Evaluation of Antibodies Induced by the Injection of Single Capsid Protein or Purified Virus Particle of Coxsackievirus B3 in Mice

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Abstract

Four capsid proteins (VP1, VP2, VP3, and VP4) of coxsackievirus B3 (CVB3) were expressed as recombinant proteins in an *Escherichia coli* expression system and used as antigens for subunit vaccines against CVB3 in ICR mice. Antigens were expressed as thioredoxin-histidine (TrxHis)-tagged protein and purified before immunization. Although all VPs other than VP4 induced anti-CVB3 specific antibodies in mice (detected by ELISA and western blotting), they did not neutralize the infectious CVB3 in a virus neutralization assay. Meanwhile, 2 virus strains were purified from CVB3 virus stock on the basis of their plaque size on HeLa cells. ICR mice were infected with the 2 purified virus strains (S-strain and L-strain) and unpurified virus stock (wild type) to analyze the difference in antibody responses against infections of purified and unpurified virus strains. The reactivity of antisera against each virus strain was tested by ELISA, and the results showed that the inoculation of purified virus strain induced a strong antibody response against the inoculated strain. As a result, the antibody response against wild-type and other virus strains was suppressed. These results suggest using unpurified virus stock as an antigen is advantageous for inducing a broad antibody response in inoculated animals.

Keywords

Coxsackievirus B3, Antibody Response, Cross Reactivity

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1. Introduction

Picornaviruses are non-enveloped, positive-sense, single-stranded RNA viruses that cause several pathogenic viruses including poliovirus, foot and mouth disease virus (FMDV), and hepatitis A virus. Although commercial vaccines are available for some viruses including poliovirus and hepatitis A virus, vaccine development for some picornaviruses is considered quite challenging. In particular, aphthovirus, enterovirus, and rhinoviruses are known to exhibit high mutation frequency [1]; this makes it difficult to completely prevent these diseases using vaccines.

To date, many research groups have used different approaches to develop effective vaccines including subunit, inactivated, and attenuated live virus vaccines [2]-[4]. Among these, subunit vaccines using recombinant proteins have a distinctive advantage in safety because they do not require the production of a large amount of infectious live virus in the manufacturing process. Although subunit vaccines might not stimulate the immune response to the same extent as live attenuated vaccines [5], they would be an effective tool to control picornavirus disease epidemics without risking the spread of infectious live viruses during production.

Picornavirus particles comprise 4 structural proteins: VP1, VP2, VP3, and VP4. Among these, VP1, VP2, and VP3 are exposed to the surface of the virus particle, forming an icosahedral particle shape. VP1 is recognized as the most potent antigen for picornavirus vaccines because it can establish protection in cows inoculated with VP1-derived peptides [6]. Therefore, many investigators used VP1 as antigens to develop vaccines against different picornaviruses. They also demonstrated these vaccines could induce neutralizing antibodies and protect animals against lethal challenge [4] [5] [7] [8]. However, this raises concerns about the emergence of escape mutants, because picornaviruses in circulation exhibit quasispecies due to the high mutation rate of their RNA polymerases [1] [9].

Therefore, we hypothesize that the application of unpurified virus stock in quasispecies as antigens is useful, because it contains viruses with varying antigenicity and may able to activate large numbers of B-cells in repertory. To test this hypothesis, we compared the antigenicity of 4 capsid proteins of coxsackievirus B3 (CVB3) expressed in *Escherichia coli* (*E. coli*) as well as 2 plaque purified and unpurified CVB3 strains. CVB3 is an important human pathogen that induces acute and chronic viral myocarditis characterized by the infiltration of immune cells and results in cardiomyopathy [10] [11]. It circulates among children as well as other coxsackieviruses and sometimes causes infection in adults. It exhibits a high mutation frequency and viral quasispecies during replication in the infected animal host [1]. Furthermore, because many mouse strains are reported to be susceptible to CVB3 challenge [11]-[14], CVB3 is a suitable virus for a vaccine development model for Picornaviridae.

