Expression of Envelope Protein (E2) of Bovine Viral Diarrhea Virus in Insect Cells

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ABSTRACT. The gene encoding the envelope glycoprotein (E2) of bovine viral diarrhea virus (BVDV) was expressed in a baculovirus. The expressed protein was detected on the surface of infected cells by immunofluorescence. Western blotting analysis showed the presence of the expressed protein of a similar molecular size to the E2 protein. The antigenicity of expressed protein were tested in guinea pigs and cattle. The immunized animals developed neutralizing antibodies against BVDV. — KEY WORDS: BVDV, envelope protein (E2), expression.

Bovine viral diarrhea virus (BVDV), a pestivirus, is the causative agent of bovine viral diarrhea-mucosal disease in cattle throughout the world [2]. Besides clinically observed diarrhea, it frequently causes fever, oral ulceration, cough, leukemia and persistent infection in some cases [1, 15]. BVDV like other pestiviruses consists of a single stranded RNA of positive polarity, which encodes all the structural proteins and replication components [4, 5, 16, 17, 24]. Recently, pestiviruses were classified the genus of Flaviviridae mainly based on their genetic organization and translational properties [7].

Although the exact translational mechanisms of pestivirus are not completely understood, the front part of the genome translates one polyprotein and processes to E0 (gp 48), E1 (gp 25) and E2 (gp 53–58), respectively [21]. Among these structural proteins, the E2 is known to be a major envelope protein related to neutralizing activity in vivo [6, 14].

The location of E2 on the BVDV genome has been initially mapped by antiserum raised against fusion proteins expressed in E. coli [5]. In addition, the comparison of pestiviruses indicated that processing of E2 involved typical hydrophobic cleavage sites for signal peptidase [21]. This information indicated that the N-terminus of E2 started from amino acid 693 (the numbers indicate amino acid positions described by Collett et al. [5]). Although the C-terminal region of E2 is not still clear, it had been suggested to be around amino acid 1130.

Previously we cloned a 1443 bp cDNA fragment of BVDV (NADL strain), which corresponded to the amino acid sequence from 660 to 1140, into the multiple cloning site of a pUC19 plasmid [9].

For introduction of an ATG initiation codon, the Bam HI site that located in front of amino acid 660 sequence was cleaved and then repaired by Klenow treatment as shown in Fig. 1, A. The repaired end was then ligated with commercially available NeoI linker (5′ CCCATGGG 3′, NEB) and followed by transformation into competent E. coli cells. The NeoI linker-inserted plasmid was isolated and sequenced to confirm the correct reading frame by the dideoxy-chain termination method [22]. For the construction of transfer vector, the NeoI linker ligated DNA fragment was digested with NeoI and blunt-ended by Klenow treatment. The site directed DNA fragment was then generated by digestion with XbaI that located downstream of 1140 of BVDV. The DNA fragment was inserted into Bombyx mori nuclear polyhedrosis virus (BmNPV) transfer vector pBK283, kindly provided by Dr. Maeda [13]. The transfer vector was first digested with EcoRI and repaired by Klenow treatment because there was no compatible restriction site. This transfer vector was further digested with XbaI to make a site directed ligation.

For derivation of recombinant baculovirus, BmN cells were cotransfected with wild-type baculovirus DNA and recombinant transfer vector DNA by calcium phosphate method. Recombinant baculoviruses without polyhedral occlusion bodies were screened under light microscopy and a plaque without inclusion bodies was purified twice by the limiting dilution assay [12].

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The expression of E2 by recombinant virus was initially screened by indirect immunofluorescent antibody (IFA) method with positive bovine sera from USDA (Ames, Iowa). BmN cells grown on glass coverslip were infected with a recombinant baculovirus at a multiplicity of 0.1–1 per cell. The infected cells were then incubated for 48 hr and examined by IFA method. The cells were fixed with cold acetone for 10 min and reacted with a 1:50 dilution of positive bovine sera for 1 hr at 37°C followed by washing with PBS (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8.1 mM Na2HPO4). FITC conjugated anti-bovine immunoglobulin (KPL) was reacted under the same conditions before examination for fluorescence. For the examination of surface immunofluorescence, cells were reacted with antibodies before fixation with acetone. In these experiments, cells infected with recombinant virus, but not with the control, showed positive reactions on the surface of the cells (Fig. 2, A & B).

