大韓獸醫學會誌(1992) 第32券 第1號
Korean J Vet Res (1992) 32(1) : 83~90

Monoclonal antibodies against structural proteins of bovine viral diarrhea virus

Cang-hee Kweon*, Zee Yuan Chun**, Hee-jong Woo***
*Veterinary Research Institute, Anyang, Korea **
*Department of Veterinary Microbiology and immunology University of California Davis, CA 95616, U.S.A.***
*Laboratory of Cancer Biology, Harvard Medical School, Boston, Massachusetts. 02115 U.S.A.****
(Received Dec 5, 1991)

소 설사병 바이러스 구조단백에 대한 단크론항체 성상에 대한 연구

권창희*·Zee Yuan Chun**·우희종***
가축위생연구소*
미국 캘리포니아 수의과 대학**
미국 하바드 의과대학***
(1991. 12. 5 접수)

초록 : 소 설사병 바이러스 구조단백에 대한 단크론항체를 작성하여 혈청중화시험, 전기영동, 면역침전반응을 이용하여 분석하였던 바 다음의 결과를 얻었다. 중화능력이 있는 항체의 경우 56K내지 54K의 구조단백에 대응하였다. 그외 중화력을 나타내지 않는 항체는 45K와 36K의 바이러스 항원과 대응하였다. 순수정제된 바이러스의 전기영동 분석결과 12종 이상의 바이러스 단백성분이 구조단백질로서 검출되었으며 중화능력을 나타내는 항체를 이용한 면역침전 결과는 이들의 존재를 뒷받침하였다. 중화단백성분의 세포내 전구물질의 검출은 불가능하였으나 방사선동위원소 부착즉시 세포배지에서 바이러스의 존재를 확인할 수 있었다.

Staphylococcus aureus V_8효소를 이용한 항원의 부분소화 분석결과 45K와 36K의 바이러스 항원은 서로 상관이 있는 것으로서 입증되었다.

Key words : Monoclonal antibodies, bovine viral diarrhea virus, neutralizing epitope, structural proteins, pulse labeling, partial peptide mapping.

Introduction

Bovine viral diarrhea virus(BVDV) is the causative agent of viral mucosal disease in cattle.[1] Besides clinically observed diarrhea, it frequently causes fever, oral ulceration, cough, leukemia as well as persistant infection in mild cases. Abortion, stillbirth and congenital defects were also confirmed in the cases of pregnant cows.[2] BVDV has a single stranded RNA of positive polarity with about 12kb in length.[3~5] Recent studies on viral proteins indicated that the sum of viral proteins in infected cell was more than its maximum coding capacity of viral genome, which suggesting the precursor-product relationship among those viral proteins.[6] However, numerous studies of the viral proteins of BVDV showed deviations, both in numbers of proteins and in molecular weight.[6~12] The controversies with respect to structural proteins of BVDV seem to be mainly due to the difficulties in virus purification as well as low viral yield in cell culture. In this study, the monoclonal antibodies against structural proteins of BVDV were derived and applied for

characterization of structural proteins of BVD virus.

Materials and Methods

Virus and cell : The plaque purified NADL strain of BVDV was propagated in Madin-Darby bovine kidney(MDBK) cells grown in Dulbecco's modified Eagle's minimal essential medium(DMEM) supplemented with 10% fetal bovine serum. The maintenance medium for infected cells was DMEM supplemented with 5% horse serum of human serum. The procedures for purification of BVDV have been described by Chu et al.[13] The protein concentration was determined by the procedures of Bio-Rad.[14]

Production of monoclonal antibodies : Four week old Balb/c mice were immunized intraperitoneally with 100 μg of purified BVDV emulsified with Freund incomplete adjuvant. At regular three week intervals, these mice received the same antigen without adjuvant for a total of three times until three days before cell fusion. Monoclonal hybridomas were produced according to standard methods using P3 × 63Ag 8.653.[15] After fusion, the supernatants from hybridomas were tested for the presence of antibodies against BVDV by the indirect fluorescent antibody method(IFA) using BVDV infected MDBK cells, which were fixed with 4% paraformaldehyde in PBS or cold acetone.[16] The positive cells were cloned twice by the limiting dilution procedures. The monoclonal ascites were produced by inoculating mice with 10^7hybridoma cells. Immunoglobulin subclasses were determined by the Ouchterlony double diffusion method.[17]

Virus neutralization test : Two fold dilutions of heat inactivated mouse ascites were mixed with an equal volume of 50~100 PFU BVDV and incubated at 37℃ for 2hours. Each sample was inoculated to duplicate of 5mℓ flask with actively growing MDBK cells when 90% of monolayer was made. Cells were inoculated with virus 4~5hrs after subculture with 2.5×10^5cells. After 2hrs inoculation, 5mℓ of overlay medium containing DMEM, 0.5% agarose and 5% horse serum was added to each flask followed by incubation at 37℃ for 4days. Plaques were visualized by staining cells with 0.01% neutral red. Neutralizing antibody titers were determinded by 50% plaque reduction.

