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Biophysical characteristics of a noncytopathic bovine viral diarrhea virus

Chang-hee Kweon*, Castro E Anthony**, Hee-jong Woo***
*Veterinary Research Institute, Anyang, Korea**
*California Veterinary Diagnostic Laboratory(CVDLS) University of California Davis, CA 95616, U.S.A.***
*Laboratory of Cancer Biology, Harvard Medical School, Boston, Massachusetts. 02115 U.S.A.****
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세포 비병원성 소 설사병 바이러스의 이화학적 성상 조사

권창희*·Castro E Anthony**·우희종***
가축위생연구소*
미국 캘리포니아 수의진단 연구소**
미국 하바드 의과대학***
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초록 : 세포 비병원성 소 설사병 바이러스가 감염된 우태아 폐세포를 이용 바이러스를 순수분리하여 이화학적 성상을 검사하였던 바 다음의 결과를 얻었다. 바이러스의 비중은 1,090~1,114 g m/㎤로 검출되었으며 1,098 g m/㎤에서 최대 감염가를 나타내었다.

면역 전자현미경을 이용한 형태학적 분석결과 30~80nm의 바이러스성 입자를 관찰할 수 있었으며 유전인자의 추출 분석결과 병원성 바이러스와의 전기영동상 유전자 규모에 있어서 차이는 인정되지 않았다. 그러나 비세포 병원성 소 설사병 바이러스는 병원성 바이러스에 비하여 세포외 배출이 적었으며 세포내에서 보다 많이 존재하는 것으로 관찰되었다.

Key words : Noncytopathic bovine viral diarrhea virus, fetal bovine lung cells, persistant infection buoyant density, Immunoelectron microscopy, cell associated characteristics.

Introduction

Bovine viral diarrhea virus, an enveloped virus with a plus-stranded RNA genome of about 12 Kb in length is the causative agent of viral mucosal disease in cattle.[1~4] BVDV strains have been classified into two biotypes based on the pathology in cell cultures. Recent studies on BVDV infection indicated that cytopathic strains were usually coisolated with serologically related noncytopathic(NC) strains in clinical mucosal disease.[5~7]

Although the etiological roles as well as pathological significance of noncytopathic BVDV has been reported, studies to characterize such strains were few. Main reason of this situation is mainly due to fact that noncytopathic virus do not produce any detectable cytopathic effect as well as low viral yields following passage *in vitro*.[8] We have propagated a NC BVDV cell culture of bovine fetal lung(BFL), which persistantly infected with NC BVDV. Herein, we described certain of the biophysical and morphological chracteristics of the NC BVDV.

Materials and Methods

Virus and cells : Primary bovine fetal lung(BFL) cells, which were persistently infected with NC BVDV, were used

for preparation and characterization of NC BVDV. The cell, which derived from a normal bovine fetus obtained from slaughter house, was identified as containing NC BVDV by and indirect immunofluorescence method(IFA) using polyclonal antibody conjugate against BVDV and was supplied by the central viral diagnostic station(CVDS) of the school of veterinary medicine, University of California, Davis, CA. The cell passages used in this study were between 4 and 10. For the assay of the NC BVDV of the propagation of cytopathic BVDV(NADL), established MDBK of bovine fetal lung(BFL) line cells were prepared using the same methods for primary BFL cells. Briefly, cells were grown as monolayers in flasks for three or four days in Dulbecco's modified Eagle's minimal essential medium(DMEM) supplemented with 10% fetal bovine serum and maintained in the same media with 5% human serum of horse serum after inoculation of the virus.

Purification of noncytopathic BVDV : Procedures for purification of noncytopathic BVDV were modified from those described by Chu et al.[9]

Briefly cells and tissue culture fluids were frozen and thawed three times and clarified by centrifugation(8000 × g) at 4℃ for 30 minutes.

The clarified fluid was then layered onto a 3㎖ cushion of 15%(W/W) potassium tartrate in TEN buffer(0.01M Tris, 0.001M EDTA, 0.1M NaCl, pH 7.2) and centrifuged at 70,000 × g for 90 minutes in type 30 rotor. The pellet was resuspended to 1/100 of original volume with TEN buffer and centrifuged for 5minutes by eppendorf. The supernatant was then layered onto 15% to 35%(W/W) potassium tartrate gradients and centrifuged to equilibrium(60,000 g for 24hrs at 4℃) in a SW 25.1 rotor. Serial fractions(0.5㎖) were collected by automatic density gradient fractionator(ISCO. INC, Lincoln, Nebraska, U.S.A).

