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The heptide repeat 2 and upstream region of TGEV induces potent cross-neutralizing antibodies against group I coronaviruses

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SUMMARY

The coronavirus heptide repeat (HR) region in the spike protein induces neutralizing antibodies that block the post-fusion core formation and inhibit virus entry into target cells. The HR2 regions for coronaviruses of the same serogroup share high homology.

We found that polyclonal antibodies derived from transmissible gastroenteritis coronavirus HR2 and upstream region were cross-reactive with the S proteins of the same serogroup in western blotting. The polyclonal antibodies also potently cross-neutralized viruses from the same serogroup. This study provides new insight for designing vaccine and therapeutic reagents against coronavirus infections.

KEY WORDS: Coronaviruses, Heptide repeat 2, Polyclonal antibodies, Cross-neutralization.

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Coronaviruses are enveloped RNA viruses that infect and cause disease in a broad array of avian and mammal species (Graham and Baric, 2010). The global outbreak of severe acute respiratory syndrome coronavirus (SARS-CoV) in 2002/2003 resulted in more than 8000 cases, with a fatality rate of about 10% (He *et al.*, 2006). The coronaviruses are classified in three groups on the basis of serological and genetic properties. Groups I and II comprise coronaviruses that infect mammalian species, including humans. Group III coronaviruses infect only avian species.

Coronavirus S protein belongs to class I viral fusion protein and plays an important role in virus entry into host cells (Bosch *et al.*, 2003). It binds to the cellular receptor and mediates fusion between viruses and the cell membrane through interaction of two heptide repeat (HR) fragments (Eckert and Kim, 2000). Studies on SARS-CoV, HCoV-NL63 and mouse hepatitis virus (MHV) have shown that the HR1 or HR2 peptides effectively block virus entry into susceptible cells (Bosch *et al.*, 2004; Liu *et al.*, 2004; Pyrc *et al.*, 2006; Taguchi and Shimazaki, 2000; Yuan *et al.*, 2004). High titers of antibodies are elicited by the HR region for MHV and SARS-CoV and neutralize viral infection *in vitro* (Keng *et al.*, 2005; Lip *et al.*, 2006; Taguchi and Shimazaki, 2000). The HR region in coronavirus spike (S) glycoprotein is a promising therapeutic target (Keng *et al.*, 2005; Lip *et al.*, 2006).

In influenza A virus, HA2-specific antibodies demonstrate broad cross-reactivity among different subtypes (Varecková *et al.*, 2002, 2003). Monoclonal antibody C179 recognizes a conformational epitope in HA protein and cross-neutralizes both H1 and H2 viruses (Okuno *et al.*, 1993). However, no information is available for the cross-reactivity and cross-neutralizing activity of the coronavirus anti-S antibodies.

We compared the amino acid sequence alignment in the HR2 and upstream region for group I, II and III coronaviruses. High homology was found

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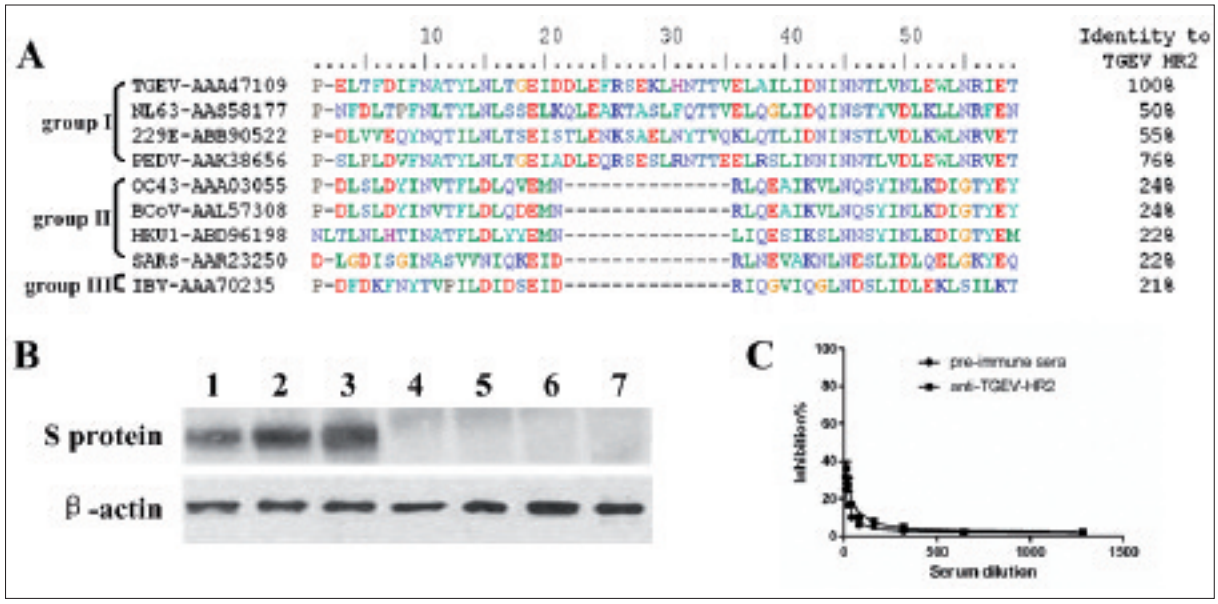

