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High-titred neutralizing antibodies to human enterovirus 71 preferentially bind to the N-terminal portion of the capsid protein VP1

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Summary

Human enterovirus 71 has emerged as an important pathogen of children in the Asia Pacific region, and it may be important to consider the development of a vaccine against this virus. Human cord serum was used as a source of neutralizing antibodies to determine whether the N- or C-terminal half of the VP1 capsid protein was more likely to harbour neutralizing determinants. Cord sera from 205 individuals were tested for neutralizing antibodies against human enterovirus 71 in an indirect ELISA against recombinant VP1 antigen as well as the N- and C-terminal portions of VP1 antigen. High-titred human neutralizing antibodies were significantly more reactive with the N-terminal half of VP1 than weak or negative sera. The N-terminal half of human enterovirus 71 is likely to have important neutralizing antibody determinants and should be investigated further in vaccine development efforts.

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Introduction

Human enterovirus 71 (HEV71) is recognized as an emerging virus in the Asia Pacific region, having been implicated since 1997 in a number of large outbreaks of hand, foot and mouth disease (HFMD) associated with severe neurological disease [5]. The molecular epidemiology of recent HEV71 strains has been described by several groups, and all data point to a number of different genogroups and subgenogroups co-circulating in the region [1]. Due to the observation of continued HEV71 transmission in many countries, including, Malaysia, Singapore, Taiwan and Japan [6], it is important to study aspects of the neutralization of HEV71 and to investigate the antigenic determinants associated with neutralizing antibody responses in order to guide vaccine design efforts, as it is known that neutralizing antibodies confer protection against HEV71 [10].

This study utilizes cord blood collected in Sarawak in 1999 as a source of human serum with different titres of neutralizing antibodies against a local strain of HEV71. We have produced recombinant fusion proteins of the complete VP1 capsid protein as well as the N- and C-terminal halves of the VP1 capsid protein, and we have used these in an indirect enzyme-linked immunosorbent assay

(ELISA) to determine if neutralizing antibodies against HEV71 preferentially bind to one of the truncated capsid proteins.

Materials and methods

Cloning of VP1 and expression and purification of VP1 protein

The source virus was a strain of HEV71 isolated from the throat swab of a child who succumbed to brainstem encephalitis in Sarawak, Malaysia, in 1997 [4]. The complete VP1 gene was amplified using the primer pair VP1-1: 5′ATTTTACAGACAGGATCCATTCAGGGGGAT3′ and VP1-2: 5′AGATTGCTGACCAAGCTTTTAAAGGGTAGT-3′, which contain the engineered restriction sites for *Bam*HI and *Hin*dIII, respectively. The primers anneal to positions 2418–2447 and 3353–3324 with respect to the complete sequence of HEV71 strain MS/7423/87, GenBank accession number U22522). This amplified product was cloned into the vector pCR-Blunt (Novagen), and plasmids with inserts were sequenced to verify the identity of the gene inserted. A pCR-Blunt-VP1 plasmid was henceforth used as the source of the VP1 gene in subsequent plasmid construction.

Three different constructs were prepared with coding sequences for the complete VP1 capsid protein (891 nucleotides), the N-terminal half (405 nucleotides), and the C-terminal half (492 nucleotides) inserted into the multicloning site of the pET30a expression vector, using the *Bam*HI and *Hin*dIII restriction sites engineered into the pCR-Blunt-VP1 construct. The truncated versions of VP1 were obtained by using the naturally occurring *Eco*RI site in VP1.

Protein was overexpressed in *E. coli* BL21 (DE3) and purified by nickel-chelate chromatography as described in the manufacturer's instructions (pET System Manual: Novagen, Madison, WI, USA). All expressed products were found in inclusion bodies and were solubilized in 6 M urea before subjecting them to column chromatography. Refolding was attempted following elution from the column using standard methods described in the manufacturer's instructions.

Serum specimens

Sera from cord blood collected in 1999 were coded and used as a source of human antibodies against HEV71. Due to a large outbreak of HEV71 in Sarawak in 1997, it was expected that a large proportion of these sera would be from individuals exposed to HEV71. In this study 205 sera were utilized from one township that was affected in 1997.

