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Phage-displayed peptides having antigenic similarities with porcine epidemic diarrhea virus (PEDV) neutralizing epitopes

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Received 10 January 2006; returned to author for revision 21 February 2006; accepted 21 April 2006

Available online 1 September 2006

Abstract

Seven-mer phage random peptide libraries were panned against 2C10, a monoclonal antibody that showed neutralizing activities against PEDV. Recombinant M13 phages displaying the peptides SHRLP(Y/Q)(P/V) or GPRPVTH on the g3p minor coat protein showed strong binding affinity with 2C10 (70% and 30% of recovered phages, respectively) after multiple panning. Sequence analysis suggested that these peptides are similar with ¹³⁶⁸GPRLQPY¹³⁷⁴ found at the carboxy-terminal of the S protein. In neutralization inhibition assays, the two peptide motifs and a 24-mer synthetic peptide corresponding to the C-terminal endodomain of PEDV S protein were observed to compete for the antigen binding site of 2C10, as demonstrated by the loss or reduction of neutralizing activity of the monoclonal antibody. This new finding suggests that the newly discovered peptide motifs mimic a neutralizing epitope PEDV.

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Keywords: PEDV; Spike protein; Phage display library; Neutralizing epitope; Synthetic peptides

Introduction

Porcine epidemic diarrhea virus (PEDV) belongs to the family Coronaviridae and is closely related with human coronavirus 229E, transmissible gastroenteritis virus and feline infectious peritonitis virus (Bridgen et al., 1993; Yaling et al., 1988). This virus causes porcine epidemic diarrhea, an enteric disease affecting swine of all ages. It is characterized by acute watery diarrhea and dehydration (DeBouck and Pensaert, 1980). The clinical and pathological symptoms are similar to those of another swine coronaviral disease, transmissible gastroenteritis, making it difficult to differentiate between the two (Pospischil et al., 1981; Pritchard et al., 1999).

During the past decade, PED infection has been a major concern in the swine industry, particularly in the Asian region, resulting in large economic losses (Chae et al., 2000; Sueyoshi et al., 1995). Because of the economic importance of PED in the swine industry, measures to control the spread of the disease have led to the development of live attenuated vaccines. It began with the work of Hofmann and Wyler (1988) when they

successfully propagated PEDV in Vero cells. Later, Bernasconi et al. (1995) reported that after multiple passages of PEDV CV777 in Vero cells, the cell adapted strain differed significantly from the parental strain both in terms of genomic sequence and pathogenicity. In a similar study, Kweon et al. (1999) reported that another cell adapted strain of PEDV induced protective immunity in pregnant sows that were transferable to lactating piglets. However, in spite of these developments, PED still persist in several provinces in the Asian region. Thus, there is a need to understand the mechanism of viral entry and pathogenesis. Such information would be valuable in developing more effective vaccines in the future.

In this study, we screened a phage displayed peptide library for peptides with sequence or structural similarity with a PEDV neutralizing epitope. Candidate phage-displayed peptides selected by multiple panning were tested for inhibition properties against a monoclonal antibody with known neutralizing activity against the KPEDV-9 vaccine strain. The peptides displayed on these phages were compared with known sequence of PEDV. A peptide corresponding to the region of the PED S protein containing the motif similar with the candidate phage peptides was synthesized and also analyzed for inhibition properties against the monoclonal antibody.

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Results

Selection of PEDV-neutralizing monoclonal antibodies

In this study, four different monoclonal antibodies (2D11, 2E4, 3H8 and 2C10) were tested for neutralizing activities against KPEDV-9. As shown in Fig. 1, 1:100 dilution of MABs 2D11 and 2C10 were able to neutralize more than 50% of KPEDV-9 (55% and 84%, respectively), while 2E4 and 3H8 had lower neutralizing activities (30% and 11% neutralization, respectively). At higher dilutions, however, only 2C10 exhibited neutralizing activities (58% neutralization at 1:400 dilution, 27% at 1:1600 and 19% at 1:6400). The other MABs had an average neutralizing activity of 8% at 1:400 and less than 1% at 1:1600. Based on these results, 2C10 was selected for bio-panning of the phage peptide library.