SDS-PAGE and immunoblotting analysis : Purified BVDV(20 μg) or infected cell lysates, prepared by the methods of Swack et al[18], were subjected to SDS-PAGE in 10% slab gel at 4℃.[19] The electrophoresis pattern of purified BVDV was stained with Coomassie brilliant blue and scanned by a video densitometer(Biomed Instrument, INC). For immunoblotting, the SDS-PAGE separated proteins were electrophoretically transferred to nitrocellulose paper as described.[20] Following transfer, the nitrocellulose paper was blocked in 3% bovine serum albumin containing 0.15M NaCl, 0.05M Tris-HCl, pH 7.4 for 30minutes at room temperature. The subsequent immunodetection procedures were as previously described.[21,22]

Immunoprecipitation : Monolayers of MDBK cells in 25㎠ flasks were inoculated with virus at a multiplicity of infection(MOI) of 1.0 PFU/cell. At 24hrs post infection the cells were labeled with 10 μCi/mℓ of ^{35}(S)-methionine with a specific activity(s.a) 1117Ci/mℓ. After 8hrs incubation, the infected cell supernatant was collected and centrifuged at 16,000 × g for 5minutes to remove cell debris. The collected supernatant was mixed with 1% SDS PBS(0.14M NaCl, 2.7mM KCL, 1.5mM KH_2PO_4, 8.1mM NaH_2PO_4) to final concentration of 0.1% in total volume.

After 30 minutes incubation, the supernatant was added with 10%(v/v) Pansorbin *Staphylococcus aureus* cells(Calbiochem. San Diago, CA) and incubated for 30 minutes at room temparature to remove nonspecific binding components. The supernatant was then centrifuged for 5minutes to remove *Staphylococcus* cells. The collected supernatant was used for the immunoprecipitation experiment according to the procedures previously described.[22] The fluorography of gels was prepared by the procedues of Chamberlain.[23]

Pules-Chase experiment : After inoculation of virus in MDBK cells, the cells were incubated with methionine free media for 30 minutes and then added with 200 μCi of ^{35}(S)-methionine/mℓ for 30 minutes. The cells were washed once with normal media and then replaced with a medium containing excessive methionine(150mg/mℓ) and 5% human serum. AT various time, the cells were scraped off and washed once with DMEM media and mixed into 1mℓ of lysis buffer(0.05M Tris-HCl pH 8.0, 0.5M NaCl, 0.1% SDS and 10 μM phenylmethylsulphonyl fluroride). The cells were incubated for 30 minutes on ice and the cell lysates were collected after centrigugation at 16,000 × g for 15minutes. The collected cell lysates were treated with *Staphylococcus aureus* cells to remove nonspecific binding proteins and used for immunoprecipitation. For immunoprecipitation of the whole virus particle the supernatant from virus infected cells was directly absorbed with *Staphlococcus aureus* cells and then used with monoclonal antibody according to the immunoprecipitation procedures described above. In

this case, the whole immune complex was washed with PBS twice at 5000 g and mixed with SDS sample buffer before loading into SDS-PAGE.

Partial peptide analysis : After SDS-PAGE of purified BVDV (20 μg), the gel was briefly stained with Commassie brilliant blue and each viral polypeptide was cut into a 3mm fragment according to its corresponding molecular weight. The gel slices were then placed into 7.5~17.5% Density gradient gel with 3cm stacking gel. An aliquot of 20 $\mu\ell$ of Cleveland's sample buffer(0.125M Tris, 1% SDS, 1% 2-mercaptoethanol, 1mM EDTA and 20% glycerol) was added to each well. Twenty $\mu\ell$ of digestion buffer[sample buffer containing 10% glycerol, 0.05% BPB and 20 $\mu\ell$/mℓ of *Staphylococcus aureus* protease V 8(Miles)]was overlaid into each well[24], and the gel was electrophoresed at 15mA until dye marker reached the bottom of the gel. The gel were stained with Coomassie brilliant blue followed by silver staining.[25]

Results

Derivation of monoclonal antibodies : A total of 714 supernatants of hybridoma cultures were screened by the indirect immunofluorescent antibody(IFA). The 6 hybridoma cells showing specific immunofluorescent reactions against BVDV-infected MDBK cells were established. Among these, one antibody(115~3)showed neutralizing activities. The general characteristcs of the monoclonal antibodies are listed in Table 1.

