Virology 509 (2017) 185–194

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

The S2 glycoprotein subunit of porcine epidemic diarrhea virus contains immunodominant neutralizing epitopes

Faten A. Okda[a,b], Steven Lawson[a], Aaron Singrey[a], Julie Nelson[a], Kyle S. Hain[a], Lok R. Joshi[a], Jane Christopher-Hennings[a,c], Eric A. Nelson[a,c], Diego G. Diel[a,c,*]

[a] Animal Disease Research and Diagnostic Laboratory, Department of Veterinary and Biomedical Sciences, South Dakota State University, Brookings, 57007 SD, USA
[b] National Research Center, Giza, Egypt
[c] South Dakota Center for Biologics Research and Commercialization (SD-CBRC), South Dakota State University, Brookings, 57007 SD, USA

ARTICLE INFO

Keywords:
Porcine epidemic diarrhea virus (PEDV)
Spike
Neutralizing antibodies

ABSTRACT

The porcine epidemic diarrhea virus (PEDV) spike (S) protein is the major target of neutralizing antibodies against PEDV. Here immunodominant neutralizing epitopes of PEDV were identified using a panel of S-specific monoclonal antibodies (mAbs). Ten of eleven S-specific mAbs successfully neutralized PEDV infectivity *in vitro*. Notably, epitope mapping by peptide ELISAs revealed that nine of these mAbs recognized linear neutralizing epitopes located in the N-terminus of the S2 glycoprotein subunit (amino acids [aa] 744–759, 747–774 and/or 756–771). Additionally, one mAb recognized a neutralizing epitope located in the C-terminus of S2 (aa 1371–1377), while only one neutralizing mAb reacted against a region of the S1 glycoprotein subunit (aa 499–600). Notably, mAbs that recognized epitopes within the S2 subunit presented the highest neutralizing activity against PEDV. Together these results indicate that the S2 glycoprotein subunit contains major antigenic determinants and, perhaps, the immunodominant neutralizing epitopes of PEDV.

1. Introduction

Porcine epidemic diarrhea virus (PEDV) is a member of the genus *Alphacoronavirus* of the family *Coronaviridae*. The virus replicates primarily in enterocytes of the small intestine causing villous atrophy and malabsorptive diarrhea which lead to electrolyte imbalance, metabolic acidosis and death (Alvarez et al., 2015; Annamalai et al., 2015). The characteristic clinical signs of PEDV infection are watery diarrhea, vomiting, anorexia, and dehydration which are followed by high mortality rates (50–100%) in suckling piglets or weight loss due to diarrhea in older pigs (Pensaert and Martelli, 2016).

The PEDV genome consists of a large (~28 Kb) single-stranded, positive sense RNA molecule which contains seven open reading frames (ORF1ab, and ORFs2-6) (Huang et al., 2013; Lawrence et al., 2014). It is organized in a central coding region that is flanked by 5′- and 3′-untranslated regions (UTRs). The first gene ORF1ab encompasses approximately 2/3 of the viral genome and encodes large polyproteins (pp1a and pp1b), that are cleaved by viral encoded proteases into 16 non-structural proteins (nsp1-16) (Huang et al., 2013). In addition, four structural proteins are encoded by ORFs2, −4, −5 and −6, including the S glycoprotein (180–220 kDa), membrane (M; 27–32 kDa), envelope (E; 7 kDa), and the nucleocapsid proteins (N; 55–58 kDa), respectively. ORF3 encodes for a non-structural accessory protein (Huang et al., 2013; Lee, 2015; Song and Park, 2012).

The S protein is the major envelope glycoprotein responsible for virus attachment, receptor binding, cell membrane fusion and entry (Cruz et al., 2008; Sun et al., 2008; Wicht et al., 2014). The S protein is expressed as a 1386 amino acid (aa) precursor protein (180–200 kDa) that is cleaved by host proteases into two major subunits: the S1 subunit (residues 20–729) that mediates virus attachment to the cell surface receptor; and the S2 subunit (residues 730–1386) involved in virus and host cell membrane fusion. Like other coronavirus' S proteins, the PEDV S is a type I membrane glycoprotein that forms homotrimeric projections (spikes) on the virion surface and contains an N-terminal signal peptide (residues 1–18), a large extracellular region, a single transmembrane domain (residues 1328–1350) and a short cytoplasmic tail (residues 1351–1386) (Li et al., 2016). The S1 subunit has been shown to have a modular architecture with four discrete domains, including an N-terminal domain (NTD; residues 19–233) that exhibits sialic acid binding activity and a C-terminal domain (CTD; residues 477–629) that can interact with protein receptor(s) (Li