Screening of 2C10-binding phages

In bio-panning the 7-mer phage peptide library with 2C10, a total of 8395 recombinant phages were initially recovered. On second panning, Polysorbate 20 (Tween-20, Sigma) concentration in the washing buffer was elevated from 0.1% to 0.5% to increase selection by removing phages having only weak to moderate binding affinities. This resulted in the recovery of only 960 strong binding recombinant phages. In subsequent panning, the number of strong binding phages recovered increased dramatically. Third panning resulted in the recovery of approximately 290,950 recombinant phages while fourth panning recovered approximately 393,300 recombinant phages. The significant increase in the number of recovered phages after third and fourth panning indicated that these phages have a relative strong binding affinity to the 2C10 monoclonal antibody.

Table 1

Predominant peptides displayed by selected phages on their g3p minor coat protein

Recovered phages ^a	Displayed random peptide ^b
PED-PhD 4-01	S H R L P Y P
PED-PhD 4-02	S H R L P Y P
PED-PhD 4-03	S H R L P Y P
PED-PhD 4-10	S H R L P Y P
PED-PhD 4-11	S H R L P Y P
PED-PhD 4-12	S H R L P Y P
PED-PhD 4-06	S H R L P Q V
PED-PhD 4-04	G P R P V T H
PED-PhD 4-05	G P R P V T H
PED-PhD 4-07	G P R P V T H

These displayed peptides show high binding affinity to the anti-PEDV monoclonal antibody 2C10 after multiple panning.

^a Isolated recombinant phages after 4 sets of bio-panning.

^b Seven-mer peptide displayed on the g3p minor coat protein of the recombinant phage.

Sequence analyses of recovered phages

After fourth panning, recombinant phages that exhibited relative strong binding affinity to 2C10 were eluted, amplified, and sequenced. In Table 1, the sequence of the heptapeptide-gIII-fusion region of 10 randomly selected phages revealed the predominance of 2 peptide motifs: SHRLP(Y/Q)(P/V) accounts for 70% (7 out of 10) of the selected phages while GPRPVTH accounts for 30% (3 out of 10). In the SHRLP(Y/Q)(P/V) motif, the 6th position is occupied either by tyrosine or glutamine, amino acids that share similar biochemical properties (neutral-polar and hydrophilic). The 7th position is occupied either by proline or valine. These two amino acids also share similar biochemical properties (neutral-nonpolar and hydrophobic). The relatively strong binding affinity of these two peptides

