped by addition of 1.25 M sulfuric acid. Optical density (OD) were read at the 492 nm wavelength. The steady-state equilibration affinity constant, Kd, was estimated from the concentration (μg/ml) of each Mab corresponding to 50% maximal binding.

Competitive binding assay

MaxisorpTM ELISA plates were coated with 50 μl of

purified viral antigen (final 1.0 μg/ml) for 1 h at 37 ℃. All buffers used were the same as those for the ELISA affinity analysis above. After washing the plates with PBST, the antigen-coated plates were incubated with 50 μl of serial dilutions of un-labeled competing Mabs for 30 min at 37℃. Without wash step, an equal volume of the biotinylated Mab of maximum absorbance was added into all wells of the plates and further incubated for 45 min at 37℃. Following washing step, the plates were incubated with 50 μl of peroxidase-labeled streptavidin (Kirkegaard-Perry Laboratories, Inc.) for 1 h at 37 oC. The subsequent steps were carried out as described above. The reaction was considered as competition positive when the OD of the labelled Mab in the presence of unlabelled Mabs showed 50% or greater reduction of that of the labeled Mab alone.

Titration ELISA

Titration ELISA was performed using two different procedures; 1) The Mab dilution method: MaxisorpTMELISA plates were coated with 50 μl of viral or recombinant N proteins at pre-determined concentration for 1 h at 37℃. After washing step, the antigen-coated plates were incubated with 50 μl of serial dilutions of the Mab for 1 h at 37℃. 2) The antigen dilution method: recombinant N protein was treated with SDS at final concentration of 0%, 0.2% and 2%, respectively for 30 min at room temperature. The treated antigens were two-fold diluted, starting from saturating dilution and then absorbed onto ELISA plates for 1 h at 37℃. After washing step, the antigen-coated plates were incubated with 50 μl of pre-determined dilution of Mabs for 1 h at 37℃.

Following antigen-antibody reaction step, the subsequent steps were carried out as described above. The wells giving an absorbance greater than 0.2 were considered as positive.

Blocking ELISA

Viral antigen (1.0 μg/ml) coated ELISA plates were incubated with test sera at a dilution of 1:10 for 30 min at 37℃. Without washing, the plates were then further incubated with each Mab at a saturating concentration for 1 h at 37℃. Following washing step, the subsequent steps were carried out as described above. The OD values were used to calculate the percent inhibition (PI) induced by serum antibodies using the following formula: PI = [1-(ODserum/ODMab)] × 100, where ODserum is the mean OD of wells with serum plus Mab, and ODMab is the mean OD of wells with Mab alone. The cut-off values were taken as the mean PI ± 3 standard deviations (SD) of nine negative control sera.

Results

Production and characterization of N protein-specific Mabs

A total of 10 Mabs were raised against N protein of RPV-LATC strain and characterized by ELISA, IFA and Western immunoblotting as summarized in Table 1. All Mabs consisted of immunoglobulin G heavy chains and kappa light chains. The subclass of the Mab R-3E-03 was classified into IgG1, R-2G-10, R-3A-08, R-5C-07 and R-5D-03 into IgG2a and R-3B-04, R-4B-04, R-4D-05, R-8A-04, and R-8C-04 into IgG2b. Concentration of immunoglobulins in ascitic fluid ranged from 38.7 mg/ml (R-4D-05) to 17.0 mg/ml (R-8C-04). In affinity analysis, the viral N protein showed very low affinity with Mabs R-2G-10 (Kd = 1.0 μg/ml) and R-3E-03 (Kd = 1.86 μg/ml) and moderate affinity with Mab R-8C-04 (Kd = 0.33 μg/ml). The affinity of other Mabs was high (Kd <0.2 μg/ml). All the Mabs bound to viral N protein of RPV-LATC strain by IFA and ELISA. Titers of Mabs in IFA and ELISA were not consistent with their affinity to the antigen (Table 1). Only three Mabs (R-3A-08, R-4B-04 and R-5C-07) reacted with denatured viral antigen in Western immunoblotting (Fig. 1).