Immunoblotting and immunoprecipitation analysis : The neutralizing monoclonal antibody 115~3 reacted with 56kd(M.W) protein of the infected cell lysates(Fig 1, lane 2). However, a cross reactive pattern with 80kd protein was observed when the purified virus was used as a reactive

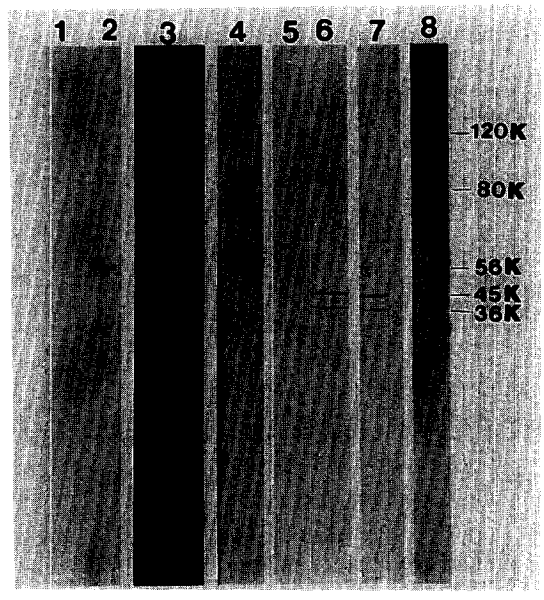

Fig 1. Immunoblotting analysis of monoclonal antibodies.
Lane 1, uninfected and Lane 2, NADL infected MDBK cell lysate were reacted with monoclonal antibody 115~3(1000×)
Lane 3 and 4, purified NADL reacted with monoclonal antibody 115~3(lane 3 : 2000×, lane 4 : 1000×, respectively.)
Lane 5, uninfected and lane 6, NADL infected MDBK cell lysate were reacted with monoclonal antibody 2~4(2000×)
Lane 7, purified NADL was reacted with monoclona antibody 2~4(2000×)
Lane 8, purified NADL was reacted with monoclonal antibody 63~6(1000×)

antigen, but this cross reaction was diminished with higher dilution of monoclonal ascites(Fig 1, lane 3 and 4). When this monoclonal antibody was reacted with ^{35}(S)-methionine labeled virus infected cell supernatants lysates(ICSLS), three viral proteins of 56kd, 54kd and 45kd were immunoprecipitated(Fig 2a, lane 2). In order to determine if the 45kd protein was detected through close association with the 56kd to

Table 1. Characteristics of monoclonal antibodies

Clones	Isotype	Proteins recognized		IFA*	Virus ⓐ
		immnoblotting	immuneprecipitation	neutralization	
115~3	IgG1	56~54Kd	56~54Kd	+	+
2~4	IgG1	45, 36Kd	45Kd	+	−
63~6	IgG1	120Kd	NR	+	−
15~1	IgG1	NR	NT	+	−
38~7	IgG2a	NR	NT	+	−
40~1	IgG2a	NR	NT	−	−

NR : Not reacted.
NT : Not tested.
• : Indirect fluorescent antibody method using BVD virus infected cells.
ⓐ : 50% plaque reduction test using 50~100pfu(=32).

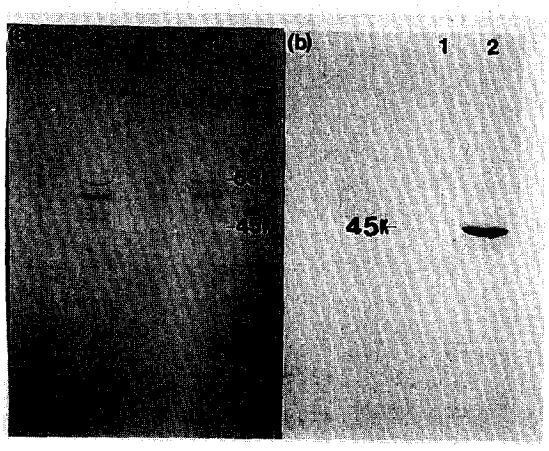

Fig 2. Immunoprectipitation of BVDV polypeptides using monoclonal antibodies.