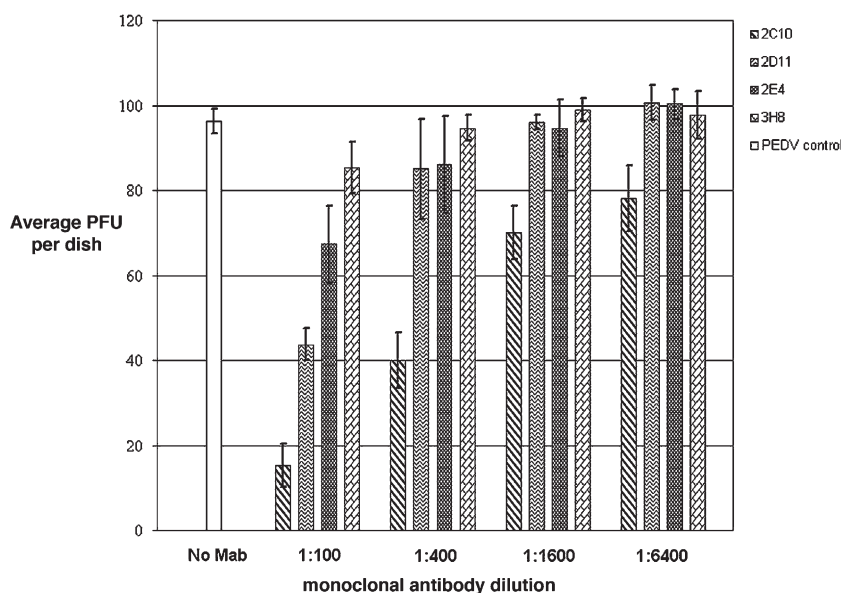


Fig. 1. Plaque reduction neutralization test. Monoclonal antibodies (2C10, 2D11, 2E4, 3H8) at various dilutions were mixed with PEDV to determine neutralizing activity. KPEDV-9 (1×10^3 pfu/ml) mixed with pre-immune mouse sera (1/100) served as negative control.

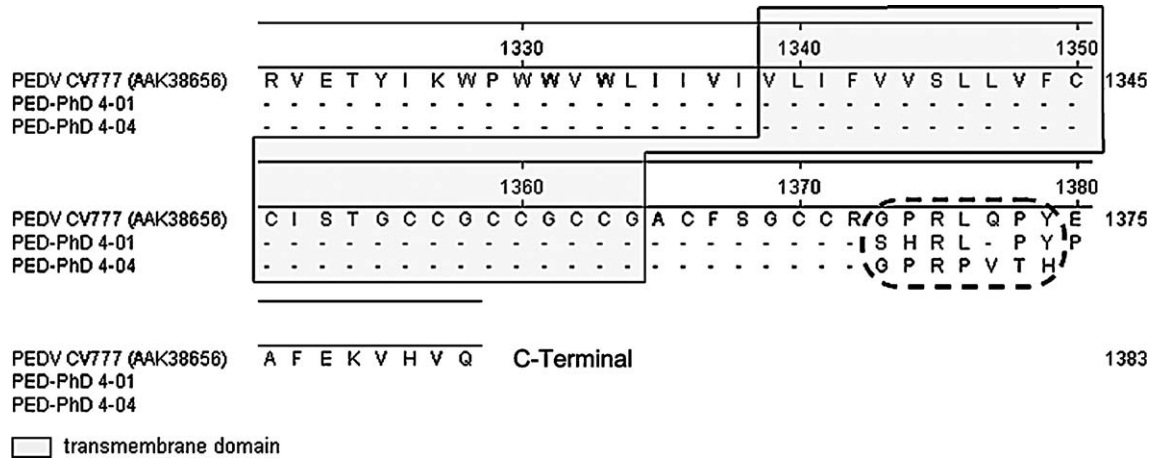


Fig. 2. Sequence alignment of PEDV spike protein. The peptide motif SHRLPYP (represented by PED-PhD 4-01) and GPRPVTH (represented by PED-PhD 4-04) were aligned with PEDV CV777 (NCBI accession number AAK38656) using Lipman–Pearson approach. The transmembrane domain was identified by SOSUI (<http://sosui.proteome.bio.tuat.ac.jp>).

suggests that they share some properties with a native neutralizing epitope on PEDV, either in terms of sequence similarity, structural similarity, or both.

Mapping the peptide motifs on known PEDV sequence

The probable location of SHRLPYP and GPRPVTH on PEDV was determined by pair-wise alignment using the Lipman–Pearson approach. A Ktuple value of 2 and a gap penalty length of 12 revealed a single location on the PEDV S protein where both peptide sequences gave the highest match (Fig. 2). SHRLPYP showed a similarity index of 50% to the native sequence GPRLQPY (aa 1368–1374), while the first 3 amino acids of GPRPVTH were identical with the native sequence. Analysis on the membrane topology of the PED S

protein revealed the location of ¹³⁶⁸GPRLQPY¹³⁷⁴ at the C-terminal region of the glycoprotein.