2. Materials and Methods

2.1. Cell Culture and Virus

HeLa cells and CVB3 Nancy strain were obtained from National Institute of Infectious Diseases of Japan. Cells were maintained in Dulbecco's modified Eagle medium (DMEM, Nissui) supplemented with 10% fetal bovine serum (FBS), l-glutamine (0.6 g/L), sodium bicarbonate (0.1%), penicillin (50 U/mL), and streptomycin (50 μg/mL). Viruses were propagated on HeLa cells prepared in 10-cm cell culture dishes (Falcon, BD) at 80% confluence. Cells were infected with pre-titrated virus stock at a multiplicity of infection of 0.001 and harvested 48 hours post-infection by disrupting infected cells with a freeze/thaw cycle. Virus titer was determined according to the 50% cell culture infective dose ($CCID_{50}$) and plaque assay method. The $CCID_{50}$ assay was performed as described in the WHO Polio Laboratory Manual (4th edition, 2004) with slight modifications. Briefly, 2×10^4 HeLa cells were prepared in 96-well plates and inoculated with 100 μL serially diluted CVB3; 8 wells were used for each dilution, and cells were incubated for 48 hours. Living cells were stained with crystal violet solution (0.1% crystal violet, 10% formaldehyde, and 20% methanol), and the percentages of CPE-positive wells were determined at each dilution point. Final $CCID_{50}$ was calculated using the Kärber formula as follows: $\log_{CCID50} = L - d(S - 0.5)$, where L is the log of the lowest dilution used in the test, d is the difference between log dilution steps, and S is the sum of the proportion of CPE (+) tests. The plaque assay was performed on HeLa cells as described previously [15]. In brief, confluent cell culture in 6-well plates were infected with serially diluted virus samples and overlaid with DMEM containing 1% Bacto Agar and 1% FBS. After 48 hours of incubation, live cells were stained with crystal violet solution, and the number of visualized plaques was determined.

2.2. Construction of Expression Plasmids for CVB3 Structural Proteins, Protein Expression, and Purification of Tagged CVB3 Structural Proteins

cDNA fragments encoding each structural protein (*i.e.*, the P1 region) were amplified by RT-PCR with SuperScript™ III reverse transcriptase and HiFiTaq polymerase (Life Technologies) using CVB3 genomic RNA extracted from virus stock as a template. The amplified product was cloned into pGEM™ easy-T vector (Promega), and the resultant plasmid, pGEM/CVB3-P1, was used as a template for the subsequent PCR with specific primer sets for each VP to obtain cDNAs for individual VPs. The oligonucleotide primers used for RT-PCR and PCR were based on the published nucleotide sequences of CVB3 Nancy strain (GenBank accession: M16572) (**Table 1**). The amplified fragments were purified from 1% agarose gel and cloned into pET32 vector (Novagen) to express each protein as thioredoxin and 6× histidine-tagged (TrxHis) fusion proteins. After confirming the nucleotide sequence, plasmids were introduced into *E. coli* Rosetta 2™ (DE3; Novagen), and a single colony was picked up. Protein expression was induced by the addition of 1 mM isopropyl-*β*-thiogalactoside (IPTG), and expression was confirmed by SDS-PAGE followed by western blotting using anti-His tag.

After confirming the target proteins, bacterial cells were resuspended in TritonX-100 lysis buffer (1% TritonX-100, 150 mM NaCl and 50 mM Tris-Cl [pH 7.4]), and fusion proteins in the insoluble fraction were solubilized by using urea followed by refolding process. Solubilized TrxHis-VP2 and TrxHis-VP4 were affinity purified using Ni-NTA Agarose (Qiagen). TrxHis-VP1 and TrxHis-VP3 were purified by ultrafiltration with Amicon® Ultra-4 Centrifugal Filter Units 10,000 MWCO (Merck Millipore). The concentration of purified proteins was determined by a Thermo Micro BCA™ Protein Assay Kit (Thermo Scientific).

2.3. Establishment of CVB3 Clones and Identification of Amino Acid Substitutions in Structural Proteins

CVB3 clones were purified by the plaque purification method. In brief, HeLa cells confluent in 6-well plates were infected with diluted CVB3, and plaques were formed by overlaying DMEM containing 1% FBS and Bacto Agar. The large plaque (>5 mm diameter) and small plaque (<1 mm diameter) were picked up, mixed with DMEM and added to HeLa cells to form new plaques. This procedure was repeated until the plaque size was fixed.

To determine differences in the amino acid sequences of structural proteins in each clone, total RNA was extracted from purified clones using ISOGEN (Nippongene) and chloroform. The cDNA coding the P1 region was obtained by RT-PCR using P1-s and P1-as primers. The nucleotide sequence was determined by a direct sequencing method using an agarose gel-purified RT-PCR product as a template and the oligonucleotide primers listed in **Table 2**.

2.4. Mouse Antisera Production

All animal experiments were performed with the approval of the Committee of Animal Experiments of the Tokyo

Table 1. Oligonucleotide primers used for the construction of VPs expression plasmids.