(a), lane 1 and 3 : uninfected controls, lane 3 and 4 : BVDV infected cell supernantants, which were reacted with monoclonal antibody 115~3.

(b), lane 1 : uninfected control and 2 : BVDV infected cell supernatant were reacted with monoclonal antibody 2~4.

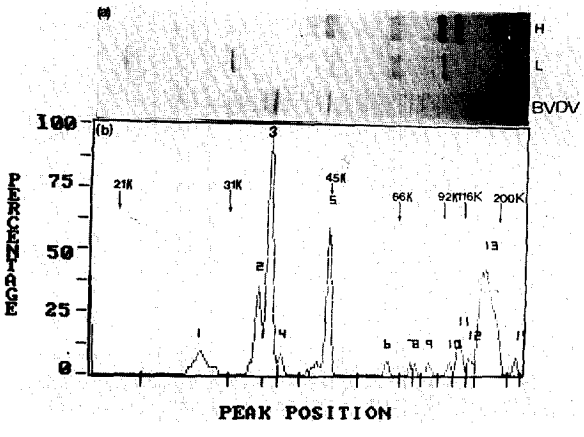

Fig 3. Polypeptides profile of purified BVDV.

a. H : high molecular markers
L : Low molecuar markers
BVDV : purified virus(NADL)

b. Scanning of the separated viral polypeptides numbers and arrows at the top of the graph represent the position and sizes in deltons of the polypeptides used for molecular weight markers(MW × 10^3)

54kd viral polypeptides, virus infected cell supernatant was directly incubated with monoclonal antibody and reacted with anti-mouse IgG. The mixture was then reacted with *Staphylococcus aureus* cells. The whole immune complex was treated with 0.1%SDS for 30minutes and washed with 0.1%SDS in PBS before loading into SDS-PAGE. Since only the 56kd and 54kd proteins were detected in this experiment, the 45kd protein band, which was detected in immunoprecipitation experiment using ICSLS, comes from the result of coimmunoprecipitation with those two polypeptides(Fig 2a, lane 4).

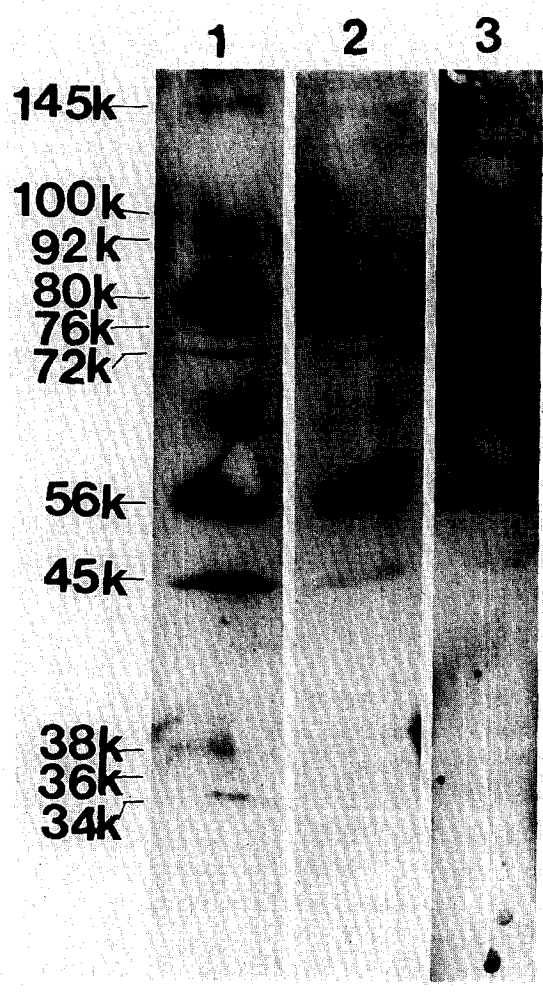

Fig 4. Immunodetection of BVDV polypeptides with the positive bovine serum.

Lane 1. purified viral polypeptides reacted with 50 × bovine serum.