Characterization of selected phages

To determine whether the recovered phages displaying SHRLPYP or GPRPVTH on their g3p minor coat protein have similar properties with a neutralizing epitope on PEDV, these phages were tested for inhibition of MAb 2C19’s neutralizing activity. Fig. 3 compares the neutralizing activity of 2C10 on KPEDV-9 in the presence of varying amounts of phages. In the absence of recombinant M13 phages, 2C10 neutralized more than 90% of pre-titered KPEDV-9 (data not shown). However, phages displaying the SHRLPYP motif completely inhibited the neutralizing activity of 2C10 at a dilution of 1:100. Higher

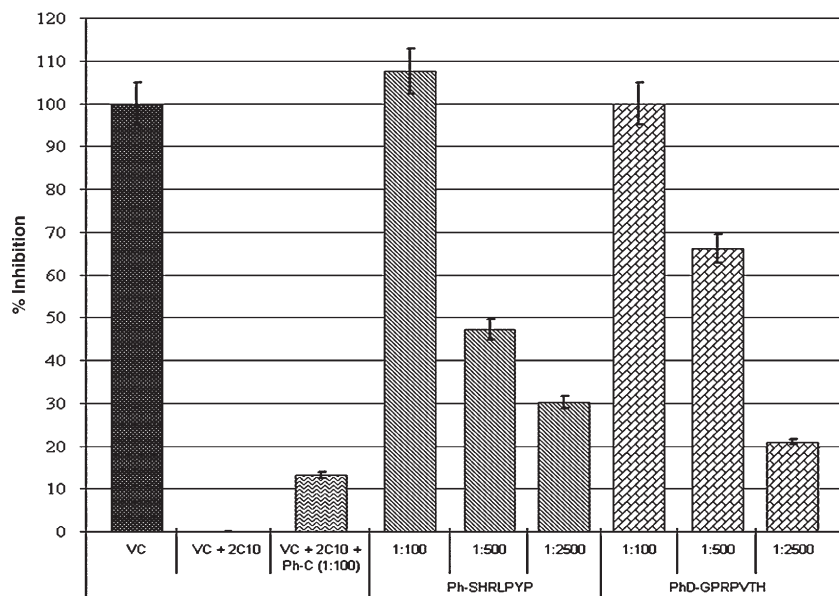


Fig. 3. Neutralization inhibition assay. PRNT of KPEDV-9 by 2C10 in the presence of 2C10 binding phages. Percent inhibition represents the reduction of neutralizing activity of 2C10 against KPEDV-9 (VC = KPEDV-9; VC + 2C10 = KPEDV-9 + MAb2C10; VC + 2C10 + Ph-C = KPEDV-9 + MAb2C10 + nonselected M13 phage; Ph-SHRLPYP = VC + MAb 2C10 + phage-SHRLPYP; Ph-GPRPVTH = VC + MAb2C10 + phage-GPRPVTH).

dilutions of this phage (1:500 and 1:2500) also demonstrated inhibiting properties against 2C10 (47.2% and 30.2%, respectively). A similar trend was observed for phages displaying GPRPVTH. Phage dilution of 1:100 completely inhibited neutralizing activity of 2C10 while higher dilutions of this phage (1:500 and 1:2500) resulted in lower inhibitions (66.1% and 20.8%, respectively). In contrast, when nonspecific M13 phage was pre-incubated with 2C10, only 13.2% of the neutralizing activity of the antibody was inhibited. These data suggest that phages specifically displaying the SHRLPYP and GPRPVTH peptides are able to block the neutralizing activity of 2C10 against KPEDV-9.