Primer	Sequence (5'-3')
P1-s	ctattggattggccatccgg
P1-as	ggtccttcaaacgaaattggg
VP1-s	cggatccaggcccagtggaagacgcg
VP1-as	atagttagcggccgctcattgccaatcag
VP2-s	cgaattctccccccacagtagaggag
VP2-as	ccgctcgagtcactggtgccctgcta
VP3-s	cgggatcccaggcttaccaaccatgaatac
VP3-as	atagtttagcggccgctcactggaagaagt
VP4-s	ggaattcggagctcaagtatcaacgc
VP4-as	ccgctcgagtcagttgagagctggta

University of Agriculture and Technology. To test antibody responses against CVB3 proteins, ICR mice were inoculated with purified TrxHis-VPs or infected with CVB3 clones, respectively. The immunization and infection schedules are summarized in **Figure 1**. For immunization with recombinant VPs, 6-week-old female ICR mice were used. In brief, mice were injected intraperitoneally (i.p.) with TrxHis-VP1, TrxHis-VP2, TrxHis-VP3, or TrxHis-VP4 (50 μg/mouse) with an equal volume of Freund's complete adjuvant. Two weeks later, the booster injection was administered with equal amounts of antigens mixed with Freund's incomplete adjuvant. The final injection was performed 4 weeks after the initial injection, with 20 μg/mouse antigen without adjuvant. Antibody production was monitored using blood samples. Whole blood samples were collected 3 days after the final injection, and antibody titer was tested (**Figure 1(a)**).

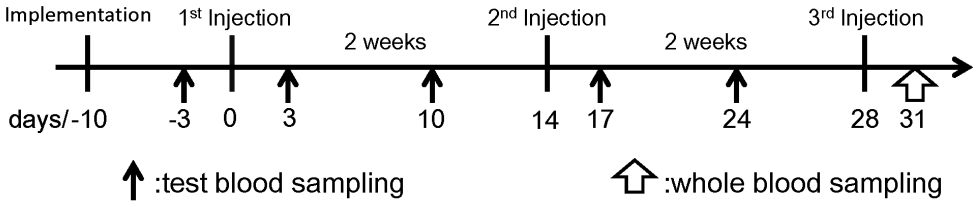
The antibody response against purified CVB3 clone infection was tested in 7-week-old male ICR mice. As shown in **Figure 1(b)**, mice were infected i.p. with 1.8×10^5 CVB3 plaque forming units (pfu) per mouse, and whole blood samples were collected 3 weeks post-infection. Anti-CVB3 antibody production in immunized or infected mouse sera was confirmed by ELISA using inactivated CVB3 as an antigen as described below.

2.5. Screening of Antibody Production in Mouse Sera by ELISA and Western Blotting

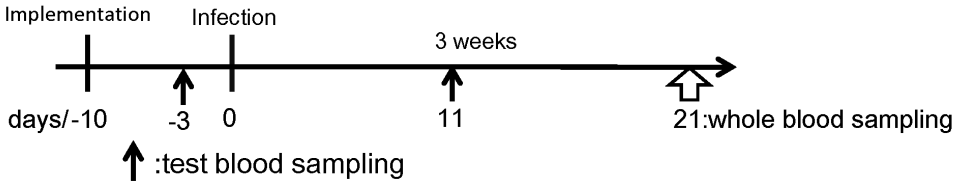
ELISA was performed to detect anti-CVB3 antibodies in collected mouse sera using formaldehyde-inactivated

Table 2. Oligonucleotide primers used for CVB3 genome sequencing.

Primer	Sequence (5'-3')
s573	cctatactggctgcttatgg
s701	ctattggattggccatccgg
s1317	gtagtgtgtgtaccggaagc
s1565	acaccaacagtgtacctatgg
s1929	atggaagcataccagatacc
s2386	gcaatgacttctctgtcagg
s2945	agtgtgttttggaccgaggg
as1951	tgccatatagaccatacgaagg
as2548	cgtcactcacgaccatatcg
as3428	ccagcacgagtgattcttctcc
as3529	ggtccttcaaacgaaattggg



(a)



(b)

Figure 1. Immunization and infection schedule. (a) Schematic diagram of mouse immunization by TrxHis-VPs and (b) infection by purified CVB3 strains.

CVB3 as described previously [16]. In brief, CVB3 virus stock was mixed with formaldehyde to a final concentration of 0.1% and incubated for 16 hours at 37˚C. Each well of a 96-well ELISA microtiter plate (Sumilon) was coated with inactivated CVB3, corresponding to 4×10^4 pfu/well in 0.1 M carbohydrate buffer (0.1 M Na_2CO_3 and 0.1 M $NaHCO_3$ [pH 8.7]) overnight at 4˚C. Plates were blocked with 5% skim milk in PBS (−) before adding diluted mouse sera. After washing with PBS (−) containing 0.05% PBS-Tween 20 (PBS-T), plates were incubated with anti-mouse IgG secondary antibody conjugated with horseradish peroxidase (HRP) (Jackson Immuno Research). The plates were subsequently washed with PBS-T and substrate solution (4.1 μM 3,3',5,5'-tetramethylbenzidine in citric buffer [0.13 M citric acid and 0.068 M trisodium citrate {pH 4.0}]). The enzyme reaction was stopped by 1 M H_2SO_4, and the absorbance at 450 nm was read with an iMark™ microplate reader (Bio-Rad).

