Identification of conformational neutralizing epitopes on the capsid protein of canine calicivirus

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Two neutralizing monoclonal antibodies (MAbs) against canine calicivirus (CaCV), which has a distinct antigenicity from feline calicivirus (FCV), were obtained. Both MAbs recognized conformational epitopes on the capsid protein of CaCV and were used to identify these epitopes. Neutralization-resistant variants of CaCV were selected in the presence of individual MAbs in a cell culture. Cross-neutralization tests using the variants indicated that the MAbs recognized functionally independent epitopes on the capsid protein. Recombinantly expressed ORF2 products (capsid precursors) of the variants showed no reactivity to the MAbs used for the selection, suggesting that the resistance was induced by a failing in binding of the MAbs to the variant capsid proteins. Several nucleotide changes resulting in amino acid substitutions in the capsid protein were found by sequence analysis. Reactivities of the MAbs to the revertant ORF2 products produced from each variant ORF2 by site-directed mutagenesis identified a single amino acid substitution in each variant capsid protein responsible for the failure of MAb binding. The amino acid residues related to forming the conformational neutralizing epitopes were located in regions equivalent to the 5’ and 3’ hypervariable regions of the FCV capsid protein, where antigenic sites were demonstrated in previous studies. The recombinant ORF2 products expressed in bacteria failed to induce neutralizing antibody, suggesting that neutralizing antibodies were only generated when properly folded capsid protein was used as an antigen. In CaCV, the conformational epitopes may play a more important role in neutralization than do linear epitopes.

Introduction

Canine calicivirus (CaCV) No. 48 strain was isolated from a 2-month-old Japanese domestic dog with fatal diarrhoea (Mochizuki et al., 1993). Characteristics of the No. 48 strain are similar to those of the first strain reported as CaCV (Schaffer et al., 1985). CaCV was shown to have an antigenicity distinct from feline calicivirus (FCV), which belongs to the genus Vesivirus, containing swine vesicular exanthema virus as prototype. Sequence analysis of a region from the RNA polymerase gene to the 3′ poly(A) tail of the positive-sense RNA genome of CaCV demonstrated the presence of three open reading frames (ORFs) in its genome (Roerink et al., 1999a). Homology analysis of the RNA polymerase genes of caliciviruses suggested that CaCV No. 48 strain was a new clade in the genus Vesivirus (Roerink et al., 1999b). The second ORF (ORF2) encoded a 75 kDa capsid precursor that was cleaved by a viral proteinase and was processed into the mature 57 kDa capsid protein (Matsuura et al., 2000).

Capsid precursors of caliciviruses can be divided into six regions, designated as A to F (Neill, 1992). As shown in Fig. 1, in both FCV and CaCV, the A regions are cleaved by the proteinase encoded in ORF1 of FCV (Sosnovtsev et al., 1998; Matsuura et al., 2000). The A regions were identified as a 14 kDa polypeptide in the FCV-infected cells (Tohya et al., 1999), and as a 22 kDa polypeptide in CaCV (Matsuura et al., 2000). The B regions contain sequences that are highly conserved among all the caliciviruses (Neill, 1992; Roerink et al., 1999a). In CaCV, little is known about regions C to F.
However, the C and E regions of FCV have been proposed to contain antigenic determinants (Milton et al., 1992; Guiver et al., 1992; Shin et al., 1993). The E region of FCV is further divided into 5’ and 3’ hypervariable regions (HVRs) separated by a conserved central domain (Seal et al., 1993; Geissler et al., 1997; Radford et al., 1999). The D and F regions are moderately conserved among caliciviruses. The F region is thought to be at least partially exposed on the surface of the virion (Milton et al., 1992).

Monoclonal antibodies (MAbs) are useful for analysing antigenic properties of viruses. In previous studies on FCV, the MAb mapping experiments by Tohya et al. (1991) demonstrated the presence of seven neutralizing epitopes on the FCV capsid. The sequence analysis of MAb neutralization-resistant variants indicated four linear epitopes localized in the 5’ HVR and two conformational ones in the 3’ HVR (Tohya et al., 1997). Although little is known about neutralizing epitopes on the CaCV capsid protein, San Gabriel (1996) succeeded in producing a neutralizing MAb (LD8) against CaCV No. 48 strain. It has been difficult to establish more MAbs against CaCV because of a high incidence of false positives and the presence of antibodies against cellular constituents in contaminated purified CaCV preparations. In this study, to eliminate such undesired antibodies against foreign constituents, the mice were immunized after induction of tolerance against cytoplasmic antigens. Neutralizing MAbs against the No. 48 strain of CaCV were used for the selection of neutralization-resistant variants. Based on sequence analysis of the variant ORF2s, we attempted to map neutralizing epitopes on the CaCV capsid protein. 