FIGURE 1 - *Epitope homolog, cross-reactivity and cross-neutralizing activity of anti-TGEV-HR2 polyclonal antibodies. A, amino acid sequence homolog of TGEV HR2 and upstream region to other coronaviruses belonging to serogroup I, II and III, respectively. The identity with TGEV HR2 and upstream region are indicated. B, detection of coronavirus S proteins from viruses infected or plasmid transfected cell lysates. Lanes 1-5, coronavirus 229E-, NL63-, TGEV-, OC43- and BCoV-infected cell lysates; lane 6, SARS protein-S-transfected cells; lane 7, mixture of MRC-5, LLC-MK2, ST, RD and 293T cell lysates. The S proteins detected were ~200 kDa according to the protein markers. The internal control protein β-actin was used for harmonization of cell protein loads. C, inhibition of SARS-CoV pseudoparticles by serially diluted anti-TGEV-HR2 polyclonal antibodies, preimmune sera were used as a control. Error bars represent standard deviation of triplicate wells in three independent experiments.*

for sequences from the same serogroup (Figure 1A). The group I coronaviruses shared 50–76% identity with transmissible gastroenteritis coronavirus (TGEV) HR2 sequence. Therefore, we cloned the HR2 and upstream region of TGEV, as indicated in Figure 1, and purified the *Escherichia-coli*-expressed recombinant protein for immunization of Balb/c mice. After five injections with Freund's adjuvant at 15-day intervals, the average antibody titer in the serum reached 1:20 000 in ELISA against the purified HR2 fragment. These antibodies were used in the following experiments.

To investigate whether these anti-TGEV-HR2 antibodies were cross-reactive with other S proteins of the same or different serogroups, we performed western blotting on cells infected with group I coronaviruses 229E, NL63 or TGEV, group II coronaviruses bovine coronavirus (BCoV) or OC43, or transfected with plasmid coding for Flag-tagged S protein of SARS-CoV. The infection dose was MOI 50 for each virus. Cells were collected until an obvious cytopathic effect appeared. Expression of SARS S protein was confirmed by anti-Flag antibodies (data not shown). Anti-TGEV-HR2 antibodies steadily detected a band of ~200 kDa in all cell lysates infected by group I coronaviruses 229E, NL63 and TGEV, which corresponded to the glycosylated full length S protein. This specific band was not detected in cells infected by group II coronaviruses BCoV or OC43 or cells transfected by SARS S protein. By contrast, this band was not detected in control cell lysates. Like influenza virus HA2 antibodies, antibodies against coronavirus HR2 region were cross-reactive with the S protein of other coronaviruses in the same serogroup (Figure 1B).

We further investigated whether the anti-TGEV-HR2 antibodies neutralized TGEV and other coronaviruses from the same or different serogroups. The neutralization assay was performed by standard $TCID_{50}$ assay. Antibodies were preincubated with 50 $TCID_{50}$ viruses before

TABLE 1 - *ND50 values of anti-TGEV-HR2 antibodies against group I coronaviruses TGEV, NL63 or 229E, and group II coronaviruses BCoV or OC43. Preimmune sera were used as a control.*

ND_{50}	*TGEV*	*NL63*	*229E*	*BCoV*	*OC43*
anti-TGEV-HR2	1:640	1:320	1:320	<1:10	<1:10
Pre-immune sera	<1:10	<1:10	<1:10	<1:10	<1:10

infection of cells in a 96-well-plate. The cytopathic effect was observed 3 days later for TGEV and 229E, 5 days later for BCoV and OC43, and 7 days later for NL63. The inhibition titer was calculated by Reed-Muench method (Reed and Muench, 1938). As shown in Table 1, the anti-TGEV-HR2 antibodies potently neutralized TGEV infection in ST cells at an ND_{50} titer of 1/640, which is similar to neutralization of SARS-CoV by anti-SARS-HR2 antibodies (Keng *et al.*, 2005). We also found that the anti-TGEV-HR2 antibodies potently neutralized other two group I coronaviruses 229E and NL63 at ND_{50} titers of 1/320. However, no apparent neutralization effect was observed against OC43 and BCoV. As controls, no neutralization effect was observed for preimmune sera against each virus. Inhibition of SARS-CoV pseudoparticles was also determined by measuring the luciferase reporter activities (Yang *et al.*, 2004). Compared to preimmune sera, no obvious inhibition effect was observed for anti-TGEV-HR2 antibodies against SARS pseudovirus entry into Vero E6 cells.

In summary, our study demonstrated that the anti-TGEV-HR2 antibodies cross-reacted and cross-neutralized other group I coronaviruses. The cross-reactivity profile in western blotting corresponded to the cross-neutralization profile, suggesting that linear epitopes in HR2 and its upstream region may be involved in the fusion process. Monoclonal antibodies that cross-neutralize viruses of the same serogroup will be identified in future studies. The coronavirus HR2 region is moderately immunogenic, and the immunogenicity should be optimized when using the HR2 fragment as an immunogen. These results may provide important information for designing vaccine and therapeutic reagents against existing and new emerging coronavirus infections.

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