Lane 2. purified viral polypeptides reacted with 100 × bovine serum.

Lane 3. purified viral polypeptides reacted with 200 × bovine serum.

Another monoclonal antiboy(2~4) reacted with the 45kd and 36kd viral proteins in immunoblotting and immunoprecipitated the 45kd protein with ICSLS(Fig 1, lane 6~7 and Fig 2b, lane 2). Monoclonal antibody 63~6 reacted with the 120~115kd proteins in immunoblotting(Fig 1, lane 8) but it was not reactive with ICSLS. The other monoc-

Table 2. Comparison of the molecular weights of the identified BVDV polypeptides

Present study	Donis et al. (6)	Magar et al. (8)	Pocock et al. (10)	Purchio et al. (12)	Ocria et al. (7)	Matthaeus et al. (9)	Prichett et al. (11)
Strain							
NADL	Singer	NADL	NADL	NADL	Singer	NADL	NADL
	165						
145 ·							
	153						
120 ·	118*	115	120	115			
100 ·							110~93
92 ·			87				
80 ·	80	80		80			
76 ·	75*				75*		
72 ·			69*				70
66		65			66		
62	62						
56~54	58~56*	55~53*	57*	55*	54*	57*	59~50
45 ·	48*	47*	49*	45*		44*	
42							
41							
40							
38 ·	37	38	37	38			
36 ·							
34 ·	35					34	
33	32		33				
28 ·		26			26		
25			23*				25
	19						

· : Detected in SDS-PEGE and RIP. * : Reported as a glycoprotein.

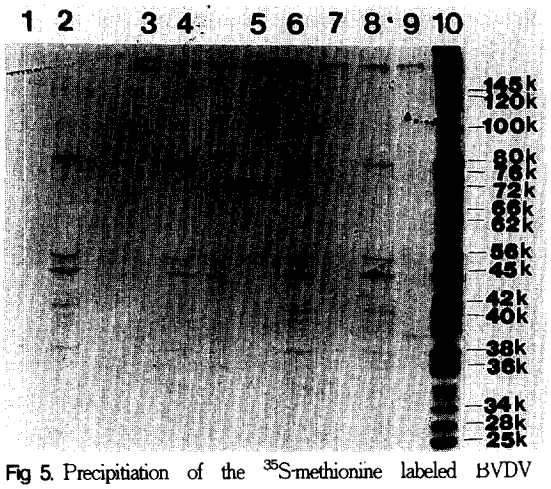

Fig 5. Precipitiation of the ^{35}S-methionine labeled BVDV through the immune complex using the neutralizing monoclonal antibody.

Lane 1, 3, 5, 7, 9 : uninfected cell supernatants
Lane 2, 4, 6, 8, 10 : virus infected cell supernatants, which were reacted at 0 hr (lane 1 and 2), 2hr(lane 3 and 4), 4hr(lane 5 and 6), 6hr(lane 7 and 8) after pules labeling.
Lane 9 and 10 : 8hrs' pulse labeling

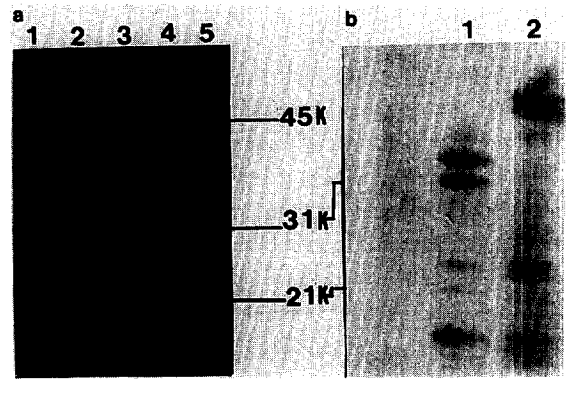

Fig 6. The partial peptibe mapping of the 45kd and 36kd(M.W.) of BVDV structural polypeptides.

a : Silver stained gel, lane 1(115kd), lane 2(80kd) lane 3(56kd), lane 4(45kd) and lane 5(36kd), respectively.
b : Coomassie blue stained gel, lane 1(45kd), lane 2(36kd)

lonal antibodies were not reactive with either immunoblottig or ICSLS.