### Methods

**Virus and cells.** CaCV No. 48 strain was propagated in Miyazaki University canine mammary gland mixed tumour (MCM-B2) cells (Prioxsoreyanto et al., 1995) and stored at $-80\, ^\circ C$ until needed. CaCV-infected cells for indirect fluorescent antibody assay (IFA) and immunoblot analysis were prepared from cells incubated at $37\, ^\circ C$ for 4 h after infection. CaCV purification was performed as previously described (San Gabriel et al., 1997).

**Production of MAbs against CaCV.** To diminish undesired antibodies against antigens from the canine cells, which were present even in the purified virus preparation, immunotolerance against uninfected cells was induced in BALB/c mice according to the methods described previously (Matthew & Sandrock, 1987; Weiland et al., 1989; Wieczorek-Krohmer et al., 1996). Three weeks after the last immunotolerizing treatment, the first immunization with the purified CaCV was administered, followed by the second immunization 2 weeks later. Using IFA with the CaCV-infected MCM-B2 cells, antiviral antibodies were evaluated in sera collected 15 days after the second immunization. Seropositive mice, which showed a high titre of antibodies against CaCV, were inoculated with the purified CaCV by the intravenous route as the third immunization. Three days after the third immunization, spleen cells of the mice were taken out and fused with P3U1 myeloma cells (Tohya et al., 1990). Hybridomas secreting antibodies against CaCV were detected by IFA and a neutralization test against 100 TCID$_{50}$ of CaCV. Cultures producing virus-specific antibodies were subcloned by limiting dilution, followed by cloning and preparation of ascitic fluid. Immunoglobulin subtypes of the MAbs were determined by a MAb typing kit for mouse tissue culture supernatants (The Binding Site).

**IFA with the products expressed from CaCV ORF2.** The CaCV ORF2 expression plasmid (pDCV-II) constructed as described previously (Matsuura et al., 2000) was transfected into COS-7 cells according to the previously described method with minor modifications (Seed & Aruffo, 1987; Shin et al., 1993). The transfected COS-7 cells were smeared on glass slides, air-dried and then fixed in acetone. The fixed cells were reacted with the MAbs and then stained with anti-mouse IgG sheep antibody conjugated with fluorescein isothiocyanate. The cells were mounted in buffered glycerol and examined by fluorescence microscopy.

**Isolation of neutralization-resistant variants.** Neutralization-resistant variants of CaCV No. 48 were selected by the methods described for FCV (Tohya et al., 1990). CaCV No. 48 was allowed to react with asctic fluids for 1 h at $37\, ^\circ C$ and appropriate dilutions of the mixture were inoculated onto monolayers of MCM-B2 cells. Following incubation at $37\, ^\circ C$ for 90 min, the cells were overlaid with 0.8% agar medium containing ascitic fluids. After incubation at $37\, ^\circ C$ for 2 days, the monolayers were stained with 0.01% neutral red in agar overlay medium. Variant plaques were picked up and subjected to two more cycles of selection. Frequencies of variants resistant to neutralization by the MAbs in cloned virus stocks were measured as previously described (Smith & Inglis, 1987; Tohya et al., 1997). Neutralization-resistant variants were designated ‘res’ followed by the name of the neutralizing MAbs.

**Cross-neutralization test with the variants.** The cross-neutralization test was performed according to the microneutralization test procedure (San Gabriel et al., 1997). Briefly, the ascitic fluids of MAbs, and anti-CaCV serum obtained from hyperimmunized mice, were serially twofold-diluted with Dulbecco’s modified Eagle medium containing
Table 1. PCR oligonucleotide primer sequences for site-directed mutagenesis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Location in ORF2</th>
<th>Sequence*</th>
<th>Reverted amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>pR8GDR</td>
<td>1332–1312</td>
<td>gccaatACtctgtgtggtttgc</td>
<td>442 G → D</td>
</tr>
<tr>
<td>pR8KTR</td>
<td>1436–1413</td>
<td>gttTgtGtctgtgtgtgaaacc</td>
<td>477 K → T</td>
</tr>
<tr>
<td>pR2DGR</td>
<td>1556–1537</td>
<td>ttagacCcaccgcgtgtacc</td>
<td>478 R → Q</td>
</tr>
</tbody>
</table>

* Capitalized letters show nucleotides that result in reversion to the amino acid of the parent.
† Reversion of the mutated amino acid of variants to the amino acid of the parent virus.
‡ Deduced amino acid position in capsid precursor protein.