Each fraction was assayed for its viral infectivity by IFA, corresponding density and absorbance by methods described previously.[9]

Monoclonal antibody : Derivation of monoclonal antibody with neutralizing activity against NADL strain and its characterizations are described in previous paper.[10]

Immunoelectron microscopy : The method for immune electron microscopy(IEM) was based on the procedures of Thangue et al.[11] With slight modifications. A 500㎕ of partially purified virus preparation prior to the steps equilibrium centrifugation was reacted with 5㎕ of monoclonal ascites overnight at 4℃. The immune complex was further incubated with 10㎕ of rabbit-antimouse IgG(Pel-Freez biologicals, Rogers, AR 72756) for 2hrs at room temperature before centrifugation for 1hr at 15,000 g. The resulting pellet was resuspended to 1/10 of original volume with TEN buffer and dropped onto a carbon coated grid(200mesh), then stained negatively with 4% phosphotungstic acid(PTA) and examined by electronmicroscopy.

Radioimmunoprecipitation : Monolayers of cell in 25㎠ flasks were labeled with 100㎕Ci/㎖ of ^{35}(S)-methionine with a specific activity(s.a) 1117Ci/㎖. After 6hrs incubation, the supernatant of infected cell as well as the control were collected and centrifuged at 16000 × g for 5 minutes to remove cell debris. A freeze-thaw lysate from virus infected cells and control were prepared at same way. The procedures for immunoprecipitation of whole virus particles using the monoclonal antibody were described in previous paper.

The mixture were then subjected to SDS-PAGE in 10% slad gel.[12]

RNA extraction and gel electrophoresis : For a comparison of the RNA genome of noncytopathic BVDV with a cytopathic strain(NADL), cytoplasmic cellular RNA from each virus infected cell was extracted from the four day old primary BFL cells, NADL infected cells and uninfected control cells according to the methods of Perbal.[13]

The extracted RNA(20㎍) were then glycosylated and separated in 0.8% agarose gel according to the procedures of Maniatis et al.[14]

Results

Purification and Identification of a noncytopathic BVDV : After equilibrium centrifugation of viral samples in potassium tartrate gradient, eight fractions were collected and individually examined for their infectivity by IFA, UV light absorbance and corresponding density. Infective virus were found to have a density of 0.09 g m/㎤ to 1.11 g m/㎤ with the maximum infectivity located at 0.089 g m/㎤(Fig 1). When a partially purified virus sample was examined by immunoelectron microscopy with monoclonal antibody, spherical shaped virus aggregates with 30~80nm in diameter see(Fig 2).

To determine if a cytopathic BVDV was present, purified virus samples were inoculated onto different cells and the BFL cells were further subcultured up to 20 passages. Cytopathic effects suspicious for BVDV were not observed in either experiment. When NC BVDV was compared to cytopathic strain(NADL), it was found that NC BVDV was

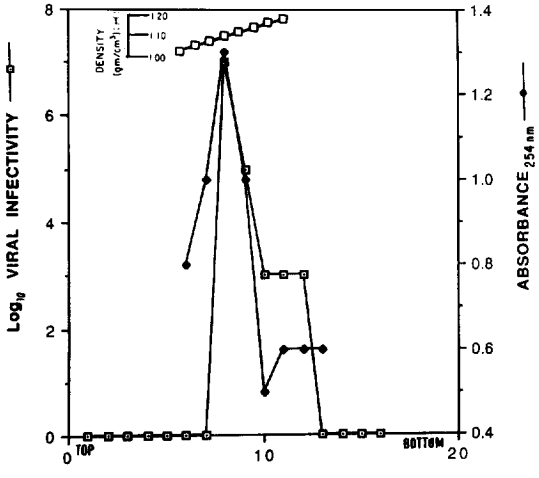

Fig 1. Purification profile of NC BVDV in density gradient.

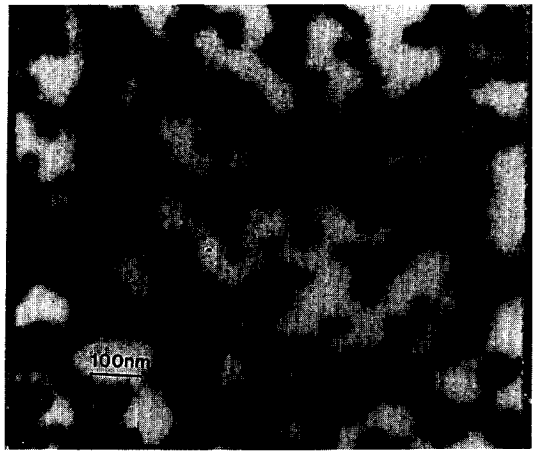

Fig 2. immunoeletron microscopy of NC BVDV.
Magnification : × 125,000

cell associated NC BVDV frequently did not yield any detectable viral bands by density gradient using concentrated samples with PEG from infected cell culture supernatant.

