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Synthetic peptides for efficient discrimination of anti-enterovirus antibodies at the serotype level

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ABSTRACT

Enteroviruses are important human pathogens, causing a broad spectrum of diseases from minor common colds to fatal myocarditis. However, certain disease syndromes are caused by one or few serotypes. Serotype identification is difficult due to the laborious neutralization tests that lack of sensitivity, while in commercial ELISAs homotypic antibodies' activities are largely masked by the recognition of genera-specific epitopes by heterotypic antibodies. In the present study homotypic assays were developed with the ability to discriminate different enterovirus serotypes. Seventy-three children sera, positive for IgM antibodies against enterovirus genus and 49 healthy children were examined for the presence of antibodies against 14 synthetic peptides derived from a non-conserved region of the VP1 protein of coxsackieviruses B2, B3, B4, B5, A9, A16, A24, echoviruses 6, 7, 9, 11, 30, enterovirus 71 and parechovirus 1. 50% of the anti-enterovirus IgM positive sera (>150 BU) reacted with the peptides with the majority of them to preferentially recognize one of them, supporting the homotypic nature of our assay. Inhibition studies yielded homologous inhibition rates 67–95% suggesting that specific peptide recognition actually occurred. The diagnostic value of our assay was tested in blood samples drawn over a 1.5-year period from a 5-year old patient. The anti-enterovirus reactivity was clearly attributed to echovirus serotype 11. The IgM/IgG antibody ratio was reversed 4 months later and subsequently IgM antibodies dropped below the cutoff point. In this paper we demonstrate that our assay can be used to discriminate between antibodies targeting different enterovirus serotypes.

Introduction

Human enteroviruses are ubiquitous viruses found throughout the world [28]. The non-polio enteroviruses include group A and B coxsackieviruses, echoviruses and "newer" enteroviruses. Coxsackieviruses are divided based on their antigenic differences in two serogroups, A (CVA) and B (CVB), comprising 23 and 6 serotypes, respectively, while echoviruses include 32 serotypes and "newer" enteroviruses with 5 serotypes [1,28,39]. The non-polio enteroviruses are responsible for a wide spectrum of disease in humans of all ages. The majority of non-polio enteroviruses infections are asymptomatic [39,41] but in some cases, can lead to severe diseases, such as aseptic meningitis [6], pancreatitis [15,24], hepatitis [9,17,38] and myocarditis [18]. Although the disease spectra of different serotypes overlap considerably, some syndromes are caused by one or a few serotypes [27]. CVB3 is responsible for 63% of myocarditis cases and may lead to dilated cardiomyopathy [7,13,19]. CVB4 and CVB5 are associated with many cases of central neural diseases, while CVA24 has been associated with respiratory illness and acute hemorrhagic conjunctivitis. CVA16 is responsible for most cases of hand, foot and mouth disease (HFMD) [10]. Additionally, CVB4 has been implicated in the development type 1 diabetes and pancreatitis [12,16].

Laboratory diagnosis of enteroviral infection is laborious as it involves isolation and characterization of the causative virus. However, some enterovirus serotypes particularly in the coxsackievirus A group, do not grow in cell cultures [22]. In addition, the opportunity to isolate a virus from a clinical specimen in cell culture is low. Alternatively, diagnosis can be performed by the detection of neutralizing IgM antibodies that inhibit the cytopathic effect caused by the virus [27,33]. Despite its wide use, the serum neutralization test is relatively insensitive and has several limitations since it requires the use of live virus and cell cultures (if it is a

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serotype that grows in cells) and is time-consuming. Molecular methods such as polymerase chain reaction (e.g. nested PCR) are also used for the detection of viral ribonucleic acid [8]. Although these tests possess increased sensitivities in CSF and blood specimens, their utility is limited to the viremia phase of the infection (usually over a few days). Nasopharyngeal secretions and feces have no diagnostic value since they are extremely liable to contamination. Enteroviral infection can also be documented by serological detection of antibodies using ELISA assays. These tests are sensitive, independent of cell cultures and do not require the isolated virus. In addition, they provide diagnostic information within a few hours and the experiments are relatively inexpensive to perform. However, the frequent cross-reactions among antigens from different enteroviruses (e.g. coxsackie, echo, enterovirus, etc.) limit the diagnostic value of ELISA [11,25,26,37]. In addition, the recognition of serotype-specific epitopes by homotypic antibodies is largely "masked" by the recognition of "genera-specific" cross-reactive epitopes by heterotypic antibodies, preventing the identification of specific viral serotypes.

In this study, we develop a homotypic immunosorbent assay (ELISA) which can discriminate between different enteroviruses and human parechoviruses serotypes. This assay is based on selected peptide epitopes from the non-conserved regions of VP1.