Construction of revertant ORF2 expression plasmids by site-directed mutagenesis. In order to confirm the amino acid residues related to forming the epitopes of the MAb, nucleotide sequences of mutated amino acids of the variants were reverted to those of the parental virus by site-directed mutagenesis. Initially, the BamHI fragment containing the ORF2 of a variant was excised from the variant ORF2 expression plasmid and ligated into pBlueScript II KS(+) (Stratagene). The reversion of amino acid position 442 (Gly for Ala) and the reversion of Lys(177) (AAA to ACA for Thr) in the capsid precursor of resLD8, and the Arg(154) reversion (CGA to CAA for Gln) in the Asp(312) reversion (GAT to GTG for Gly) of res272G were introduced into the fragments with the use of synthetic oligonucleotides (Table 1) and the TaKaRa LA PCR in vitro Mutagenesis kit (TAKARA) according to the manufacturer’s instructions. After all reversions in the fragments were confirmed by sequence analysis, the reversed fragments were ligated into the Xhol–Xbal site of pME18S. The COS-7 cells transfected with each of the constructed plasmids were analysed by IFA with MAb.

Immunoblot analysis. Cells transfected with ORF2 expression plasmids, CaCV-infected cells and purified CaCV were separated by 10% SDS–PAGE and electro-transferred onto a polyvinylidene difluoride filter (San Gabriel et al., 1997). The transferred proteins were reacted with the MAb and serum monoclonal to the CaCV capsid protein (Matsuura et al., 2000). Bound antibodies were detected with peroxidase-conjugated goat anti-mouse immunoglobulins. The bands were visualized with 3,3′-diaminobenzidine tetrahydrochloride in the presence of hydrogen peroxide.

Preparation of antiserum against fragments of the capsid protein. To find the most immunodominant region on the capsid protein, a set of four fragments of ORF2 was expressed as fusion proteins with glutathione S-transferase (GST) using the pGEX bacterial expression system (Pharmacia Biotech). The fragments of ORF2 were designated as follows: BF (nt 472–2076 in ORF2, mature capsid protein), B (nt 472–1257, aa 158–419), CDE (nt 1258–1668, aa 420–556) and F (nt 1669–2076, aa 557–692). Construction of expression plasmids was performed as described previously (Tohya et al., 1999; Matsuura et al., 2000). The fragments were amplified by PCR using pDCV-II as template and sense and antisense primers that corresponded or were complementary to sequences of CaCV ORF2. Paired of used primers, to which a restriction enzyme site (EcoRI or Xhol) was added, were as follows: (i) for BF, sense primer CaCV ORF2-B (5′ AGATTTCCTCGCATAGTTCACCCCGCTGTA 3′) and antisense primer CaCV ORF2-FR (5′ ATG-
Table 2. Characterization of MAbs against CaCV

<table>
<thead>
<tr>
<th>MAb</th>
<th>Isotype</th>
<th>Neutralization activity*</th>
<th>CaCV-infected cells</th>
<th>pDCV-II-transfected cells†</th>
<th>Immunoblot analysis‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD8</td>
<td>IgG2b</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>27G2</td>
<td>IgG2a</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

* Neutralizing test of hybridoma supernatant against 100 TCID₅₀ of CaCV.
† Cells transfected with CaCV ORF2 expression plasmid.
‡ Reactivity of MAb to the capsid protein of CaCV in immunoblot analysis.

Table 3. Neutralizing and immunofluorescence reactivities of MAbs with variants

<table>
<thead>
<tr>
<th>MAb</th>
<th>Neutralizing activity*</th>
<th>Reactivity to ORF2 product by IFA†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parent</td>
<td>resLD8</td>
</tr>
<tr>
<td>LD8</td>
<td>160</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>27G2</td>
<td>640</td>
<td>640</td>
</tr>
<tr>
<td>Anti-CaCV serum</td>
<td>95</td>
<td>110</td>
</tr>
</tbody>
</table>

* Reciprocal of 50% neutralization end-point dilution of ascitic fluids.
† Reactivity in cells transfected with ORF2 expression plasmid.