Identification of viral proteins noncytopathic BVDV : To investigate viral proteins of the NC BVD virus, purified virus samples were subjected to SDS-PAGE and coomassie brilliant blue staining. As a positive control, purified NADL was tested in parallel with noncytopathic virus. Although two structural proteins of NC BVDV with molecular weights of 45kd and 36kd were identified, further identification and comparison of other viral proteins was difficult due to the relatively low protein concentration of noncytopathic virus(data not presented).

However, since the monoclonal antibody against NADL

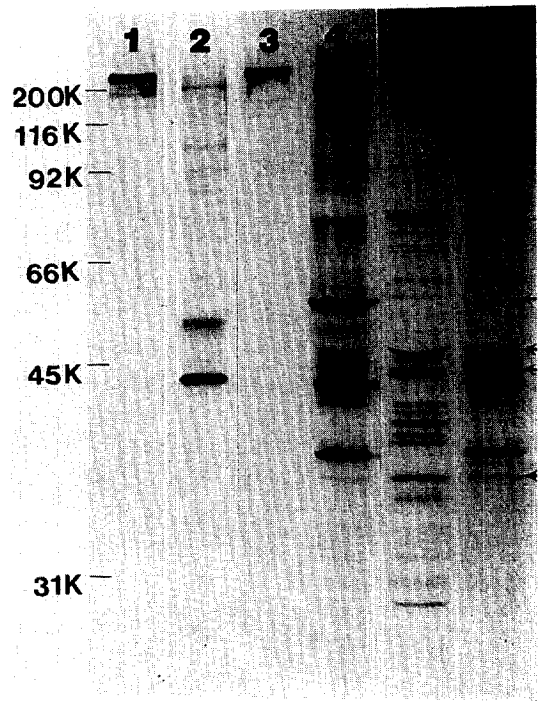

Fig 3. Comparison of the protein profiles between NC BVDV and cytopathic NADL by immunoprecipitation of whole immune complex using monoclonal antibody 115~3.

lane 1. uninfected cell supernatant
lane 2. uninfected cell lysate from freeze-thaw
lane 3. NADL infected cell lysate from freeze-thaw
lane 4. BFL cell supernatant
lane 5. BFL cell lysate from freeze-thaw
lane 6. NADL infected cell supernatant

viral protein was found to react with the noncytopathic virus from the result of immunoelectron microscopy, this monoclonal antibody was used for immunoprecipitation of whole virus particles after ^{35}(S)-methionine labeling in contrast to the immunoprecipitation results of NADL protein, no viral protein was detected from immunoprecipitation using cell culture supernatant from primary BFL cells(Fig 3, lane 4). Nevertheless, viral protein bands were detected with monoclonal antibody using cell supernatant after freezing and thawing with media, substantiating the presence of NC BVDV particles(Fig 3, lane 5). Although there was little difference between the protein profile of noncytopathic virus and the cytopathic NADL strain, different intensities of viral bands with the same molecular weight proteins between two viral strains were also observed. For instance, while the 62kd protein band was strong in the noncytopathic virus, 42kd,

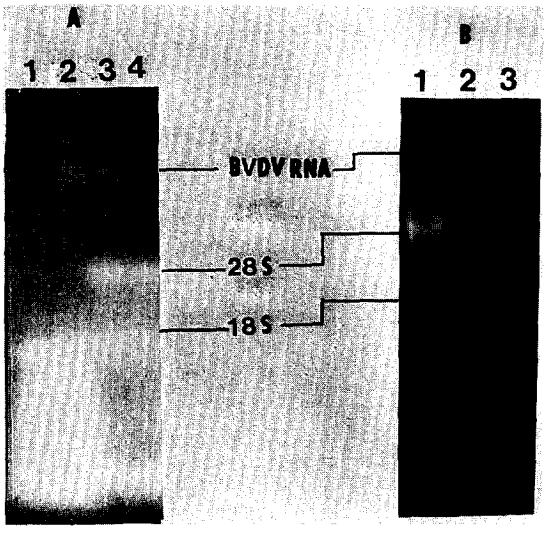

Fig 4. Identification of the NC BVD viral RNA and comparison with the cytopathic NADL.