Characterization of the ¹³⁶⁸GPRLQPY¹³⁷⁴ motif

Based on the results of protein sequence alignment, two peptides were synthesized. Peptide S-CT(24) (ACFSGCCRGPRLPQPYEAFQK VHVQ) corresponded with the C-terminal cytoplasmic domain of the S protein, including the GPRLQPY motif which showed high similarity index with the phage displayed peptides. Peptide S-CT(17) (ACFSGCCREAFQKVHVQ) is similar to the S-CT(24) but does not include the GPRLQPY motif. In the neutralization inhibition assay, 10 µg/ml S-CT(24) blocked 67.9% of 2C10's neutralizing activity. In contrast, S-CT(17) was observed to block the neutralizing activity of 2C10 by only 9.5% with the same concentration. (Fig. 4).

Discussion

Current research on alternative vaccine strategies for PEDV has focused mainly on the CO-26K equivalent (COE). The CO-26K (collagenase digested fragment) is a conserved region discovered on the S protein of TGEV and was previously shown to include most of the critical epitopes important for virus

neutralization (Delmas et al., 1986, 1990). Chang et al. (2002) expressed the recombinant COE in *E. coli* and used that to generate polyclonal antibodies in mice. The antibodies were demonstrated to possess neutralizing activity against PEDV in plaque assay experiments using Vero cells as host. However, sequence comparison among PEDV strains identified in recent outbreaks in Korea (Cruz et al. unpublished data) revealed numerous differences on the COE domain of the S protein, implying that antibodies that recognize critical epitopes on the COE of one strain of PEDV may not be able to recognize that of another strain.

In this study, we identified the peptide sequence recognized by 2C10, a monoclonal antibody that has neutralizing activity against PEDV, using a phage peptide library. Our findings revealed two peptide motifs, SHRLP(Y/Q)(P/V) and GPRPVTH. Phages displaying these peptides showed strong binding affinity to 2C10 and demonstrated their ability to block the neutralizing activity of 2C10. It is quite interesting to note that these peptides were found to share a homology with the region on the C-terminal cytoplasmic tail of the S protein. Two peptide molecules were synthesized based on this region. The S-CT(24) which contained the GPRLQPY motif inhibited the PEDV neutralizing activity of 2C10. In contrast, S-CT(17) which had the GPRQPY motif deleted, failed to inhibit the neutralizing activity of 2C10. This strengthens our hypothesis that the phage peptides SHRLP(Y/Q)(P/V) and GPRPVTH share antigenic similarities with the ¹³⁶⁸GPRLQPY¹³⁷⁴ motif found on the cytoplasmic domain of the S protein. Because of the inaccessibility of this region in intact virions to neutralizing antibodies, it raises the question of whether the C-terminal endodomain of the S protein indeed has a neutralizing epitope or that the newly identified peptide sequence share a structural similarity with a conformational epitope in another region of the glycoprotein.