CTCGAGTCATAGTGTTGTAGCGCTACC 3′); (ii) for B, CaCV ORF2-B and CaCV ORF2-BR (5′ ATTACTCGAGGGACCAGGCCAAGCTCTGT 3′); (iii) for CDE, CaCV ORF2-CDE (5′ GCGAATTCCGACCGTACAAAAGGCTATTG 3′) and CaCV ORF2-CDER (5′ CAACTCGAGGAGCGCTTTGCTCTTGGCTAT 3′); (iv) for F, CaCV ORF2-F (5′ TAGAATTCGGAGGAGCTCCCATTGGA 3′) and CaCV ORF2-FR. Amplified DNA products were digested with EcoRI and XhoI and ligated into the vector pGEX-5X-1 in-frame with the GST gene. Expression in Escherichia coli, purification by SDS–PAGE of the fusion proteins and inoculation to ddY mice were performed as described previously (Tohya et al., 1999). Obtained sera were inactivated for 30 min at 56 °C and stored at −20 °C until experiments.

Results

Production and characterization of MAbs

Fusion of spleen cells from the mice was carried out five times. Several lines of hybridomas producing MAbs against CaCV were obtained from the fusions. However, as the result of recloning, only two lines stably produced MAbs with specific reactivity to CaCV in IFA. As one of the MAbs had neutralizing activity, this one was selected for this study and was designated as 27G2. Finally, two neutralizing MAbs, LD8 (San Gabriel, 1996) and 27G2 were used in this study and characterized. LD8 and 27G2 belong to IgG2b and 2a subclasses, respectively. As shown in Table 2, both the MAbs showed positive reactivities with the ORF2 product in COS-7 cells transfected with pDCV-II in IFA, although no protein recognized by the MAbs was detected by immunoblot analysis using infected cells or purified CaCV. These results indicated that the MAbs recognized neutralizing epitopes with a conformational nature on the capsid protein.

Isolation of neutralization-resistant variants and their reactivities with the MAbs

For the purpose of analysing the epitopes of the neutralizing MAbs on the CaCV capsid protein, selection of neutralization-resistant variants of CaCV was attempted using LD8 and 27G2. The frequencies of resLD8 and res27G2 were 10⁻³⁻⁷⁴ and 10⁻⁶⁻⁷², respectively. This result indicated that the neutralizing epitope for LD8 might be more variable than that for 27G2. The variants were equally neutralized by the anti-CaCV serum (Table 3), suggesting that their mutations did not have a profound effect on their overall antigenicity. Each variant was resistant to neutralization by the homologous MAb used for its selection but not by the heterologous one (Table 3). IFA using cells infected with each variant showed that variant-infected cells did not bind the homologous MAb (data not shown). Further IFA on COS-7 cells transfected with...
Table 4. Changes in nucleotide and deduced amino acid sequences of resistant variants

<table>
<thead>
<tr>
<th>Resistant variant</th>
<th>Nucleotide change/position*</th>
<th>Amino acid change/position†</th>
</tr>
</thead>
<tbody>
<tr>
<td>resLD8</td>
<td>G → A/1325</td>
<td>D → G/442</td>
</tr>
<tr>
<td></td>
<td>C → A/1430</td>
<td>T → K/477</td>
</tr>
<tr>
<td>res27G2</td>
<td>G → A/1325</td>
<td>D → G/442</td>
</tr>
<tr>
<td></td>
<td>A → G/1433</td>
<td>Q → R/478</td>
</tr>
<tr>
<td></td>
<td>G → A/1550</td>
<td>G → D/517</td>
</tr>
</tbody>
</table>

* Nucleotide position in ORF2.  † Deduced amino acid position in capsid precursor.

variant ORF2 expression plasmids was tried with MAbs. The expressed products of the variant ORF2s were not detected with the homologous MAb although the ORF2 products were confirmed to be expressed in COS-7 cells by IFA using anti-CaCV serum and the heterologous MAbs (Table 3). Based on the reactivity pattern of IFA and neutralization between variants and MAbs, the neutralizing resistance was thought to be induced by a failure to bind MAbs. The resistant ORF2 products were detected as a 75 kDa capsid precursor by immunoblot analysis using the serum against CaCV capsid protein (data not shown).

Sequence analysis for detection of mutation in the variant ORF2s

In the previous study on neutralizing epitopes of FCV, a single point mutation was found in one of the HVRs on the capsid protein of each variant resistant to a MAb neutralizing FCV (Tohya et al., 1997). The altered nucleotide was expected to be capable of determining resistance to the neutralizing MAb. Sequence analysis of the ORF2 encoding the capsid precursor of CaCV variants indicated that two or three nucleotide changes resulting in amino acid substitutions were present in each ORF2 of the variants (Table 4). The amino acid substitutions of resLD8 were located at positions 442 (D to G; Asp to Gly) and 477 (T to K; Thr to Lys) on the capsid precursor. Those of res27G2 were located at positions 442 (D to G), 478 (Q to R; Gln to Arg) and 517 (G to D; Gly to Asp). At the other positions, no amino acid change was observed in the sequences of the two clones obtained from separate PCR experiments for each of the variants.