Western blotting was performed to test the specificity of the antisera using recombinant proteins or cell lysates of CVB3-infected HeLa cells as antigens. Briefly, proteins were separated by 12% SDS-PAGE and transferred to a PVDF membrane (Immobilon-P, Millipore). After the blocking with 2% skim milk, the membrane was incubated with antisera diluted to 1:250 for 14 hours at 4˚C. After washing with PBS-T, the membrane was reacted with HRP-conjugated anti-mouse goat IgG secondary antibody, and protein bands were visualized by a LAS-3000 (Fujifilm).

2.6. Statistical Analysis

The significance of cross-reactivities of sera obtained from mice infected with different CVB3 strains were tested by unpaired student's *t*-test.

3. Results

3.1. Expression and Purification of Recombinant CVB-VPs

The whole-cell lysate of *E. coli* expressing TrxHis-tagged CVB3 VPs was analyzed by 12% SDS-PAGE (**Figure 2(a)**). The expression of recombinant protein after IPTG induction was confirmed by Coomassie Brilliant Blue staining and western blotting using anti-His tag antibody. The approximate molecular weights of TrxHis-VP1, TrxHis-VP2, TrxHis-VP3, and TrxHis-VP4 were estimated to be 54, 49, 47, and 27 kDa, respectively, on

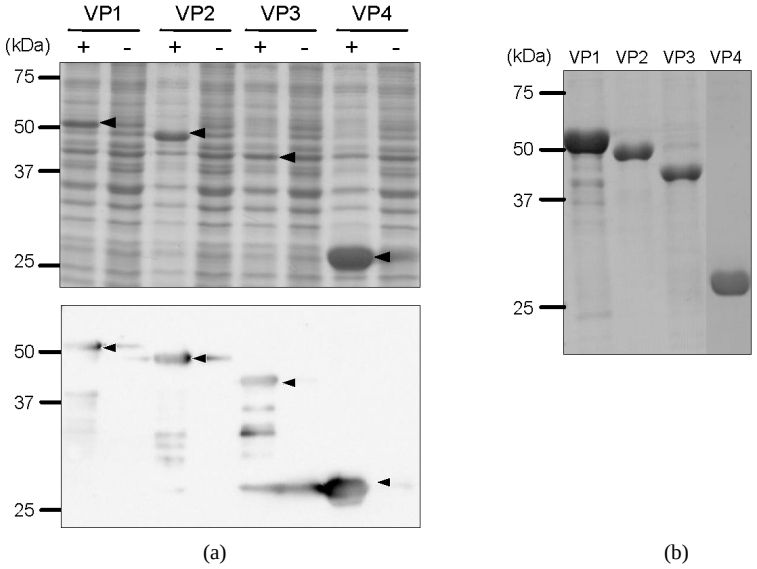


Figure 2. Expressions of TrxHis-VPs in *E. coli* and purification of recombinant proteins. (a) *E. coli*-harboring expression plasmids for each fusion VP were cultured in the presence (+) or absence (−) of IPTG. Whole bacterial lysates were separated and stained with CBB (upper panel) or western blotting with anti-His antibody (lower panel). Arrowheads indicate expressed TrxHis-VPs. (b) CBB staining of TrxHis-VPs after affinity purification or ultrafiltration.

the basis of their amino acid sequences deduced from previously published genomic RNA sequences [17]. Because recombinant TrxHis-VPs were expressed in the insoluble fraction of *E. coli*, they were solubilized with urea and subsequently refolded by stepwise dialysis. Solubilized proteins were purified by affinity purification with Ni-NTA or ultrafiltration, and pure TrxHis-VPs were obtained (**Figure 2(b)**).