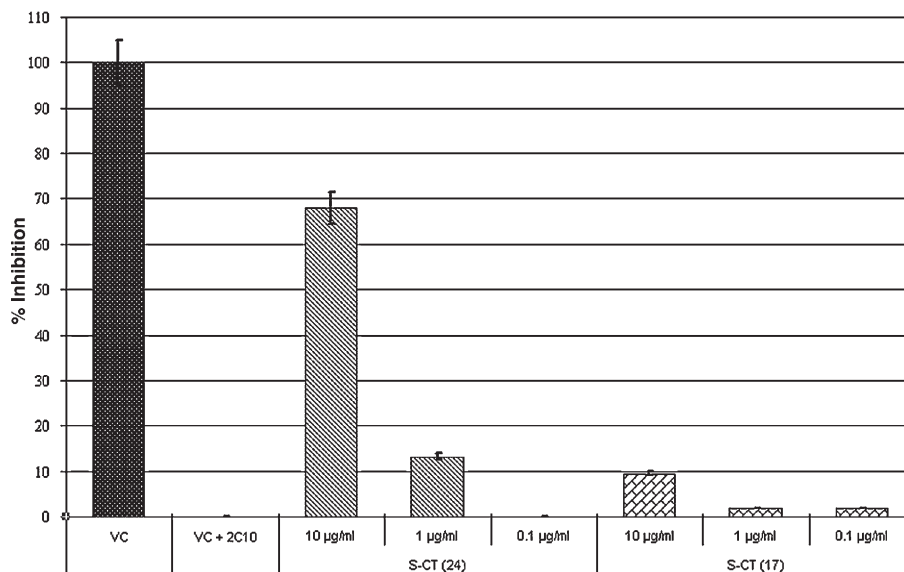


Fig. 4. Neutralization inhibition assay using synthetic peptides. Synthetic peptides S-CT(24) (corresponding to the 24 aa C-terminal cytoplasmic domain of PEDV S protein) and S-CT(17) (modified version of S-CT(24) with the GPRLQPY motif removed) were co-incubated with PEDV and MA2C10 (VC = KPEDV-9; VC + 2C10 = KPEDV9 + MA2C10; S-CT(24) = KPEDV-9 + MA2C10 + S-CT(24); S-CT(17) = KPEDV-9 + MA2C10 + S-CT(17)).

Virus neutralization involves blocking the early stages of the virus replication cycle, namely the attachment and entry into the host cell. This is usually mediated by antibodies that block the receptor binding sites on the surface of the virus or other related sites involved in virus integration into the host. Most of the neutralizing epitopes involved with virus attachment and entry are located on the surface of the virus and are easily accessible to the antibodies. However, some epitopes may exist on sites that are thought to be inaccessible to antibodies (Cleveland et al., 2003). Until now, only a few neutralizing epitopes located on the cytoplasmic domain of transmembrane proteins have been identified. The envelope glycoprotein gp41 in HIV-1 was first reported to contain the neutralizing epitope ⁷⁴⁶ERDRD⁷⁵⁰ at the C-terminal endodomain (Broliden et al., 1992; Vella et al., 1993). However, this finding has been highly controversial since antibodies cannot cross the lipid bilayer envelope membrane. Buratti et al. (1997) suggested that this discrepancy can be explained by the existence of a similar epitope exposed on the ectodomain of the p17 matrix protein which is cross-reacting with the antibodies directed to the gp41 epitope. Incidentally, this transmembrane protein is involved in virus replication and has been a target of several candidate HIV-1 vaccines (Boucher et al., 1990; Naylor et al., 1987). Cheung et al. (2005) proposed a theoretical model that suggests that the C-terminal of gp41 spans the membrane several times and that the ⁷⁴⁶ERDRD⁷⁵⁰ epitope is constantly exposed on the surface of HIV-1. At present, the exact topology of the ⁷⁴⁶ERDRD⁷⁵⁰ and how antibodies that bind specifically to this epitope are able to inhibit virus infection is not fully known.

Peptide residues of a linear or continuous epitope are located side by side on the secondary and tertiary structures of protein molecules. In contrast, peptide residues of a conformational or discontinuous epitope maybe several amino acids apart on a linearized protein but are spatially juxtaposed in a folded protein resulting from internal disulfide bonds, addition of carbohydrate side-chains and other post-translational modifications. Coronaviruses have highly complex peplomers. The S protein is about 175 to 180 kDa and forms a homotrimer with a highly glycosylated terminal. It also contains two hydrophobic (heptad) repeat regions that forms the fusion core complex (Delmas et al., 1986; Jan Bosch et al., 2003). In the case of PEDV, the S protein is a 1383- to 1386-amino-acid transmembrane glycoprotein that has 27 to 29 potential N-linked glycosylation sites, a stretch of hydrophobic residues proximal to the C-terminal believed to function as the membrane anchor, and lacks the proteolytic cleavage site to yield cleaved amino and carboxy subunits S1 and S2, similar to the TGEV subunit (Duarte and Laude, 1994; Yeo et al., 2003). There is the likelihood that the 2C10 monoclonal antibody recognizes a discontinuous epitope on PEDV that is structurally similar with the SHRLP(Y/Q)(P/V) and GPRPVTH peptides.