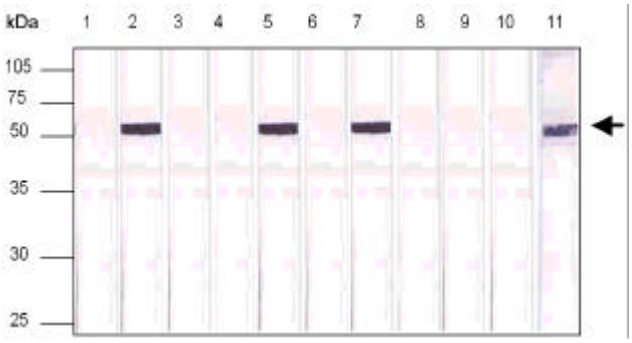

Fig. 1. Reactivity of anti-RPV N Mabs with denatured RPV N protein in Western immunoblotting. Whole viral proteins were denatured by the treatment of SDS, 2-mercaptoethanol and boiling and then subjected to the SDS-PAGE and Western immunoblotting. Arrow indicates the N protein. Each lane represents R-2G-10 (1), R-3A-08 (2), R-3E-03 (3), R-3E-03 (4), R-4B-04 (5), R-4D-05 (6), R-5C-07 (7), R-5D-03 (8), R-8A-04 (9), R-8C-04 (10) and hyperimmune RPV bovine serum (11), respectively.

Reactivities of the Mabs with different N proteins of RPV and PPRV

Indirect ELISAs using different N proteins of RPV (RPV-LATC, RPV-RBOK and RPV-RGK) and PPRV (PPRV-Nig75/1) were performed. All Mabs except R-3E-03 reacted exclusively with N proteins of RPV whereas R-3E-03 bound to N proteins of three RPV strains and the PPRV-Nig75/1 strain (Table 2).

Delineation of antigenic sites on the RPV N protein by competitive binding assay

Antigenic sites to which the Mabs bound were analyzed by competitive binding assays. Binding of biotinylated antibodies to the solid phase viral antigen was determined in the absence or presence of various concentrations of

Table 1. Characterization of N protein-specific Mabs produced in this study

Map	Isotype subclas	Conc. Ab. in Ascites (mg/ml)	Affinity (Kd μg/ml)	Reactivity with RPV-LATC		
				IFA	ELSA	WBA
R-2G-10	IgG2a	25.7	1.00 a	++ b	++	−
R-3A-08	IgG2a	36.2	0.06	++	++	++
R-3B-04	IgG2b	29.0	0.14	++	++	−
R-3E-03	IgG1	23.8	1.86	++	+	−
R-4B-04	IgG2b	33.1	0.08	++	++	++
R-4D-05	IgG2b	38.7	0.19	++	++	−
R-5C-07	IgG2a	31.9	0.16	++	++	++
R-5D-03	IgG2a	28.9	0.05	++	++	−
R-8A-04	IgG2b	30.0	0.10	++	++	−
R-8C-04	IgG2b	17.0	0.33	+	+	−

a The affinity constant, Kd, was determined from the concentration (μg/ml) of the Mab corresponding to 50% maximal binding to the N antigen.
b Anti-N Mabs at 1:1000, 1:10,000 dilutions were tested by ELISA, IFA and Western immunoblotting assay. ++, positive at 1:10,000 dilution; +, positive at 1;1,000 dilution; −, negative at 1:1,000 dilution.