BVDV polypeptides : After SDS-PAGE of purified

BVDV, the gel was stained with Coomassie brilliant blue and the seperated viral polypeptides was scanned using video densitometer. The scanning result indicated that there were at least fourteen seperated peaks(Fig 3, a and b). The estimated molecular weights of the peaks were estimated as >200kd, 145kd, 120kd, 100kd, 92kd, 80kd, 76kd, 72kd, 62kd, 45kd, 38kd, 36kd, 34kd and 28kd, repectively. Among those fourteen peaks, the 36kd and 45kd polypeptides proteins were most abundant in the purified virus. The positive bovine serum of various dilutions was also reacted with purified BVDV antigens by immunoblotting. Ten polypeptides(100kd, 92kd, 80kd, 76kd, 72kd, 56kd, 45kd, 38kd, 36kd and 34kd) were detected in this experiment. However, the 80kd and 56kd proteins were reacted with higher dilution of serum, indicating these two proteins are major immunogenic viral polypeptides(Fig 4, lane 1~3).

Pulse chase experiment : In order to get an insight on the translational processing of structural proteins of BVDV, virus infected cells were pulse chased and then immunoprecipitated with monoclonal antibodies.

Although on viral protein was detected with neutralizing monoclonal antibody(115~3)using cell lysates in pulse labeling experiment, the presence of virion was found immediately after pulse labeling through immunoprecipitation of whole virus as described above(Fig 5, lane 1~8). In addition, when the supernatant from long labeled(8 hrs) virus infected cells was tested by this method using monoclonal antibody, the results indicated that all but one of those previously detected protein bands from SDS-PAGE using purified vrus were also identified as viral specific polypeptides bands(Fig 5, lane 9 and 10). One high molecular weight of protein band(>200kd) turned out to be a nonspecific protein in this experiment because the same molecuar weight band was also detected in the control(Fig 5, lane 9). In addition, seven viral specific bands with the molecular weight of 68kd, 56kd, 42kd, 41kd, 40kd, 33kd and 25kd were detected along with those thirteen viral polypetides. In contrast to scanning result of purified virus, the 80kd, 56kd, 42kd and 38kd viral bands appeared to be a same intensity with the 45kd polypeptide band and the 92kd viral protein band was often difficult to detect.

Partial peptide mapping analysis : Since a cross reaction between the 45kd and 36kd proteins were detected in immunoblotting and immunoprecipitation, partial digestion of those viral proteins with *Staphylococcus aureus* protease V 8 was performed in order to examine and similarity in primary sequence of those proteins. In addition, three other viral protein bands(120kd, 80kd and 56kd)were also selected because they have been reported as the major structural proteins.

The digestion patterns of the 45kd and 36kd proteins showed considerable similarity, suggesting that thses two viral proteins are related(Fig 6a, lane 4, 5 and b, lane 1, 2). However, it was difficult to compare the digestion patterns of other viral proteins even after silver staining as indicated in Fig 6 a, lane 1~3.

Discussion

The molecular weight of viral proteins, which were reacted with monoclonal antibodies derived in the study, were 120~115kd, 80kd, 56 to 54kd, 45kd and 36kd, respectively. The results on the molecular weight of the viral structural proteins were in agreement with those found by others.[11,12] Among those viral proteins, the 56kd~54kd protein was identified as the glycosylated viral polypeptides for virus neutralization with monoclonal antibodies.[8,26,27] The fact that two viral proteins of similar molecular weight 56kd and 54kd were detected with neutralizing monoclonal antibody may be due to the presence of carbohydrate moieties in polypeptides as suggested previously.[27] Although a total of twelve viral proteins were identified in the virus infected cells as reported by Donis et al.[6] it is still not clear how those viral polypeptides are related to the mature virion. In this study, it was possible to demonstrate that BVDV consists of at least more than twelve structural polypeptides through the combined analysis of purified virus using SDS-PAGE and densitometer scanning. Purifying BVD virus is laborious because BVDV does not grow well in cell culture and also contains the possibility of host cell contamination.[11] In addition, there is also a loss of some of viral polypeptides during the extensive purification steps. For example, the 56kd of viral protein peak was not detected in the gel, but was detected by immunoblotting with positive bovine serum even at a higher dilution of serums. In fact, the 92kd and 80kd protein bands were identified as the minor peaks. However, the 80kd of viral band was strongly detected as a major band, but the 92kd of protein band was detectable at a low dilution of positive bovine serum in immunoblotting. The result of densitometer scanning on SDS-PAGE of the purified virus indicated rather asymmetrical distribution of previously reported viral polypeptides. For these reason, previous definitions on major stuctural proteins based on RIP, which showed the possible selection of immunodominance viral proteins using hyperim-