3.2. Anti-VP Antibodies Elicited by the Immunization of Recombinant VPs Recognized the Whole Virion as Well as Virus Proteins in Cvb3-Infected Cells

To evaluate the antigenicity of purified TrxHis-VPs, 5 groups of female ICR mice were immunized i.p. with each fusion protein and received 2 booster injections at 2-week intervals. To monitor antibody production, test blood samples were collected on the indicated days (**Figure 1(a)**) and subjected to ELISA using inactivated CVB3 as an antigen at 1:100 dilution. As shown in **Figure 3(a)**, mouse groups inoculated with TrxHis-VP1, TrxHis-VP2, and TrxHis-VP3 exhibited significantly elevated antibody production, whereas groups immunized with TrxHis-VP4 did not exhibit any antibody production. Because all recombinant proteins except TrxHis-VP4 elicited antibody production in mice, the strength of the antibody reaction was compared using sera obtained from whole blood samples take 31 days after the first injection. Antisera were serially diluted from $10^2 \times 2^0$ to $10^2 \times 2^{13}$ fold and subjected to ELISA using inactivated CVB3 as an antigen. As shown in **Figure 3(b)**, sera from TrxHis-VP1-injected mice reacted with CVB3 until a dilution of $10^2 \times 2^{13}$, whereas the sera from mice immunized with TrxHis-VP2 or TrxHis-VP3 lost reactivity at lower dilution ($10^2 \times 2^9$ and $10^2 \times 2^5$, respectively). Sera from CVB3-infected mice were used as a positive control; reactivity was apparent until a dilution of $10^2 \times 2^8$.

To confirm the reactivity of antibodies against each viral protein expressed in CVB3-infected cells, western blotting was performed using the cell lysate of HeLa cells infected with CVB3 as an antigen. **Figure 2(b)** shows that sera from mice immunized with TrxHis-VP1, TrxHis-VP2, and TrxHis-VP3 reacted with VP1, VP2, and VP3 in CVB3-infected cells, respectively, as well as the recombinant proteins used for immunization. In contrast, no protein band was detected in CVB3-infected cells with anti-TrxHis-VP4, which is consistent with the results obtained by ELISA with the whole virion as an antigen.

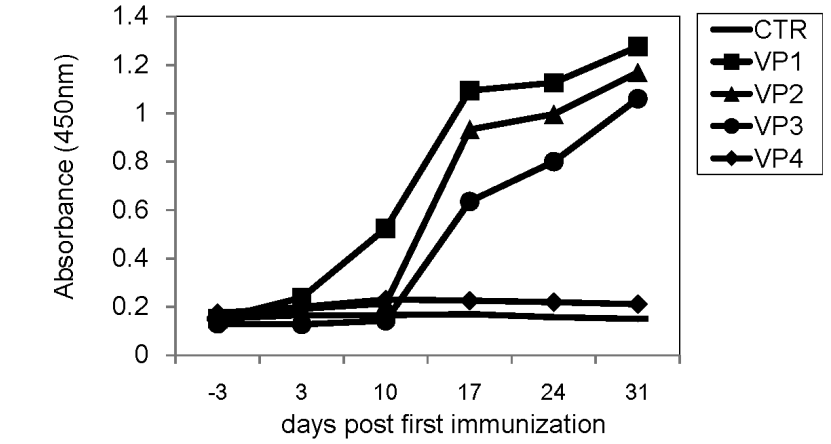
3.3. Selection and Characterization of Virus Strains Exhibiting Different Plaque Sizes

Two CVB3 strains forming plaques with diameters exceeding 5 or 1 mm were established and designated the L-strain and S-strain, respectively. The strain was established when the plaque size remained unchanged after 3 passages of purified viruses in HeLa cells. Although the L-strain was established by 3 plaque purification cycles, the S-strain required 15 cycles. The original virus quasispecies used for purification was designated the wild type (wt). The plaques formed by the 3 virus strains are shown in **Figure 4**. L-strain formed large plaques more than 5 mm in diameter, whereas S-strain formed plaques only 1 mm in diameter despite being cultured for the time after infection.

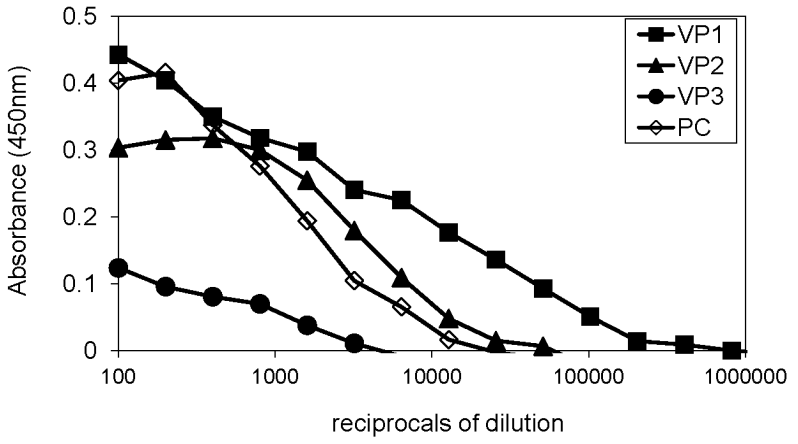
Two amino acid substitutions in VP2 (*i.e.*, D177V and T220S) are reported to have increased affinity for the second receptor in RD cells [18]. To confirm if the different plaque sizes observed in purified strains is a result of increased receptor affinity, the genomic sequence coding of structural proteins of the L-strain and S-strain was analyzed by direct sequencing and the deduced amino acid sequences were compared with that of the Nancy strain. The results show there were 17 and 21 amino acid substitutions in L-strain and S-strain, respectively (**Figure 5**); among these substitutions, 6 and 4 substitutions in the L-strain and S-strain, respectively, occurred in amino acids exposed to the surface of the virus particle when mapped on CVB3 structural data (PDB: 1COV). In addition, 2 substitutions, L469P and C516Y, were common between strains, whereas others were specific for each strain. Both strains possessed substitutions at position T220, but the resultant amino acids were different: T220S for L-strain and T220G for S-strain.