* Corresponding author at: 1155N Campus Drive, Box 2175, Department of Veterinary and Biomedical Sciences, South Dakota State University, Brookings, SD 57007, USA.
E-mail address: diego.diel@sdstate.edu (D.G. Diel).

http://dx.doi.org/10.1016/j.virol.2017.06.013
Received 4 May 2017; Received in revised form 4 June 2017; Accepted 12 June 2017

et al., 2016). The S2 subunit presents the typical structural features found in class I fusion proteins, including a hydrophobic fusion peptide (FP, residues 891–908), two heptad repeat regions (HR1, residues 978–1117 and HR2, residues 1274–1313), and a C-terminal transmembrane domain (residues 1328–1350) (aa positions based on PEDV strain CO13 S sequence, GenBank accession no. KF272920) (Li et al., 2016). The homotrimeric S proteins on the virion surface are uncleaved and undergo proteolytic processing required for fusion during entry (Shirato et al., 2011; Wicht et al., 2014).

Given its critical functions during cell entry, the PEDV S protein is the main target of host neutralizing antibodies against the virus (Song et al., 2016). Indeed, several studies have recently shown that immunization of pigs with full-length or truncated versions of the S protein elicits antibody responses and protection against PEDV (Hain et al., 2016; Makadiya et al., 2016; Oh et al., 2014). Most importantly, at least four neutralizing domains have been identified in the S protein, including: 1) a domain recently mapped to the NTD/S0 region (Li et al., 2017), 2) a domain that is homologous to the collagenase resistant fragment (CO-26K) of TGEV S (Godet et al., 1994), thus being named "collagenase equivalent" (COE) in PEDV (residues 499–638) (Chang et al., 2002), 3) an epitope that has been mapped to the S1D region (residues 636–789) and spans the S1-S2 junction region (Sun et al., 2008), and 4) an epitope mapped to C-terminus of the S protein (residues 1371–1377) (Cruz et al., 2008).

Here we have shown that immunization of BALB/c mice with purified and inactivated whole virus preparations of PEDV followed by somatic cell fusion resulted in hybridoma cell lines secreting mAbs specific for the S protein that exhibited potent neutralizing activity against PEDV *in vitro*. Epitope mapping of the resultant neutralizing mAbs led to the identification of important antigenic determinants within the S protein. Notably, most mAbs obtained here reacted against linear epitopes within the S2 subunit, indicating that this region contains immunodominant neutralizing epitopes of PEDV.

2. Results

2.1. Selection and characterization of PEDV S-specific mAbs

PEDV S-specific mAbs were generated and selected based on the reactivity of the mAbs with full length PEDV S expressed by a recombinant viral vector (ORFV-PEDV-S) (Hain et al., 2016) (Fig. 1). Approximately 25 primary hybridomas secreting S-specific mAbs were obtained and their reactivity was confirmed by immunofluorescence (IFA) and Western blot assays (data not shown). The reactivity of select (n=11) mAbs was assessed by IFA against the prototype PEDV strain USA/Colorado/2013 (Marthaler et al., 2013) and the S-INDEL strain USA/Iowa106/2013 (Oka et al., 2014) (Fig. 1). Additionally, their neutralizing activity was assessed by fluorescent focus neutralization (FFN) assay, with supernatants from nine hybridomas presenting neutralizing titers between 4 and 16 (data not shown). These hybridoma lines were subcloned by limiting dilution (10^{-1} to 10^{-12}) and further characterized in our study. One additional hybridoma cell line (SD33-1; Fig. 1) secreting S-specific mAbs that did not neutralize PEDV and another line previously established at the SD Animal Disease Research and Diagnostic Laboratory (SD37-11) (unpublished data) were also included in our study. Following subcloning one-to-two clones of each primary hybridoma line was selected and subjected to ascites production (Envigo, Inc.). A summary of the properties and characteristics of the mAbs generated here is presented in Table 1.

All mAbs specifically recognized cells infected with the recombinant ORFV-PEDV-S, expressing the full length PEDV S and cells infected with the PEDV strain CO13 (Fig. 1; Table 1). Notably, ten out of eleven mAbs recognized cells infected with the S-INDEL variant PEDV strain USA/IOWA/106/2013, with mAb SD125-2 not recognizing S-INDEL infected cells (Fig. 1; Table 1).