Combinatorial peptide libraries, like the phage peptide library, are useful tools in identifying the primary sequence of a target epitope by probing with monoclonal antibodies that recognize continuous epitopes (Liu et al., 2003; Scott and

Smith, 1990). However, when a monoclonal antibody used to probe the peptide library recognize a discontinuous epitope, the resulting peptide sequences may vary or may not match any regions on the target protein (D'Mello et al., 1999; Geysen et al., 1984). Although such characteristic limits the ability of this technique in identifying the primary sequence of discontinuous epitopes, the use of peptide libraries does offer the advantage of determining antigen–antibody interaction and structural homology between discontinuous epitopes and linear peptides (Al-Bukhari et al., 2002; Houghten, 1985). For the past decade, phage peptide libraries have been used extensively to identify the primary sequences of continuous and discontinuous epitopes of different viruses like dengue virus (Wu et al., 2003), human papillomavirus (Santamaria et al., 2001), hepatitis C virus (Petit et al., 2003), porcine reproductive, and respiratory virus (Ostrowski et al., 2002), HIV (Scala et al., 1999) and coronaviruses like MHV (Yu et al., 2000) and SARS CoV (Zhong et al., 2005). Aside from identifying probable epitopes, these functional linear epitopes can be used as mimotopes in subunit vaccines. Several candidate subunit vaccines derived from phage peptide libraries have been tested against certain diseases (Meola et al., 1995; Puntoriero et al., 1998; Tumanova et al., 2001).

Our discovery of two linear peptides cross reacting with a PEDV neutralizing monoclonal antibody offer a new perspective on the membrane topology of the S protein as well as provide valuable information regarding the characteristic of a neutralizing epitope. Because of the high degree of conformation on the S protein, using linear epitopes that elicit production of cross reacting antibodies capable of neutralizing PEDV offers a simple but effective alternative strategy in developing recombinant vaccines for PED.

Materials and methods

Cell and virus

Cell-adapted PEDV (KPEDV-9) was kindly provided by the National Veterinary Research and Quarantine Services of Korea. The virus was cultured in Vero cells supplemented by Eagle's minimal essential medium (EMEM, Invitrogen) containing 0.001% trypsin (Invitrogen) according to the method described by Hofmann and Wyler (1988). Briefly, stock virus was inoculated to a confluent monolayer of Vero cells and placed at 37 °C, 5% CO₂. After 24 h, progeny virions were harvested, and titer was determined by plaque assay as described by Vautherot (1981). Ten-fold serially diluted KPEDV-9 was inoculated in 35-mm diameter TC dishes (SPL Labware) containing confluent monolayer of Vero cells at 37 °C. After 1 h, the monolayer was overlaid with 1% agarose in EMEM supplemented with 0.001% trypsin and incubated at 37 °C, 5% CO₂. A second overlay containing 0.01% neutral red was added for staining after 24 h. When visible plaques appeared, the monolayer was fixed with 3.5% formaldehyde in phosphate-buffered saline (PBS, pH 7.2) then permanently stained with crystal violet solution (0.01% crystal violet, 2.5% ethanol in PBS) for plaque counting and titration.

Selection of monoclonal antibodies

Monoclonal antibodies 2D11, 2E4 and 3H8 were generously provided by Bioleaders Corp., Inc., Korea, while 2C10 was kindly provided by Dr. Kweon of the National Veterinary Research and Quarantine Service. The neutralizing activities of the four MABs were determined by plaque reduction neutralization test (PRNT) following the method mentioned previously (Beatty et al., 1989). Four-fold serially diluted MABs were mixed with pre-titrated KPEDV-9 (100 pfu) at 37 °C for 30 min and inoculated to 35-mm diameter TC dishes containing Vero cell monolayer. After 1-h incubation at 37 °C for 1 h, the monolayer was overlaid with 2.5 ml 1% agarose in EMEM supplemented with 0.001% trypsin and continued incubation at 37 °C, 5% CO₂. After 24 h, second agarose overlay containing 0.01% neutral red was added. The cell monolayers were fixed with 3.5% formaldehyde in PBS after 24 h, then permanently stained with crystal violet solution. The monoclonal antibody that reduced plaque formation by more than 50% at a dilution of 1:400 was used for screening of the phage peptide library.