mune serum, might not valid. Similar result was also described by Dinis et al[6], who indicated that the 37kd(M.W.) viral protein was found to be fairly abundant, but was poorly immunoprecipitated by hyperimmune serum. It is known that there is considerable heterogeneity in the viral polypeptides among BVDV strains.[8,10] When the molecular weights of purified viral proteins identified in SDS-PAGE were compared with previously reported results, there was a considerable agreement(Table 2). The presence of 100kd and 92kd viral proteins were reported by Prichett et al.[11] Who decribed those viral bands as heterogenously migrating components in SDS-PAGE. Among fourteen protein bands, which were detected in SDS-PAGE of purified virus, the specificity of nine protein bands with molecular of 100kd, 92kd, 80kd, 76kd, 72kd, 56kd, 45kd, 38kd, 36kd and 34kd were suported with positive bovine serum by immunoblotting. However, the presence of other viral polypeptides except one was also identified by the immunoprecipitation of the whole virion. In this experiment, viral proteins with molecular weights of 42kd, 41kd, 40kd, 33kd and 25kd were additionally identified. It is not clear whether these proteins bands were detected as a result of heterogenic moiety of viral proteins with simillar molecular weight. Nevertheless, it is possible to expect that the immunoprecipitation of whole virions using monoclonal antibody might alleviate the possible degradation of other surface proteins through binding to 56kd protein.

The fact that these proteins were not detected in purified virus or immunoblotting with positive serum might presumably by related to the degradation during the purification steps as well as a lack of immunogenicity.

Although the BVD viral genome is believed to encode a large polypeptide, no higher molecular weight protein as well as the corresponding viral protein band were detected with neutralizing monoclonal antibody. However, the detection of the presence of viral proteins in infected cell supernatant immediately following pulse labeling confirmed the characteristics of the rapid translational processing of viral proteins during translation in infected cells. The results of partial peptide mapping indicated that the 45kd and 36kd proteins are related, suggesting that the cross reaction between two proteins may be due to the common antigenic binding site within those two viral proteins. Since those two viral proteins are present as the structural proteins, it is of future interest to determine the exact ralationship between those viral proteins during maturation of the virus in cells. Previously, Purchio et al reported that 115kd and 80kd proteins were structually related, but no similarity exist with the 56kd protein.[12] In this study, the comparison of the digestion patterns on those viral proteins was not rewarding, mainly because those proteins were less abundant than the 45kd and 36kd proteins in purified virus. The result of immunoblotting with the neutralizing monoclonal antibody indicated that there was at certain extent of antigenic similarity between the 80kd and 56kd proteins. The result of this study, as well as previous findings on viral proteins, clearly indicated that there is potentially a precursor-product relationship among those identified structural proteins.

Further studies including precursor-product relationship in mature virion seems to be required to understand the exact translational mechanism of this virus.

Summary

Monoclonal antibodies against structural proteins of bovine viral diarrhea virus(BVDV) were derived by classical hybridoma techniques. These antibodies were characterized by serum neutralization, immunoblotting and immunoprecipitation. The neutralizing monoclonal antibody reacted with the 56kd to 54kd(M.W.) viral protein in western blotting and immunoprecipitation analysis. Although there was no neutralizing activity, another monoclanal antibody reacted with the 45kd protein by immunoprecipitation and with both the 45kd and 36kd proteins in immunoblotting analysis, respectively. Densitometer scanning of purified BVDV and the immunoprecipitation of whole virus particles with neutralizing monoclonal antibody revealed the presence of more than twelve viral polypeptides. Although no possible precursor form of protein was identified with the neutralizing monoclonal antibody, the presence of intact virion was detected in the infected cell supernatant immediatelty after pulse labeling, indicating rapid translational processing as well as packaging of the virus. The partial peptide mapping of 45kd and 36kd proteins with *Staphylococcus aureus* V 8 protease showed that these two proteins are related.

Acknowledgements : Kweon CH was recipient of fellowship from Rockefeller foundation for the research at the University of California, Davis and this work was done as the partial fulfillment of Ph.d dissertation.

References

1. Bolin SR, McClurkin AW, Cutlip RC, et al. Response

of cattle persistantly infected with noncytopathic bovine viral diarrhea virus vaccination for bovine viral diarrhea and subsequent challenge exposure with cytopathic bovine viral diarrhea virus. *Am J Vet Res* 1985;46 : 2467~2470.