3.4. Antisera from Purified CVB3 Strain-Infected Mice Exhibited Cross-Reactivities among Purified Strains

To produce antisera against each virus strain, we inoculated male ICR mice with the S-strain, L-strain, or wt virus and observed them for 21 days. During this period, no significant clinical symptoms or weight loss was observed. The reactivity of each serum against 3 virus strains was tested by ELISA using inactivated virus strains as antigens. All sera were diluted 1:25. Sera from wt-infected mice recognized all virus strains to the same extent (**Figure 6**). On the other hand, anti-L-strain sera exhibited significantly higher reactivity against the L-strain



(a)



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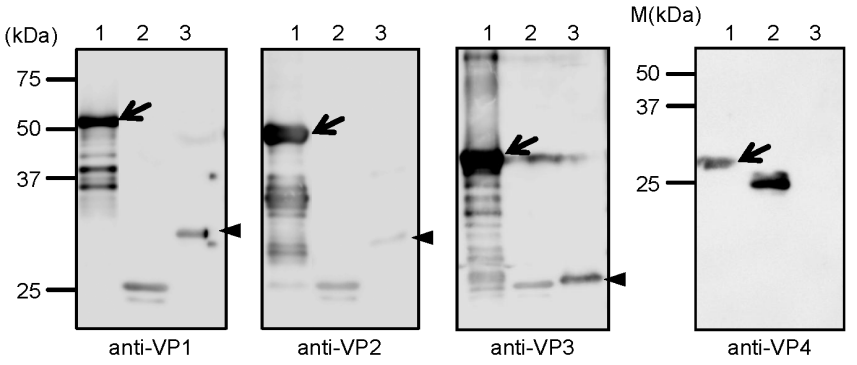
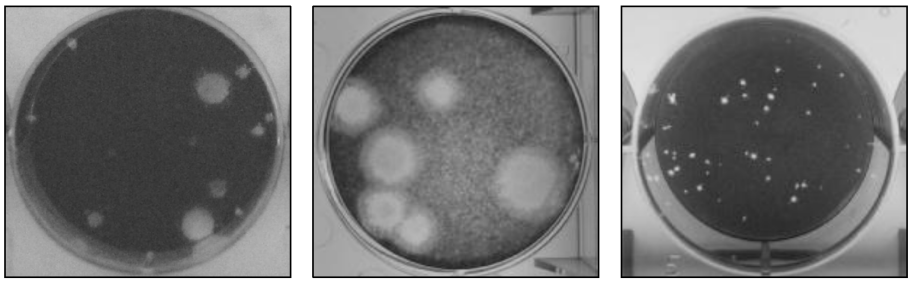


(c)

Figure 3. Evaluation of anti-CVB3 antibodies in mouse sera. (a) Anti-CVB3 antibody production in TrxHis-VP-immunized mice was monitored on the indicated days during immunization. Antisera obtained from mice injected with PBS (−) were used as a negative control (CTR). (b) Serially diluted antisera were subjected to ELISA, and the endpoint was determined as the dilution rate at which the absorbance reached zero. Antisera obtained from CVB3-infected mice were used as a positive control (PC). (c) The specificities and reactivity of obtained antisera for each VP were determined by western blotting. TrxHis-VP used for immunization (lane 1 in each blot), TrxHis (lane 2), and cell lysate from CVB3-infected HeLa cells (lane 3) were separated and detected with antisera corresponding to each VP. Arrows and arrowheads indicate TrxHis-VPs and intact virus capsids, respectively.



(a) (b) (c)

Figure 4. Comparison of the plaque sizes formed by the 2 purified virus strains. Two CVB3 strains (*i.e.*, the L-strain and S-strain) were purified on the basis of their plaque size on HeLa cells. HeLa cells were infected with (a) wt; (b) L-strain; or (c) S-strain viruses to induce plaque formation. Cells were fixed 2 days post-infection, and plaques were observed after staining with crystal violet solution.