Plaque-reduction neutralization test ($PRNT_{50}$)

This was carried out in 24-well tissue culture plates using Vero cell monolayers under a carboxymethylcellulose overlay. Equal volumes of virus dilution and heat-inactivated serum were mixed directly in duplicate wells of a 24-well plate and incubated at 37 °C for 60 min before addition of 0.5 ml of a Vero cell suspension at a density of 4×10^5 cells per ml. When the cells had adhered after 3–4 h at 37 °C, the carboxymethylcellulose overlay was added to a final concentration of 1%. The plates were incubated at 37 °C for 3 days before staining with naphthalene black to visualize plaques. Serum neutralization titres are expressed as the reciprocal of the endpoint dilution demonstrating a 50% reduction in plaques.

Indirect ELISA for VP1 and truncations

Flat-bottomed 96-well plates (Maxisorp, Nalge Nunc International, Naperville, IL, USA) were coated with 0.1 μg per well of recombinant fusion protein. A molar equivalent of the fusion moiety, S.Tag, was used as a specificity control. Sera were tested at a dilution 1:100 by incubating in coated wells for 60 min at room temperature following by washing 6 times with PBS containing 0.5% Tween 20. Bound antibody was detected using an anti-human IgG conjugated with horseradish peroxidase (Dako, Glostrup, Denmark) at the manufacturer's recommended dilution, with an incubation time of 60 min at room temperature. After washing as before, colour development was achieved by incubation for 30 min using o-phenylenediamine (OPD) and hydrogen peroxide, and stopping with acid. The optical density (OD) was read in an ELISA reader using dual wavelength mode at 492 nm and reference wavelength of 620 nm. Adjusted ODs were calculated by subtracting the OD of the specificity control from the OD of the VP1 antigens.

Statistical methods

The statistical package Prism 4 ver 4.0c (Graphpad Software, Inc.) was used to analyse data and to generate the figures.

Results

$PRNT_{50}$ titres of serum from cord blood

A large outbreak of HEV71 hand, foot and mouth disease happened in Sarawak, Malaysia, from March through August 1997. We collected cord serum from 205 individuals born in 1999 in Sarawak, Malaysia, expecting that a proportion of their mothers would have been exposed to HEV71 in 1997 and would have developed a neutralizing antibody response to the virus. Neutralizing antibody levels were undetectable in 46% of the cord

blood, while the remaining sera had plaque reduction neutralization test ($PRNT_{50}$) endpoint titres ranging from 1:10 to 1:640 (see Table 1).

Table 1. Summary of $PRNT_{50}$ results from cord blood

Reciprocal $PRNT_{50}$ titre	Number of sera	% of total
<10	94	45.9
10	35	17.1
40	41	20.0
160	26	12.7
640	9	4.4

Antibodies directed against the complete VP1 and truncated VP1 proteins by indirect ELISA

The scatterplots shown in Fig. 1 illustrate the range of optical density (OD) readings obtained for the 205 sera tested. The data show that there was no linear relationship ($r^2 = 0.03886$) between the OD readings obtained by testing against the complete VP1 protein and the C-terminal half of VP1 protein. When the OD readings obtained against the complete VP1 were compared to those obtained with the N-terminal construct, there was a better correlation, with an r^2 of 0.6772, and OD readings

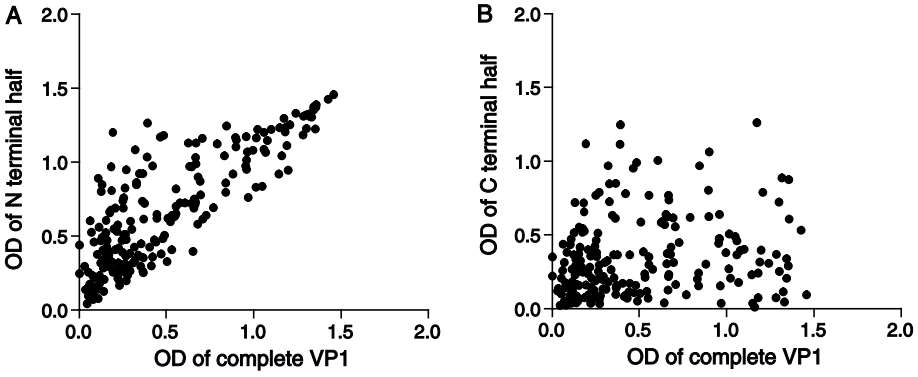

Fig. 1. Scatterplot showing adjusted optical densities (ODs) of antibody responses to the N- and C-terminal halves of the capsid protein VP1. **A** OD readings against N-terminal VP1 protein on the Y-axis compared to the complete VP1 protein on the X-axis. The r^2 is 0.6772. **B** OD readings against C-terminal VP1 protein on the Y-axis compared to the complete VP1 protein on the X-axis. The r^2 is 0.03886