A : lane 1 and 4, RNA from the uninfected MDBK cells.
lane 2, RNA from the NADL infected cells.
lane 3, RNA from the A NC BVDV infected BFL cells, 9th passage.
B : lane 1, RNA from the uninfected MDBK cells.
lane 2, RNA from the NADL infected MDBK cells.
line 3. RNA from the BFL cells, 20th passage.

41kd and 25kd protein band were hard to detect compared with those viral bands of NADL(Fig 3, lane 5 and 6). In addition, 37kd of viral specific protein bands were usually detected samples using a freeze-thaw lysate from both NC BVDV and cytopathic NADL strain infected cells, but not in the supernatants or uninfected control. The reason for this result is not clear now. However, two proteins with molecular weight of 200kd and 44kd were also detected both in control as well as virus infected cells during immunoprecipitation, indicating that these proteins were precipitated as a result of nonspecific binding of myosin and actin proteins with *Staphylococcus* cells as reported previously.[10]

Identification of noncytopathic BVDV RNA : When cytoplasmic RNAs extracted from the primary BFL cells, cytopathic(NADL) strain infected cells and uninfected control cells separated in agarose gel, one extra high molecular weight RNA band was detected in virus infected cells, but not in the uninfected control lane(Fig 4a, 1~4). The comparison of two viral RNA bands showed no big difference in molecular size because two RNA bands appeared in same position. However, no viral RNA band was detected from of BFL cells after 20 passages, indicating decreased replication of the noncytopathic virus(Fig 4b, lane 2 and 3).

Discussion

It has been suggested that a persistent infection of a fetuses by noncytopathic BVDV results in immunotolerance calves, which then develop a mucosal disease when infected or vaccinated by similar serotype of cytopathic strains.[5~7] Studies on noncytopathic strains of BVDV and their persistent behavior in infected cells has been rather limited because of low viral yields. To overcome this problem, the persistently infected cell derived from a uninfected bovine fetus was selected as a source of virus. Since no cytopathic effect(CPE) in these cells was observed for 20 passages, contamination with other cytolytic viruses appeared unlikely. The density range(1.09~1.10 g/cm³) of a noncytopathic viral strain of BVDV revealed little difference when compared with previously reported results(1.09~1.12 g/cm³) for cytopathic BVDV.[4,15,16] Differences in the genomic size of their RNA were also not found. In addition, the examination of NC BVDV by immunoelectron microscopy also indicated the presence of regular spherical particles, which can fit the morphological characteristic of BVDV strains.[9,17,18] The ability to recognize the morphological features of NC BVDV will be useful in the diagnosis of noncytopathic BVDV infection by EM because such viral particles are usually morphologically amorphorous as seen in specimens from clinical cases. Noncytopathic BVDV is characteristically associated with cells and few cell free viruses were found as compared to the cytopathic strain(NADL), which usually release cell free viruses.[16] The cell associated property of noncytopathic BVDV was also indicated in a previous study by Nuttall[8] who further suggested that the lower yield of BVDV was not due to the inability of the virus to infect cells of culture since all cells were found to be infected by the IFA test. Although the mechanisms of viral persistence is not completely understood, it may be related to the pathogenecity of these biotypes, indicating that noncytopathic BVDV may be mainly responsible for a persistent infection *in vivo* as well as *in vitro* system. It is known that viral persistence depends on an altered replication strategy in cells.[19] For example, alpha virus are reported to induce viral persistence in cells by diminishing synthesis of viral gene products, while certain picora viruses produce incomplete virus particles.[20,21] In previous studies, it was suggested that the translational processing of viral proteins from 118kd to 80kd was responsible for the cytopathogenesis in cells, whereas the antigenic variations were dependent on 56kd to 58kd glycoprotein.[22~24]

Although the comparison of protein profile of 118 kd between two biotypes was not clear in this study mainly as a result of nonspecific binding of host cell component during immunoprecipitation as reproted previously[25], it was observed that there can be different levels of expression among apparently similar molecular weight proteins between two biotypes. Further studies will be required to understand the replicative events of noncytopathic viruses in cells, which relate to a lack of pathogenecity as well as their persistence in cells.

Summary

A noncytopathic bovine viral diarrhea virus(NC BVDV) strain isolated and purified from persistently infected primary bovine fetal lung(BFL) cells was studied by biophysical methods. The buoyant density of particles of the NC BVDV strain was shown to be between 1.090 and 1.114 g /cm³ and the maximum virus infectivity occured at 1.098 g /cm³. Immunoelectron microscopic examination by using the partially purified virus revealed regular spherical particles 30 ~80nm in diameter. Differences in the genomic size of cytopathic and noncytopathic BVDV from infected cells were not found. A comparison of viral proteins of a noncytopathic and cytopathic strain(NADL) by immunoprecipitation using monoclonal antibody indicated that NC BVDV, compared to cytopathic NADL, was cell associated.