Table 2. Reactivity of the Mabs with N proteins of RPV and PPRV

Mab	Reactivity with recombinant N proteins from			
	RPV-LATC	RPV-RBOK	RPV-RGK	PPRV-Nig75/1
R-2G-10	++a	++	+	−
R-3A-08	++	++	++	−
R-3B-04	++	++	++	−
R-3E-03	+	+	+	+
R-4B-04	++	++	++	−
R-4D-05	++	++	++	−
R-5C-07	++	++	+	−
R-5D-03	++	++	++	−
R-8A-04	++	++	++	−
R-8C-04	+	+	+	−

a Anti-N Mabs at 1:1,000, 1:10,000 dilutions were tested by indirect ELISA. ++, positive at 1:10,000 dilution; +, positive at 1;1,000 dilution; −, negative at 1:1,000 dilution.

un-labelled antibodies. The competition patterns revealed that at least six distinct epitopes, denoted A-F, were recognized by the Mabs as shown in Table 3. Competition of six anti-N Mabs by homologous and representative heterologous Mabs is shown in Fig. 2. Mabs R-2G-10 (site A), R-3E-03 (site B), and R-8C-04 (site F) showed competition with homologous antibodies only. Mabs R-3A-08 recognized the site C. Mabs R-4B-04 and R-5C-07 bound to the site D and Mabs R-3B-04, R-4D-05, R-5D-03 and R-8A-04 to the site E. One-way competition was found in between site C and site D, or between in site D and site E.

Effect of SDS on antigenicity of Mab epitopes

Recombinant N protein from RPV-LATC strain was treated with various concentration of SDS to investigate whether denatured N antigen retain the antigenicity of each site. To all Mabs the N protein after exposure to 0.2% SDS exhibited higher ELISA titers than the titers by untreated N antigen (Fig. 3). However, when the N antigen was treated with 2% SDS, the sites A (R-2G-10), B (R-3E-03), E (R-8A-04) and F (R-8C-04) showed no reactivity with corresponding Mabs while sites C (R-3A-08) and D (R-5C-07) showed reduced reactivity with corresponding Mabs.

Blocking of Mab epitopes by serum antibodies

We investigated whether the binding of a Mab to corresponding epitopes was competed out by that of RPV serum antibodies to the antigen in a blocking ELISA. If it

Table 3. Mapping of antigenic sites in the N protein of RPV-LATC strain by competition binding assay

	Biotinylated Mabs					
	R-2G-10	R-3E-03	R-3A-08	R-5C-07	R-8A-04	R-8C-04
R-2G-10 (A)a	++b	-	-	-	-	-
R-3E-03 (B)	-	+	-	-	-	-
R-3A-08 (C)	-	-	++	+	-	-
R-5C-07 (D)	-	-	-	++	+	-
R-4B-04 (D)	-	-	-	++	+	-
R-8A-04 (E)	-	-	-	-	++	-
R-3B-04 (E)	-	-	-	-	++	-
R-4D-05 (E)	-	-	-	-	++	-
R-5D-03 (E)	-	-	-	-	++	-
R-8C-04 (F)	-	-	-	-	-	+

a Letters in parenthesis indicate the antigenic sites recognized by Mabs.
b The biotinylated Mabs in the presence of serially diluted un-labelled Mab showing 50% or greater reduction of the biotinylated Mab alone were considered as competition positive. ++, positive at 1:1000 dilution; +, positive at 1:100 dilution; -, negative at 1:100 dilution.