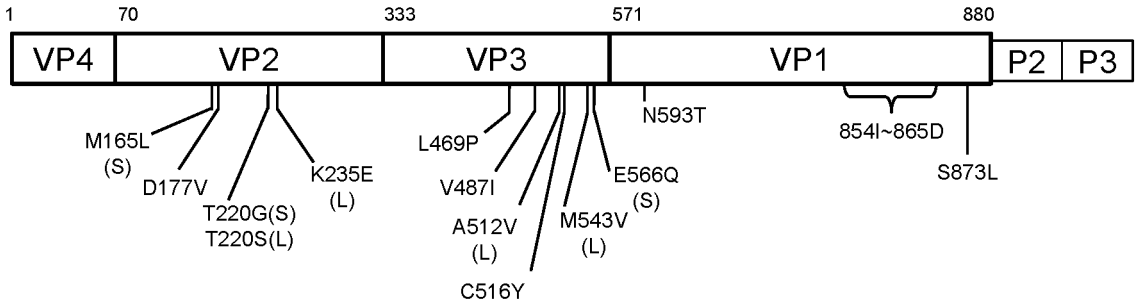
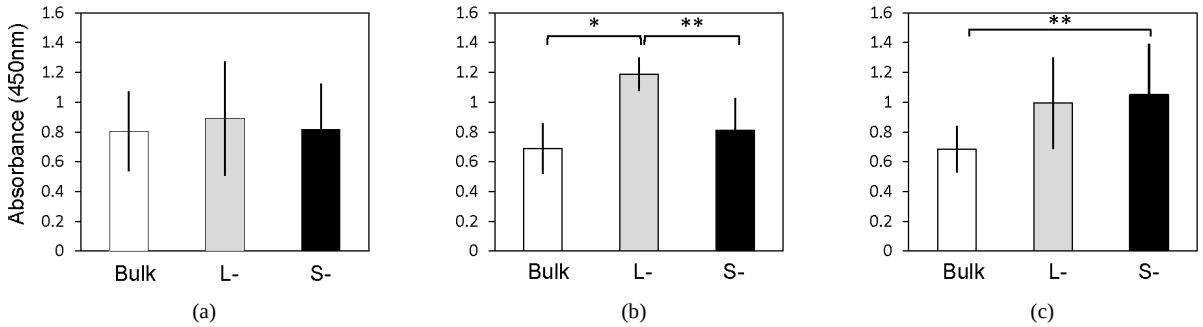


Figure 5. Mapping of amino acid substitutions identified in purified strains. The amino acid sequences of purified strains were deduced from genome RNA sequences and compared with the published sequence of CVB3 Nancy strain (M16572). The substituted amino acids are mapped in their corresponding position in CVB3 capsid proteins. (S) or (L) after the position indicate substitutions found only in the S-strain and L-strain, respectively.



(a) (b) (c)

Figure 6. Cross-reactivity of anti-CVB3 mouse sera induced by infection with purified CVB3 strains. Antisera collected from CVB3-infected mice were subjected to ELISA using 3 CVB3 strains as antigens. (a) Sera from wt virus-infected; (b) L-strain-infected; and (c) S-strain-infected mice were used as antigens and reacted with inactivated wt (open column), L-strain (gray column), and S-strain (filled column) viruses, respectively. The mean absorbance of triplicate tests is shown, and groups exhibiting significant differences are indicated by asterisks ($^{*}P < 0.01$, $^{**}P < 0.05$, student's *t*-test).

virus than the other strains ($P = 0.001$ and $P = 0.003$ vs. wt and S-strain, respectively). S-strain-induced antibodies also exhibited significantly lower reactivity to the wt virus ($P = 0.036$). However, there was no significant difference in the recognition of the L-strain virus.

4. Discussion

Previous studies suggest VP1 possesses the highest antigenicity among the 4 structural proteins of picornaviruses. In fact, 2 monoclonal antibodies out of 4 neutralizing monoclonal antibodies raised against FMDV recognize

VP1 [19], and antisera from CVB3-infected mice recognized VP1 dominantly in CVB3-infected cells in the present study (data not shown). On the basis of these observations, VP1 has been employed as the main antigen in the development of subunit vaccines against many picornavirus diseases [4]-[8] [19] [20]. However, because picornaviruses have a high mutation frequency because of the low fidelity of their RNA polymerase [1] [18], the viruses can easily escape from antibodies induced by subunit vaccines. Therefore, it is necessary to evaluate whether other capsid proteins with lower variability can induce an antibody response in inoculated animals. In fact, DNA vaccines with the VP3 gene confer good protection against lethal CVB3 infection like vaccines using the VP1 gene [12]-[14].