References

1. 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.
2. Malmquist WA. Bovine viral diarrhea-mucosal disease : etiology, pathogenesis and applied immunity. *J Am Vet Med Assoc* 1968;152 : 763~768.
3. Purchio AF, Larson R, Torborg LL, et al. Cell-free thanslation of bovine viral diarrhea virus RNA. *J Virol* 1984;52 : 973~975.
4. Renard A, Guiot C, Schmetz D, et al. Molecular cloning of bovine viral diarrhea viral sequences. *DNA* 1985;4 : 429~438.
5. Bolin SR, McClurkin AW, Coria MF. Frequency of persistent bovine viral diarreha virus infection in selected herds. *Am J Vet Res* 1985;46 : 2385~2387.
6. Bolin SR, McClurkin AW, Cutlip RC, et al. Severe clinical disease induced in cattle persistently infected with noncytopathic bovine viral diarrhea virus by superinfection with cytopathic bovine viral diarrhea virus. *Am J Vet Res* 1985;46 : 573~576.
7. Brownlie J, Clarke MC, Howard CJ. Experimental production of fatal mucosal disease in cattle. *Vet Rec* 1984;114 : 535~536.
8. Nettal PA. Growth characteristics of two strains of bovine virus diarrhea virus. *Arch Viro* 1980;66 : 365~369.
9. Chu HJ, Zee YC, Morphology of bovine viral diarrhea virus. *Am J Vet Res* 1984;25 : 103~107.
10. Kweon CH, Zee YC. Monoclonal antibodies against structural proteins of bovine viral diarrhea virus. Submitted for publication.
11. Thangue NBL, Chan WL, Almeida JD. Monoclonal antibodies to herpes simplex virus type 1 glycoproteins show that epitope location influences virus neutralization *J Med Virol* 1984;13 : 227~242.
12. Laemmli UK. Cleavage of structural proteins curin the assembly of the head of dacteriophage T4. *Nature* 1970;227 : 680~685.
13. Perbal. B. *A practical guide to molectular cloning* John-Willey & Sons, New York, NY 1984
14. Maniatis T, Fritsh EF, Sambrook J. *Molecular cloning Laboratory manual* 1982. NY : Cold harbor laboratory
15. Buxton A, Fraser G. *Animal Microbiology* Blackwell scientific publications. 1977
16. Park JB, Pritchett RF, Zee YC. Buoyant density of bovine viral diarrhea virus. *Pro Soc Exp Biol and Med* 1972;140 : 595~598.
17. Ohmann HB, Bloch B. Electron microscopic studies of bovine viral diarrhea virus in tissues of diseased calves and in cell cultures *Arch virol* 1982;71 : 57~74.
18. Stott EJ, Almeida JD, O'Reilly KJ. Characterization of mucosal disease virus as a togavirus by electron microscopy. *Microbios* 1974;11A : 79~83.
19. Michael B, Oldstone A. Viral persistence. *Cell* 1989;56 : 517~520.
20. Kristensson K, Norrby E. Persistence of RNA viruses in the central nervous system. *Ann Rev Microbiol* 1986;40 : 159~184.
21. Richardson MA, Boultion RW, Raghow RS, et al. Polypeptide synthesis in Alphavirus-infected Aedes albopictus cells during the establishment of persistant infection. *Arch Virol* 1980;63 : 263~274.
22. Corapi WV, Donis RO, Dubovi EJ, Monoclonal antibody analyses of cytopathic and noncytopathic viruses

from fatal bovine diarrhea virus infections. *J Virol* 1988;62 : 2823~2827.

23. Donis RO, Dubovi EJ, Differences in virus-induced polypeptides in cells infected by cytopathic and noncytopathic biotypes of bovine viral diarrhea-mucosal disease virus. *Virol* 1987;158 : 168~173.
24. Pocock DH, Howard CJ, Clarke MC, et al. Variation in the intracellular polypeptide profiles from diferent isolates of bovine virus diarrhea virus. *Arch Virol* 1987;94 : 43~53.
25. Hayes EC, Wright LL, Zweerink HJ. Staphylococcal protein A-Sepharose columns and the characterization of measles virus specific polypeptides in persistantly infected cells. *Analy Bioch* 1978;91 : 276~282.