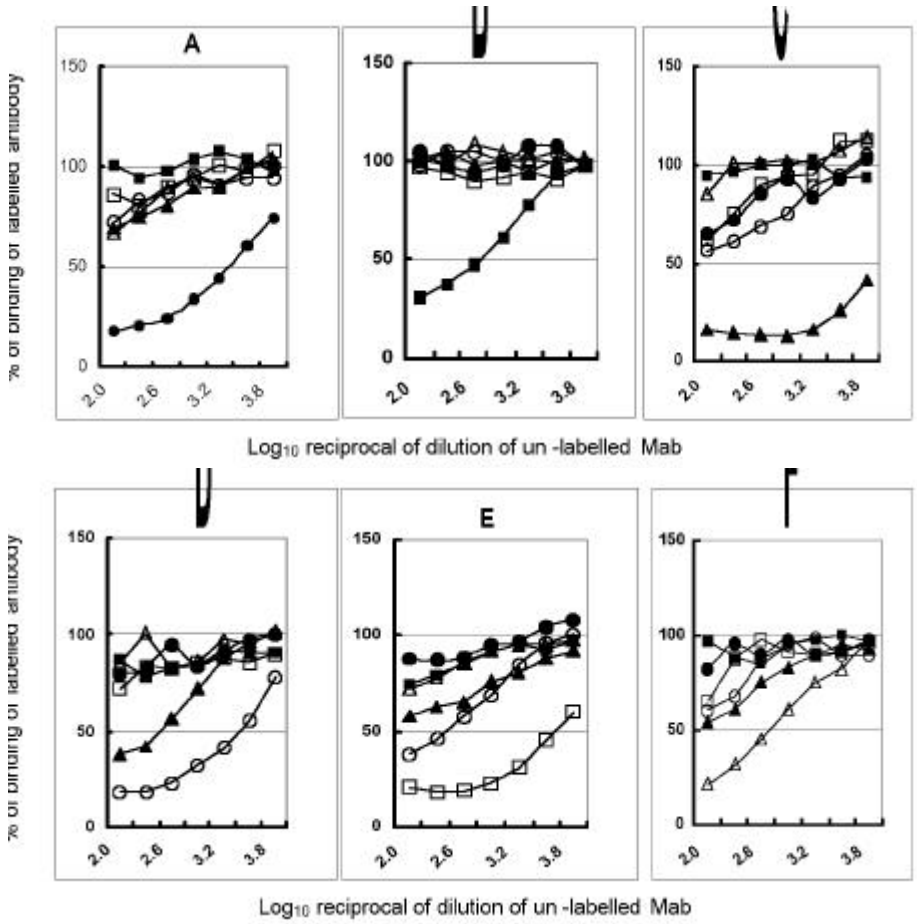

Fig. 2. Competitive binding of representative Mabs to the N protein in competitive binding assay. Absorbance values developed by a biotinylated Mab in the presence of unlabelled antibodies at the indicated dilutions and applied to the viral N protein on solid phase were expressed as a percentage of the absorbance value of unlabelled antibody. Bionylated Mab: A, R-2G-10; B, R-3E-03; C, R-3A-08; D, R-5C-07; E, R-8A-04; F, R-8C-04. Unlabeled antibody: ●, R-2G-10; ■, R-3E-03; ▲, R-3A-08; ○, R-5C-07; □, R-8A-04; △, R-8C-04.