Screening of phage displayed peptide library

Seven-mer phage displayed peptide libraries (New England Biolabs) was bio-panned with the selected MAB and titrated by plaque assay according to manufacturer's instructions. Briefly, a well of a 96-well microtiter plate was sensitized with 25 µg of selected MAB and incubated at 4 °C overnight. Blocking was performed with 350 µl of BSA (5 mg/ml) in 50 mM Tris-buffered saline (TBS) for 1 h at room temperature followed by six times washing with 50 mM TBS containing 0.1% Tween 20 (0.1% TBS-T) to remove excess antibody and blocking reagent. Approximately 2×10^{11} pfu recombinant phages were added to the well and incubated for 1 h. Unbound phages were removed by ten times washing with 0.1% TBS-T. Antibody-bound phages were eluted by treatment with 100 µl of 0.2 M Glycine-HCl (pH 2.2) followed by 15 µl of 1M Tris-Cl (pH 9.1) containing BSA. The eluted phages were amplified in log phase *E. coli* ER2738 and harvested in the culture supernatant at $12,000 \times g$ for 10 min and concentrated by PEG/NaCl precipitation. Bio-panning was repeated 3 times on amplified phage eluate using a wash buffer containing 0.5% Tween 20 (0.5% TBS-T) to enrich the pool of phages with strong binding affinity to the MAB. Eluted and amplified phages were titrated after each panning.

Sequencing of MAb specific recombinant phages

Following manufacturer's instructions, 10 phage colonies recovered after fourth panning were randomly selected and amplified. Each selected phage colony was excised from the bacterized agar and cultured in *E. coli* ER2738. Phage genomic DNA was extracted by treatment with Iodide buffer (pH 8.0) and purified by ethanol precipitation. The phage heptapeptide-gIII fusion gene was sequenced, and the corresponding amino acid sequence of the heptapeptide was deduced from the resulting nucleotide sequence using the reduced genetic code chart.

Mapping the putative PEDV neutralizing epitope

The amino acid sequences derived from the phage displayed peptides were compared with known PEDV sequence from NCBI. Sequence similarity between the phage displayed peptides and native PEDV proteins was determined using DNASTar Megalign™ 5.0. A topology prediction program (<http://sosui.proteome.bio.tuat.ac.jp>) was used to predict the location of the identified motif on the virus surface.

Neutralization inhibition assay

Synthetic peptides corresponding to the regions showing neutralizing activities against PEDV were designed and purchased from Any Gen (Korea). Both recombinant phages demonstrating relatively strong binding to the PEDV MAB and the synthetic peptides were tested for inhibition of the MAB's neutralizing activity. The selected MAB was pre-treated with four-fold serially diluted recombinant phages or various concentrations of the synthetic peptides at 37 °C for 10 min then mixed with pre-titrated KPEDV-9 (50 pfu) for 30 min. The mixture was added to 35-mm diameter TC dishes containing Vero cell monolayer and incubated at 37 °C for 1 h similar to plaque assay method described previously. Percent inhibition of recombinant phages or synthetic peptides to the neutralizing activity of the PEDV MAB was compared.

Acknowledgments

This research work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD No. R05-2003-000-11395-0).

We would like to thank the National Veterinary Research and Quarantine Service with special mention to Dr. Chang Hee Kweon for providing the cell-adapted PEDV (KPEDV-9 strain) as well as the 2C10 monoclonal antibody and Bioleaders Corp., Inc., Korea, for the monoclonal antibodies 2D11, 2E4, 3H8 used in this study.

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