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RESEARCH NOTE

Identification of immunodominant VP1 linear epitope of enterovirus 71 (EV71) using synthetic peptides for detecting human anti-EV71 IgG antibodies in western blots

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ABSTRACT

A major IgG-specific immunodominant VP1 linear epitope of enterovirus 71 (EV71) strain 41 (5865/SIN/00009), defined by the core sequence LEGTTNPNG, was identified by Pepscan analysis. Oligonucleotides corresponding to the amino-acid sequence of synthetic peptide SP32 were cloned and over-expressed in Escherichia coli as a recombinant glutathione-S-transferase (GST)–SP32 fusion protein. In ELISAs, this protein did not react with human anti-EV71 IgG antibodies, but there was significant immunoreactivity according to western blot analysis. The amino-acid sequence of SP32 was highly specific for detecting EV71 strains in western blot analysis, and showed no immunoreactivity with monoclonal antibodies raised against other enteroviruses, e.g., CA9 and Echo 6.

Keywords Detection, enterovirus 71, immunodominant linear epitope, pepscan analysis, recombinant GST–SP32 fusion protein, synthetic peptide

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Enterovirus 71 (EV71) is a single positive-stranded RNA virus [1], with infection often manifesting as hand, foot and mouth disease [2], characterised by ulcerating vesicles on the palate and vesicular lesions on the hands, feet and buttocks. The usual method for detecting EV71 in any clinical specimen involves viral isolation, followed by serotyping with serotype-specific antibodies and indirect immunofluorescence assays. An IgM-based ELISA for EV71 diagnosis has been reported to have high sensitivity and specificity. However, this approach is too laborious, as it involves the use of purified whole EV71 virions for detecting anti-EV71 antibodies [3–5]. Synthetic peptides corresponding to the VP1 protein of enteroviruses are broadly reactive in an IgG-based ELISA using sera from patients infected with enteroviruses, and this approach has the potential for serodiagnosis of a wide range of enterovirus serotypes [6]. To detect an enterovirus of unknown serotype, an assay with broad reactivity would be useful, but this would not help to identify a specific enterovirus.

In the present study, 95 overlapping synthetic peptides, designated SP1–SP95, that spanned the entire VP1 sequence of EV71 strain 41 (SIN/00009) (GenBank accession no. AF316321) were synthesised by Mimotopes (Clayton, Australia). EV71 was propagated in RD cells using minimum essential medium (Gibco, Rockville, MD, USA), based on cytopathic effects produced as a result of virus infection. Serum samples were collected from children attending a paediatric outpatient clinic at the National University Hospital of Singapore, and these were screened for the presence of anti-EV71-neutralising antibodies using an in-vitro microneutralisation assay. A serum-neutralising titre of ≥1:8 was considered to be positive for EV71 exposure. Sera containing anti-EV71-neutralising antibodies \( (n = 40) \) were pooled and designated as EV71-positive sera. Sera without anti-EV71-neutralising antibodies \( (n = 40) \) were pooled and designated as EV71-negative sera. For assigning linear epitope(s) in ELISAs, the mean OD obtained from EV71-negative sera plus twice the standard deviation of the respective mean was used as the cut-off value.

Two IgG-specific immunoreactive VP1 linear regions were identified, designated as sites I and II. The first region, represented by synthetic peptides SP14 and SP15 (amino-acid residues 40–57), was not specific for detecting human anti-EV71 IgG, as both peptides also reacted with EV71-negative sera, perhaps because of the presence of non-specific IgG elicited previously against other enteroviruses that might harbour similar antigenic determinants. The second immunoreactive region was represented by synthetic peptides SP31, SP32 and SP33 (amino-acid residues 91–111), and these were highly specific for detecting human anti-EV71 IgG. This region, defined by the core sequence LEGTTPNG, represents the major immunodominant VP1 linear epitope of EV71. When SP32 (DLPLEGTTPNGYAN) was tested against EV71-positive sera, there was significant immunoreactivity towards SP32, which contained the core sequence.

The VP1 protein of the prototype EV71 BrCr strain (GenBank accession no. U00871), when produced as a recombinant His–VP1 fusion protein, was highly insoluble and therefore required denaturation and renaturation before it could be used as a capture antigen in western blots [7]. This insolubility could be attributed to several hydrophobic transmembrane regions within VP1. When a smaller peptide without these regions was used, the immunodominant VP1 linear epitope of EV71. When a smaller peptide without these regions was used, the immunodominant VP1 linear epitope represented by synthetic peptides SP32 was revealed to be highly homologous to EV71 strains, indicating its probable high sensitivity as a capture antigen for detecting human anti-EV71 IgG. Hence, a pair of positive- and negative-sense oligonucleotides was designed, based on the nucleotide sequence of SP32 (nucleotides 280–324 of the VP1 gene). For annealing, both oligonucleotides were heated at 90°C for 3 min, and then incubated at 37°C for 15 min. Primers flanking the VP1 gene (nucleotides 2442–3352 of the complete genome of EV71 strain 41) were also constructed: GST–VP1F, 5′-TGATCCGCCAGATAGTGCGAG (the BamH1 restriction site is underlined) and GST–VP1R, 5′-GCCGAAGTCGACTCAAAGGG (the SalI restriction site is underlined). The vector pGEX-6p-1 (GE Healthcare Life
Sciences, Little Chalfont, UK) was used to construct recombinant GST–SP32 and GST–VP1 fusion proteins, following the procedure recommended by the manufacturer.

The efficacy of the recombinant GST–SP32 fusion protein as a capture antigen for human anti-EV71 IgG was evaluated in IgG-based ELISA and western blot assays. No significant immunoreactivity was detected by ELISA, but because of its native state, the antigenic site represented by SP32 could have been masked by the larger GST protein moiety, thereby hindering antigen–antibody interactions. However, the fusion protein was immunoreactive against human anti-EV71 IgG in western blots, probably because of its denatured state, in which the steric hindrance caused by the GST protein would have been removed, thereby exposing the immunogenic region represented by SP32. Before being used as a capture antigen, EV71 was heat-inactivated at 56°C and the amount of viral protein was quantified using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Both the recombinant GST–VP1 fusion protein and the whole EV71 virion were non-specific when used as capture antigens in western blots for detecting human anti-EV71 IgG. This was attributed to the presence of numerous common linear epitopes among enteroviruses [6,9–11]. It is believed that the cross-reactivity may be attributed to human anti-poliovirus IgG, present in most paediatric patients as a result of the national childhood immunisation programme.

In conclusion, the recombinant GST–SP32 fusion protein, harbouring the immunodominant VP1 linear epitope of EV71, demonstrated high specificity and sensitivity, and was a better capture antigen in western blots than the recombinant GST–VP1 fusion protein and the whole EV71 virion. This suggests that the recombinant GST–SP32 fusion protein could be further developed as a serological reagent for the detection of human anti-EV71 IgG, and could be used as a tool for identifying EV71 infection and as a confirmatory assay for recognising previous EV71 exposure.

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