The reactivity of the PEDV-S mAbs was also evaluated by Western blots and ELISAs using whole virus preparations, and recombinant S- (amino acid [aa] 630–800 or aa 499–600) and N (full length) proteins expressed in *E. coli*. On Western blots, ten of eleven mAbs recognized the full-length spike protein in whole PEDV lysates (Fig. 2) and the truncated S protein spanning the S1-S2 junction region (aa 630–800) (Fig. 2). Notably while mAb SD37-11 did not recognize the full-length spike in whole virus lysates, mAb SD125-2 did not react with the S1-S2 recombinant protein (aa 630–800) (Fig. 2). None of the mAbs reacted with the recombinant N protein (Fig. 2). The reactivity of the mAbs with the S protein under denaturing Western blot conditions indicate that they recognize linear epitopes, with most of them being specific for epitopes within the S1-S2 junction region (Fig. 2). When tested by ELISA, all mAbs reacted against PEDV strains CO13 (Fig. 3A) and ten of eleven (except for SD125-2) reacted with S-INDEL strain Iowa106 (Fig. 3A and B). These results corroborate the findings of the IFA assays, confirming the reactivity of the mAbs with PEDV.

Next, we assessed the reactivity of the mAbs against truncated versions of the S protein (aa 499–600 and aa 630–800). Notably, nine of eleven mAbs reacted against the S1-S2 region (aa 630–800), with mAb SD131-3 also reacting with the product corresponding to aa 499–600, which contains the putative receptor binding domain of the S1 subunit (Fig. 3C and D). These results confirmed the Western blots findings, indicating that most mAbs developed here specifically recognize epitopes located in the S1-S2 junction of the S protein. It is important to note that, while mAb SD125-2 reacted with whole PEDV strain CO13 (Figs. 1, 2 and 3A), it did not recognize the S1- nor the S1-S2 truncated proteins (Fig. 3C and D), suggesting that this mAb may recognize another region of the S protein. Additionally, mAb SD131-3 reacted with S1-S2 protein on Western blot and with the S1 and S1-S2 truncated proteins on ELISAs, suggesting a mixed hybridoma population.

2.2. Neutralizing activity of PEDV S-specific mAbs

The neutralizing activity of S-specific mAbs was assessed by FFN and plaque reduction neutralization (PRN) assays. For this, all mAbs were purified from ascites fluid and diluted to a working concentration of 1.5 mg/mL (in PBS). Two-fold serial dilutions of the mAbs' working stocks (1:20 – 1:2560) were tested in triplicate by FFN or PRN assays. Endpoint titers were considered the reciprocal of the highest mAb dilution capable of reducing PEDV infectivity by 90 (FFN) or 80% (PRN) *in vitro*. Ten out of eleven mAbs presented neutralizing titers ranging from 1:40 to 1:640 against PEDV strain CO13 (Table 2, Fig. 4) and from 1:40 to 1:160 against PEDV S-INDEL variant strain (Table 2). Similarly, PRN titers against PEDV strain CO13 ranged between 1:40 to 1:640 (Table 2, Fig. 4). Consistent with the results of our preliminary screening, no neutralizing activity was observed for PEDV S-specific mAb SD33-1 (Fig. 4). These results demonstrate potent neutralizing activity of ten S-specific mAbs against PEDV *in vitro*.

2.3. Epitope mapping of S-specific mAbs

To identify neutralizing domains in the PEDV S, the epitope specificity of the neutralizing mAbs developed here was assessed by peptide ELISAs. Four regions of PEDV S protein have been shown to be the targets of neutralizing antibodies, including the NTD/S0 region, the RBD (aa 499–600), the S1-S2 junction (aa 639–789) and a region in the carboxi terminus of the S protein (Chang et al., 2002; Li et al., 2017; Ostrowski et al., 2002; Sun et al., 2007). A few linear epitopes within these regions have been shown to induce neutralizing antibodies against PEDV, including epitopes at aa positions 747–774 and 1371–1377 (Cruz et al., 2006, 2008; Sun et al., 2007). Since most mAbs developed here recognized the S1-S2 (aa 630–800) recombinant protein in Western blot and/or ELISAs, they were initially screened against epitope 747–774 located in the N-terminus of S2 (Fig. 5A).