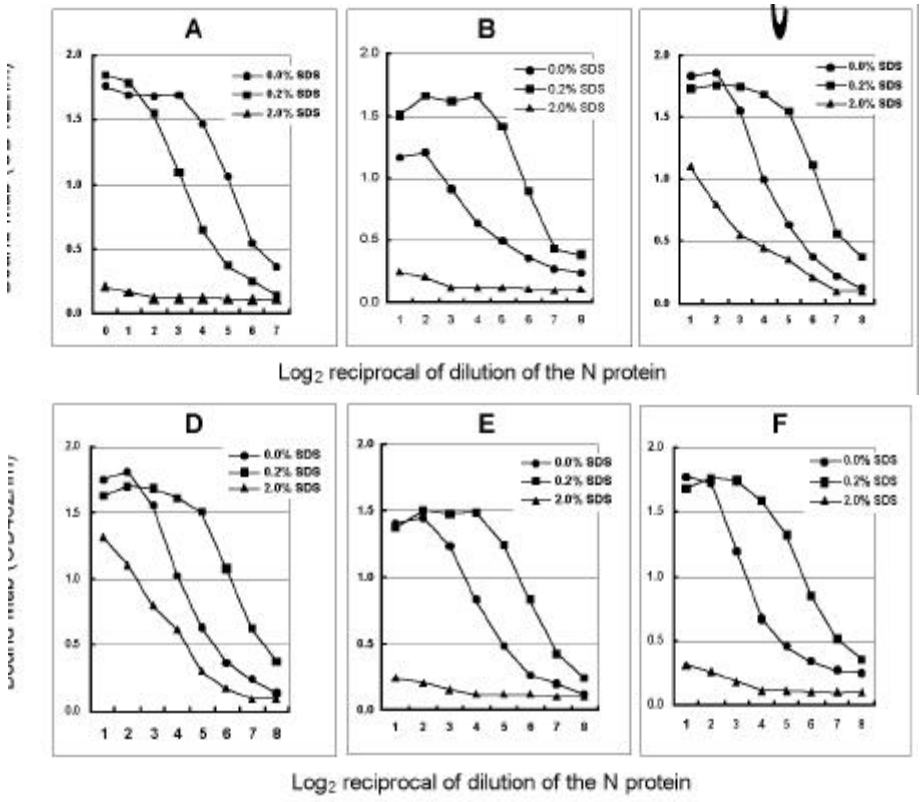

Fig. 3. Reactivity of Mabs with N protein of RPV exposed to SDS. Recombinant N protein treated with SDS of 0%, 0.2% and 2.0%, respectively was two-fold diluted, starting from saturation dilution and then tested using anti-N Mabs in ELISA. A, R-2G-10; B, R-3E-03; C, R-3A-08; D, R-5C-07; E, R-8A-04; F, R-8C-04.

is realized, the Mab could be as detecting antibody for demonstrating anti-RPV antibodies in serum samples. Six representative Mabs (bound to each antigenic site), experimental rinderpest sera and whole virus antigen were used in this study. Blocking ELISAs for competing Mabs were established by checkerboard titration (data not shown). Pre-vaccinated sera from the same individuals were used to determine cut-off values of each Mab in the blocking ELISA. The cut-off values for Mabs R-2G-10, R-3E-03, R-3A-08, R-5C-07, R-8A-04 and R-8C-04 were set at 43.6, 67.7, 24.4, 21.2, 52.4 and 63.3 percent inhibition, respectively. All Mabs exhibited competition with RPV serum antibodies in the ELISA as shown in Fig. 4.

Discussion

Ten Mabs were raised against N protein of RPV from immunized Balb/c mice. All Mabs except for Mab R-3E-03 reacted with three N proteins from strains of RPV-LATC, RPV-RBOK and RPV-RGK but was not bind to N protein of PPRV-Nig75/1, suggesting that the Mabs possibly recognize epitopes conserved among RPV strains except PPRV-Nig75/1 strain. On the other hand, Mab R-3E-03 bound to PPRV-Nig75/1 as well as RPV strains, indicating that the eptiope of the Mab can recognize antigenic site which is shared for at least key amino acid residues with the N protein of the PPRV-Nig75/1.

Epitope mapping studies [4] on the N protein of the measles virus (MV) showed that 3 antigenic sites recognized by MV-specific Mabs were located in variable regions (aa 122-150, aa 457-476 and aa 519-525). It is postulated, therefore, that our RPV-specific Mabs can recognize regions of RPV N protein corresponding to antigenic sites of MV N protein. Using these Mabs, we mapped five RPV-specific and a RPV/PPRV-common antigenic sites on the N protein of the RPV-LATC by a competitive binding assay. Similar attempts had been made previously in the N protein of the RPV-L strain [25] and the RPV-RBOK [18]. They mapped four morbillivirus common and one RPV-specific antigenic sites on the N protein of the RPV-L, and four RPV/PPRV common and two RPV-specific sites on the N protein of the RPV-RBOK, indicating that at least three additional RPV/PPRV-common antigenic sites may be present on the N protein of the RPV-LATC.