2. Malmquist WA. Bovine viral diarrhea-mucosal disease : etiology, pathogenesis and applied immunity. *J Am Vet Med Assoc* 1968;152 : 763~768.
3. Collett MS, Larson R, Gold C, et al. Molecular cloning and nucleotide sequence of the pestivirus bovine viral diarrhea virus. *Virol* 1988;165 : 191~199.
4. Purchio AF, Larson R, Porborg LL, et al. Cell-free translation of bovine viral diarrhea virus RNA. *J Virol.* 1984;52 : 973~975.
5. Renard A, Guiot C, Shumetz D, et al. Molecular cloning of bovine viral diarrhea viral sequences. *DNA* 1985;4 : 429~438.
6. Donis RO, Dubovi EJ. Characterization of bovine viral diarrhea-mucosal disease virus-specific proteins in bovine cells. *J Gen Virol* 1987;68 : 1597~1605.
7. Coria MF, Schmerr MJF, Mcclurkin AW. Characterization of the major structural proteins of purified bovine viral diarrhea virus. *Arch Virol* 1983;76 : 335~339.
8. Magar R, Minocha H, Lecomte J. Bovine viral diarrhea proteins : Heterogeneity cytopathogenic and non-cytopathogenic strains and evidence of a 53k glycoprotein neutralization epitope. *Vet Microbiol* 1988;16 : 303~314.
9. Matthaeus W. Detection of three polypeptides in preparation of bovine viral diarrhea virus. *Arch Virol* 1979;66 : 365~369.
10. Pocock DH, Howard CJ, Clarke MC, et al. Variation in the intracellular polypeptide profiles from different isolates of bovine virus diarrheas virus. *Arch Virol* 1987;94 : 43~53.
11. Pritchett RF, Zee YC. Structural proteins of bovine viral diarrhea virus. *Am J Vet Res* 1975;36 : 1731~1735.
12. Purchio AF, Larson R, Collett MS. Characterization of bovine viral diarrhea virus proteins *J Virol* 1984;50 : 666~669.
13. Chu HJ, Zee YC. Morphology of bovine viral diarrhea virus *Am Vet Res* 1984;25 : 103~107.
14. Bradford MA. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of proteindye binding. *Anal Biochem* 1976;72 : 248~254.
15. Goding J. Antibody production by hybridomas. *J immunol Meth* 1983;39 : 285~308.
16. Singer RH, Lawrence JB, Villnave C. Optimization of in situ hybridization using isotopic and non-isotopic detection methods. *Biotechniques* 1986;4 : 230~246.
17. Ouchrerlony O, Nilsson LA. Immunodiffusion and immunoelectrophoresis. In. Weir DM(ed), *Handbook of Experimental Immunology* 1978.
18. Swack JA, Nakatsuji T, Ito K, et al. Preservation of membrane glycoprotein labile epitopes during western blotting with monoclonal antibodies. *Biotechniques* 1987;5 : 564~570.
19. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227 : 680~685.
20. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets : Procedure and some applications. *Proc Nat Acad Sci USA* 1979;76 : 4350~4354.
21. Bush CE, Pritchett RF. Immunologic comparison of the proteins of pseudorabies(Aujeszky disease) virus and bovine herpesvirus-1. *Am J Vet Res* 1986;47 : 1708~1712.
22. Chang LWS, Zee YC, Pritchett RF, et al. Neutralizing monoclonal antibodies directed to infectious bovine rhinotracheitis virus. *Arch Virol* 1986;88 : 203~215.
23. Chamberriain JP. Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. *Anal Biochem* 1979;98 : 132~135.
24. Cleveland DW, Fischer SG, Kirschner MW, et al. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J Biol Chem* 1977;252~1102~1106.
25. Merril CR, Goldman D, Sedman SA, et al. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in Cerebrospinal fluid proteins. *Science* 1981;211 : 1437~1438.
26. Donis RO, Corapi W, Dubovi E. Neutralizing monoclonal antibodies to bovine viral diarrhea virus bind to the 56k to 58k glycoprotein. *Gen Virol* 1988;69 : 77~86.
27. Donis RO, Dubovi EJ, Glycoproteins of bovine viral diarrhea mucosal disease virus in infected bovine cells. *J Gen Virol* 1987;68 : 1607~1616.