The expressed protein was further analyzed by SDS-PAGE and immunoblotting methods. For the identification
of expressed protein, the infected cells were treated with lysis buffer (50 mM Tris-Cl pH 8.5, 0.2% Na-deoxycholate, 2% Mega 10, 1 mM PMSF) in sonicator. The cell lysates were clarified by centrifugation for 1 hr at 100,000 g. The supernatants were subjected to SDS-PAGE as described [11]. The result indicated the presence one extra protein band with molecular weight of 55–58 kDa in SDS-PAGE, but not in the control (Fig. 3, A; lane 1).

For immunoblotting, SDS-PAGE separated proteins were electrophoretically transferred to nitrocellulose filter as described by Towbin et al. [23]. Following transfer, the nitrocellulose filter was blocked in TBS (0.15 M NaCl, 0.05 M Tris-HCL, pH 7.4) containing 10% skim milk for 60 min at room temperature. The filter was then incubated for 1 hr with a 1:1,000 dilution of mouse sera raised against BVDV [10]. The filter was washed with TBS containing 0.05% Tween 20 (TBST) and then incubated with alkaline phosphatase-conjugated goat anti-mouse antibodies for 1 hr at room temperature. After washing, the filters was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

As expected, the protein band with similar molecular weight was also detected in this experiment (Fig. 3, B; lane 2 & 3). However, another extra protein band with higher molecular size was detected. Although the reason for this result was left for further characterization, it was suspected that the band might be a doublet form of E2 on the way of intracellular processing as shown in other pestiviral envelope expression in insect cells [8].

In order to test immunogenicity, BmN cells infected with
Fig. 2. Immunofluorescence of BVDV envelope protein expressed in *Bombyx mori* cells. The cells are reacted with positive bovine sera against BVDV after (A) or before (B) fixation.

Fig. 3. Detection of BVDV E2 envelope protein expressed in *Bombyx mori* cells by SDS-PAGE (A) and immunoblotting (B). (A) Lane 1: wild BmN cell lysate, lane 2: recombinant BmNPV infected cell lysate, lane 3: prestained molecular marker. (B) Lane 1: wild BmN cell lysate, lane 2: recombinant BmNPV infected cell lysate, and lane 3: BVDV infected MDBK (Madin-Darby bovine kidney) cell lysate, respectively. Molecular weight calibration (kDa) is indicated.
recombinant BmNPV were directly tested in guinea pigs. At the same time, the amount of protein of the supernatant from infected cell lysate was estimated by the procedures of commercially available assay kit (Pierce, U.S.A.). The supernatant was then adsorbed onto aluminium (20% v/v) before inoculation. In addition, the supernatant was roughly filtered to remove higher molecular weight proteins than 100 kDa using commercially available concentrator (Centriprep-100, Amicon). The filtered supernatant was then used for the preparation of immunostimulating complex (ISCOM) [3].

Groups of four guinea pigs were inoculated intramuscularly with BmN cells, 100–500 µg of gel absorbed antigen or ISCOM at three or two week intervals. In addition, the supernatant was roughly filtered to remove higher molecular weight proteins than 100 kDa using commercially available concentrator (Centriprep-100, Amicon). The filtered supernatant was then used for the preparation of immunostimulating complex (ISCOM) [3].

Paired sera was collected two weeks after inoculation in guinea pigs immunized with supernatant and three weeks in calves, respectively. The collected sera were tested for the presence of neutralization antibodies against BVDV.