Reactivities of the MAbs with revertant ORF2 products in IFA

In order to identify the location of substitutions capable of conferring resistance of the variants, ORF2 expression plasmids with amino acid reversions were constructed by site-directed mutagenesis. IFA using COS-7 cells transfected with the revertant ORF2 expression plasmids was performed. As shown in Table 5, the ORF2 expression product of the Lys417 revertant of resLD8 had reactivity with LD8 in IFA, but the Gly442 revertant had no reactivity. In res27G2, the product of the Asp478 revertant recovered reactivity with 27G2, but the Arg478 revertant product did not. The mutations at positions 442 and 478 may have been spontaneous mutations of the single-strand RNA virus and not responsible for the antigenic characteristics of the virus.

Reactivities of the sera against GST fusion proteins with fragments of the capsid protein

Production of fusion proteins with fragments of the CaCV capsid protein was analysed by Coomassie blue staining after 10% SDS–PAGE. The level of expression was high in every case. All sera obtained from immunized mice, except serum against the GST–F fusion protein, showed strong reactivity.
with the 57 kDa capsid protein in the CaCV-infected cells by immunoblot analysis (Fig. 2). However, no virus-neutralizing activity was observed in neutralization tests against 100 TCID_{50} of CaCV using any of the sera. The anti-GST serum prepared as a control showed no reactivity with the viral proteins in the infected cells and no neutralizing activity.

Discussion

In the previous preparation of MAbs using ELISA as the screening method, MAbs from most of the hybridomas reacted against cellular components contained in the purified virus preparation (San Gabriel, 1996). Schaffer et al. (1985) reported that CaCV virions were strongly cell-associated and difficult to purify completely. In this study, the induction of tolerance against cellular components results in a low background reactivity of antibodies in the screening of hybridomas. This attempt seemed useful for the production of MAbs against the CaCV virion. However, the number of established MAbs in this experiment was two and only one of them showed neutralizing activity. It is likely that screening methods might need to be more sensitive in order to obtain a panel of MAbs against CaCV.

The mapping study of neutralizing epitopes recognized by the MAbs was performed using antigenic variants of CaCV. Cross-neutralization tests using the variants and the IFA reactivities of MAbs to variant ORF2 products suggested that there were at least two functionally independent epitopes on CaCV. No apparent deletion in the variant ORF2s was shown although the three-dimensional structure of CaCV has not been analysed.

In FCV studies, the 5’ HVR of region E seemed to be significant as a major antigenic determinant and as an important target for neutralizing antibodies (Guiver et al., 1992; Milton et al., 1992; Shin et al., 1993; Tohya et al., 1997; Radford et al., 1999). In MAb mapping experiments on FCV using neutralization-resistant variants (Tohya et al., 1997), the mutations disrupting each of four linear epitopes located in the 5’ HVR of region E (Fig. 3). The region involved in the formation of the linear neutralizing epitopes was recognized by antibodies developed by FCV-infected cats (Radford et al., 1999).
1999). A region of CaCV capsid protein that approximates to the 5’ HVR of FCV appeared to be missing in CaCV (Fig. 3), although the extreme variability in this region might make such line-ups difficult. Indeed, the GST fusion polypeptides containing fragments BF and CDE failed to induce neutralizing antibodies in spite of inducing antibodies to the mature capsid protein in immunoblot analysis, suggesting that neutralizing antibodies were only generated when properly folded capsid protein was used as an antigen. In a recent study on FCV, Neill et al. (2000) reported that the conformational epitopes might play a major role in antigenicity and neutralization. The 3’ HVR has been implicated in the formation of at least two conformational epitopes (Tohya et al., 1997). The position of CaCV Gly$_{517}$ was mapped in a region corresponding to the 3’ HVR of FCV (Fig. 3). As Thr$_{517}$ of CaCV was revealed to be involved in the formation of another conformational epitope, each of the 5’ and 3’ HVR corresponding regions might constitute a conformational antigenic site, playing an important role in neutralization of CaCV. The conformational epitopes on the surface of the CaCV particle seemed to be more significant targets for neutralizing antibodies against CaCV than the linear epitopes, although a panel of MABs that had neutralizing activity against CaCV may be required to determine the antigenic structure of the virus in more detail.

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References


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