In this study, we obtained 4 CVB3 capsidproteins tagged with TrxHis; 3 of these proteins induced antibody production in mice. As expected, VP3 and VP2 also worked as antigens in mice, although fewer antibodies were induced by these antigens than by VP1 (**Figure 3(a)**, **Figure 3(b)**). These antibodies recognized whole virus particles in ELISA and virus proteins expressed in CVB3-infected cells in western blotting, suggesting VPs expressed in *E. coli* may be potential antigens for subunit vaccines against CVB3. However, it should be noted that all TrxHis-VPs expressed in this study were expressed as insoluble proteins; therefore, solubilization with strong denaturing reagents was required to purify these proteins. Picornavirus VPs are reported to be extremely hydrophobic, making them difficult to purify them after expression in *E. coli* [21] [22]. To solve this problem, modifications by some tags such as SUMO (Smt3) were tested and several water-soluble virus capsid proteins including FMDV-VP3 were produced [23]. However, because SUMO and TrxHis tags have particular molecular weights (12 and 16 kDa, respectively), it is still possible that this tag part possesses antigenicity in addition to the VPs and weakened antibody response. Therefore, antigen preparation should be approached carefully.

In parallel to the evaluation of antibody response against the 4 VPs of CVB3, we isolated 2 independent virus strains from CVB3 virus stock on the basis of plaque size. Sequence analysis of these 2 strains identified several amino acid substitutions in each structural protein, including amino acids responsible for receptor affinity in cell culture systems (**Figure 5**) [18]. In particular, the combination of D177V and T220S observed in L-strain are reported to be related to increased affinity to cellular receptors on RD cells [18]. The fact that this combination was not observed in S-strain suggests altered receptor affinity in L-strain determines the plaque size. Antisera obtained from mice infected with different virus strain exhibited different cross-reactivity against other virus strains when tested in ELISA using whole virions as antigens. In particular, the lower reactivity of L-strain-induced antibodies against the other 2 strains (**Figure 6**) suggests the existence or emergence of dominant epitopes on the L-strain due to the amino acid substitutions. In the case of natural virus circulation in quasispecies, it is possible that a strain that has acquired certain mutations advantageous for survival will be a dominant strain and induce a strong antibody response. However, even after one strain becomes dominant in the circulating population, some strains will remain from the parental virus stock, which could establish infection, because antibodies against the main strain would partially react with them. This creates further diversity in circulating viruses and makes it more difficult to control virus spread using vaccines based on whole virus particles isolated from among circulating viruses or vaccine strains whose antigenicity matches with circulating viruses. This tendency was observed in the sera obtained from S-strain-infected mice. Although the wt virus induced antisera maintained reactivity against the L-strain virus, its recognition was significantly lower, suggesting the induction of a strong antibody response against particular epitopes on purified strains.

The present results indicate the structural proteins of CVB3 except VP4 elicit antibody responses in mice when independently used as antigens. However, when the neutralizing activities of sera from immunized mice were tested, no neutralizing activities were detected even though whole virus particles were recognized in ELISA. Neutralizing epitopes of picornavirus are thought to comprise amino acids in different VPs [24]. Therefore, it might be necessary to use whole virions or virus-like particles made up of recombinant proteins such as immunogens as suggested previously [2] [23] [25]. In addition, CVB3 DNA vaccines using expression plasmids for VP1 and VP3 are reported to protect mice against lethal challenge without inducing neutralizing antibodies [14]. Although the explanations for these results are merely speculative, the involvement of cellular immune response—especially the induction of virus-specific cytotoxic T-lymphocytes—has been suggested; this is because cytotoxic T-lymphocytes are reported to play a critical role in protection against picornavirus infection [26]. In addition, the fact that the reactivity of wt virus-induced antibodies against the L-strain and S-strain was unaltered highlights the potential of multivalent vaccines. Therefore, combining large-scale antigen production systems using recombinant protein with multivalent antigen inoculation would be a powerful tool for establishing solid and long-lasting protection against picornavirus diseases.

5. Conclusion

In this report, the potential of individual CVB3 capsid proteins as immunogens was shown but the importance of using multiple VPs as antigens to induce neutralizing antibodies *in vivo* was also proved. Furthermore, we found that antisera obtained from infected with purified virus strain showed less reactivity against unpurified wild type virus stock. The decreased cross-reactivity suggested the benefit of un-purified viral stock as immunogens since it might induce broader protection in immunized animals than using purified virus strain.

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