Non-reciprocal competition in competitive binding assay has been observed in competitive binding assays by several investigators [8, 15, 18, 22, 24, 25, 29]. In this study, such unidirectional competition was found among the sites C, D and E on the N protein of RPV-LATC. The sites C and D were sequential while the site E was conformation

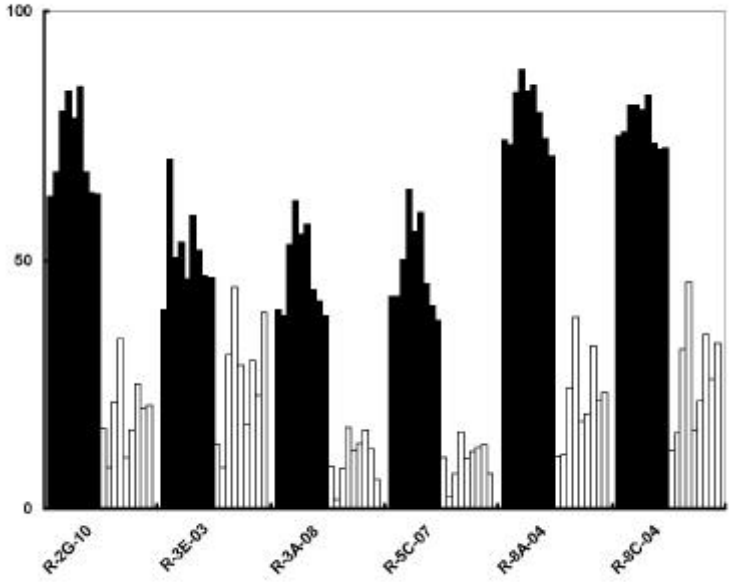

Fig. 4. The reactivity of Mab-based blocking ELISA with a collection of vaccinated cattle sera. Eighteen paired serum samples from vaccinated cattle were tested using Mab-based blocking ELISA. Black bars represent vaccinated cattle sera and blank bars represent pre-vaccinated cattle sera. Each dotted line in the box indicates cut-off value of each Mab-based blocking ELISA.

dependent. These findings may be explained in three ways: 1) These sites were so close to each other as the binding of an antibody to one of the epitopes can be sterically hindered by the binding of another antibody to the other epitope (steric hindrance), 2) the sites share some of the residues (partial overlapping) or 3) the binding of one Mab with higher affinity may induce a change in conformation that alters the binding site of other Mabs with lower affinity (conformational change of antigenic site) as demonstrated in tick-borne encephalitis virus [9].

We investigated three-dimensional antigenic structures of the sites on the N protein by treating the N antigen with SDS and then examining reactivity with Mabs. Reactivity of each site with corresponding Mab were dependent on the concentration of SDS as shown in Fig. 3. All the Mabs exhibited higher ELISA titers after treatment with 0.2% SDS than those with untreated antigen, suggesting that low concentration of SDS was allowed to expose antigenic sites on the antigen, presumably by breaking up aggregation of the protein [11], more accessible to the antibodies. All Mabs except R-3A-08 and R-5C-07 lost completely reactivity with antigen after treatment with 2% SDS, indicating that they recognize conformational epitopes. Results obtained from N antigen treated with SDS (2%) were consistent with those of Western immunoblotting (Table 1). It indicates that disulfide bonds involved in N-N or N-P interactions are unlikely to contribute to the conformation of antigenic sites, considering that SDS breaks up disulfide bonds on the protein under mercaptoethanol.

The binding of all Mab except R-3E-03 to whole viral (native N) protein was competed out by that of rinderpest cattle serum antibodies to the antigen in a blocking ELISA. This suggests that these Mabs can be used as potential competitors in a blocking ELISA for detecting RPV antibodies from serum samples. Five sites of the N protein of RPV-LATC strain are antigenic in cattle since competition in the ELISA is the result of an immunological interaction among Mabs and serum antibodies (from RPV-infected cattle) to the same epitope on the antigen.

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