The neutralizing antibodies were detected from all the guinea pigs immunized with BmN cells infected with recombinant baculovirus, but not in control (Table 1). In guinea pigs immunized with the supernatant from E2 expressed cell lysate, the neutralizing antibodies were only detected in three groups, but dose dependent as shown in Table 1.

In the experiment in calves, five calves have already had the antibodies before inoculation, nevertheless, all showed the negative status after first inoculation, indicating that antibodies originated from passive immunity (Table 2). In fact, seven from ten calves showed the positive antibody titers with fluctuation from 2–64 after the second inoculation. However, higher titers were observed in the groups of animals immunized with ISCOM compared to animals immunized with the supernatant from infected cell lysate. This result was not surprising if the effect of concentration through the process of size fraction as well as immunostimulating effect of ISCOM is considered [19]. Since baculovirus expressions of envelope protein of hog cholera virus strains were also found to be functional as the diagnostic reagent or the protective antigen, it is of interest to elucidate the amount of purified E2 for the induction of protective level of antibody response in cattle [18, 20].

Nevertheless, it was demonstrated in this study that E2 expressed in insect cells like other pestivirus were immunogenic as a candidate for subunit vaccine in future.

### Table 1. Immune responses of guinea pig to E2 expressed cell lysate and ISCOM

<table>
<thead>
<tr>
<th>Immunized with Protein concentration</th>
<th>No. of guinea pigs tested</th>
<th>Dosage</th>
<th>Antibody titers after inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control BmN cells&lt;sup&gt;b)&lt;/sup&gt;</td>
<td>4</td>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>&lt;2</td>
</tr>
<tr>
<td>E2 expressed BmN cells&lt;sup&gt;c)&lt;/sup&gt;</td>
<td>4</td>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>16,32,128,128</td>
</tr>
<tr>
<td>Gel absorbed 200 µg/ml</td>
<td>4</td>
<td>0.5 ml</td>
<td>&lt;2</td>
</tr>
<tr>
<td>400 µg/ml</td>
<td>4</td>
<td>0.5 ml</td>
<td>&lt;2</td>
</tr>
<tr>
<td>1000 µg/ml</td>
<td>4</td>
<td>0.5 ml</td>
<td>&lt;2</td>
</tr>
<tr>
<td>ISCOM 400 µg/ml</td>
<td>4</td>
<td>0.5 ml</td>
<td>&lt;2</td>
</tr>
<tr>
<td>1000 µg/ml</td>
<td>4</td>
<td>0.5 ml</td>
<td>&lt;2, 2, 2, 4</td>
</tr>
</tbody>
</table>

a) The neutralizing antibody titers is expressed as the reciprocal of the serum dilutions against 200 TCID<sub>50</sub> of BVDV (NADL).

b), c) Guinea pigs were immunized three times at 3 week intervals with frozen and thawed control BmN cells (b) and infected with E2 expressed BmNPV (c). Cells were mixed with Freund’s complete adjuvant first and Freund’s incomplete adjuvant second. Last inoculations were conducted without adjuvant and the animals were bled 2 weeks after final injections.

### Table 2. Immune responses of calves to E2 expressed from recombinant baculovirus

<table>
<thead>
<tr>
<th>Immunized with</th>
<th>Calf no.</th>
<th>Neutralizing antibody titers after inoculation&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel absorbed 1</td>
<td>2</td>
<td>&lt;2, 2, 2, 4</td>
</tr>
<tr>
<td>2</td>
<td>&lt;2</td>
<td>&lt;2, 2</td>
</tr>
<tr>
<td>3</td>
<td>&lt;2</td>
<td>&lt;2, 8</td>
</tr>
<tr>
<td>4</td>
<td>&lt;2, 8</td>
<td>&lt;2, 2</td>
</tr>
<tr>
<td>ISCOM 1000 µg/ml</td>
<td>2</td>
<td>&lt;2, 8, 32, 32</td>
</tr>
</tbody>
</table>

a) The neutralizing antibody titers is expressed as the reciprocal of the serum dilutions of 200 TCID<sub>50</sub> of BVDV (NADL).
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REFERENCES