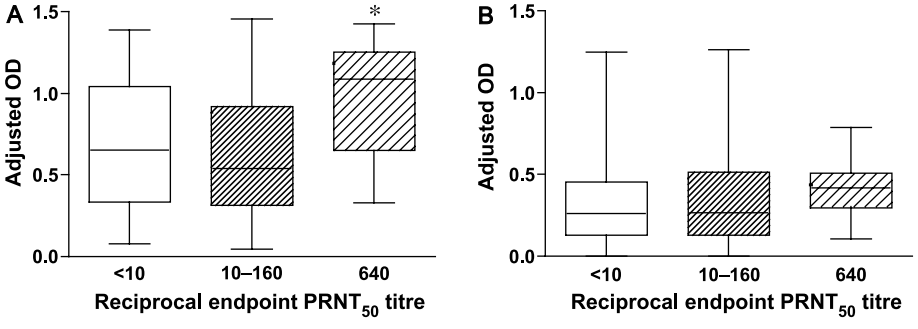

Fig. 2. Boxplot showing the reactivities of different categories of neutralizing antibodies against the N- and C-terminal halves of the capsid protein VP1. **A** Comparison of negative, weak and strongly neutralizing antibodies in an indirect ELISA using the N-terminal VP1 protein. **B** Comparison of negative, weak and strongly neutralizing antibodies in an indirect ELISA using the C-terminal VP1 protein. The asterisk (*) denotes statistically significant difference. In both panels, the boxes denote the interquartile range, and the line in the box denotes the median value. The short horizontal lines across the top and bottom of each plot denotes the highest and lowest value (the range). The boxplots were generated using the statistics software package Prism

Table 2. Unpaired *t*-test comparing different categories of neutralizing sera

$PRNT_{50}$ category	N-terminal VP1	C-terminal VP1
<10 vs. 10–160	$P = 0.3096$	$P = 0.6242$
10–160 vs. 640	$P = 0.0110$	$P = 0.4295$
<10 vs. 640	$P = 0.0300$	$P = 0.3225$

tended to be higher against the N-terminal construct than against the complete VP1 protein.

Comparison of $PRNT_{50}$ titres and OD readings of the N- and C-terminal VP1 proteins

The 205 sera were stratified into 3 groups based on $PRNT_{50}$ titres, with a negative group (<1:10), a weakly neutralizing group (1:10–1:160), and a strongly neutralizing group (1:640). A comparison of the OD readings of the ELISAs performed with the N- and C-terminal constructs showed that the strongly neutralizing group (1:640) was significantly more reactive than the negative or weakly neutralizing groups in the N-terminal VP1 ELISA (Fig. 2A), but there was no significant difference in the reactions of any of the groups in the C-terminal VP1 ELISA (Fig. 2B). Table 2 shows the results obtained using an unpaired *t*-test comparing the different neutralization categories.

Discussion

Natural infection by enteroviruses leads to the generation of a protective antibody response characterized by the presence of neutralizing antibodies. Knowledge of where neutralization determinants are located on the virus can be useful in making decisions about vaccine design, and thus studies attempting to locate these determinants can provide useful guidance. Some studies on neutralization epitopes of specific enteroviruses, for example, swine vesicular disease virus (SVDV), have shown that these determinants can be found on VP1, VP2, VP3 as well as VP4 [3]. However, neutralization epitopes on the VP1 protein have been the best characterized for poliovirus [8], SVDV [2] and Coxsackievirus A9 [7]. Furthermore, studies on the protective response against HEV71 in mice have provided evidence that HEV71 VP1 capsid protein generates neutralizing antibodies associated with protection against challenge [9, 10].

In this study, we have expressed HEV71 VP1 as a complete capsid protein as well as 2 non-overlapping fragments of VP1. Human cord sera with high titres of neutralizing antibodies against HEV71 were found to react strongly with the N-terminal fragment of the VP1 protein. Thus, in searching for neutralizing epitopes of HEV71 it would be useful to zoom in on this portion of the VP1 gene product. We believe that the information generated in this study may be useful in dissecting the antigenic determinants important in the protective antibody response after a natural infection.

Acknowledgements

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