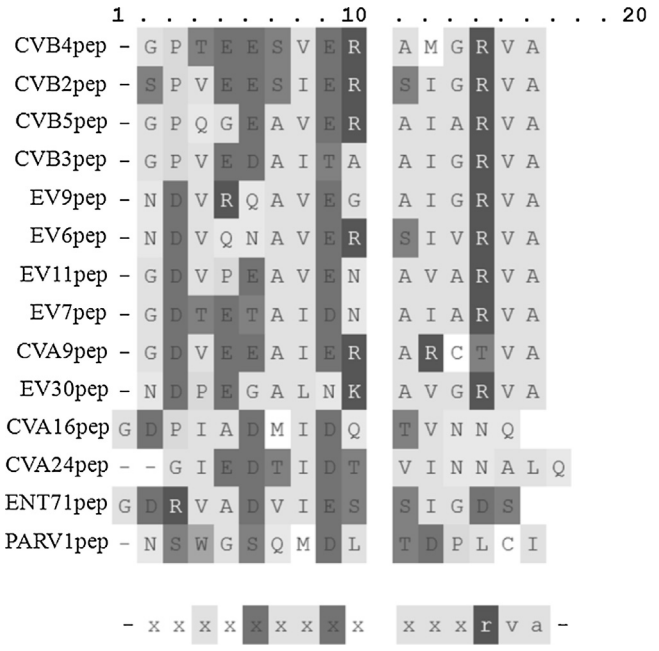

Fig. 1. Homology of amino acid sequences of synthetic peptides derived from VP1 protein from the viruses *Coxsackie B4* (CVB4pep), *Coxsackie B2* (CVB2pep), *Coxsackie B5* (CVB5pep), *Coxsackie B3* (CVB3pep), *Echo 9* (EV9pep), *Echo 6* (EV6pep), *Echo 11* (EV11pep), *Echo 7* (EV7pep), *Coxsackie A9* (CVA9pep), *Echo 30* (EV30pep), *Coxsackie A16* (CVA16pep), *Coxsackie A24* (CVA24pep), *Enterovirus 71* (ENT71pep) and *Parecho 1* (PARV1pep). Different gray tones represent differences in sequence similarities.

Materials and methods

Sera

73 child sera, positive for IgM antibodies against enterovirus genus (as assessed by a commercial anti-enterovirus assay, Serion Immunodiagnostica, Wórzburg, Germany) were collected in the Panagiotis & Aglaia Kyriakou Children's Hospital over a period of 2 years, 2011–2012. All children had symptomatic manifestations such as pleurodynia, symptoms of respiratory tract infection, exanthemas, and symptoms of CNS infection (aseptic meningitis). Unfortunately, detailed patient information was not available. 49 sera, negative for IgM antibodies against enterovirus, were obtained from healthy children in the same hospital. All sera were tested for the presence of IgG and IgM antibodies by enzyme-linked immunosorbent assay (ELISA) against synthetic peptides derived from coxsackieviruses B2, B3, B4, B5, A9, A16, A24, echoviruses Ech6, Ech7, Ech9, Ech11, Ech30, enterovirus 71 and parechovirus 1.

Synthetic peptides

Synthetic peptides derived from a non-conserved region of VP1 protein of coxsackieviruses B2, B3, B4, B5, A9, A16, A24, echoviruses 6, 7, 9, 11, 30, enterovirus 71 and parechovirus 1 (previously designated as echovirus 22) were synthesized using automated Fmoc (N-[9 fluorenyl] methoxycarbonyl) solid-phase synthesis [2] (Bio-Synthesis, Lewisville, TX). The peptides were purified by high-pressure liquid chromatography (HPLC) and their identities and purities (>90%) were confirmed by mass spectroscopy (MS).

ELISAs

The reactivity of sera against enterovirus genus was investigated using a commercially available assay for IgM and IgG antibodies (Serion ELISA classic enterovirus; Serion Immunodiagnostica, Wórzburg, Germany). The basis of the antigen preparation used in this assay is a mixture of heat-denatured coxsackievirus type B5 and echovirus 6 virions. This antigen preparation was reported to be widely cross-reactive, facilitating the detection of infections due to other enterovirus species and serotypes [36]. The assay was performed according to the manufacturers and extinction signals were converted to quantitative values of antibody concentrations using 4 parameter logistic-log-model (4 PL) curve fitting.

The evaluation of sera reactivity against synthetic peptides derived from 14 different enterovirus serotypes was performed using a custom ELISA assay. Briefly, high-binding microplates (Costar TM; Corning Life Sciences, Acton, MA) were coated with synthetic peptides in PBS, pH 7.2 at concentrations 2–10 μg/mL (optimal concentration was defined separately for each peptide in preliminary experiments), for 3 h at room temperature (RT). After blocking the free binding sites with 200 μL blocking buffer (2% BSA-PBS) for 1 h at RT, the wells were washed with PBS containing 0.05% Tween 20 (PBS-T). Sera were preincubated for 15 min with RF absorbent (1:100, Serion Immunodiagnostica, Wórzburg, Germany) and thereafter added to the wells at 1:200 dilution factor in blocking buffer and incubated overnight at 4 °C. After three washes with PBS, alkaline phosphatase-conjugated anti-human IgG (Jackson ImmunoResearch, West Grove, PA), diluted 1:1100 in blocking buffer, was added and incubated for a period of 1 h at room temperature. The plates were washed with PBS-T and optical densities (ODs) were quantified at 405 nm with an ELISA reader (Bio-Tek Instruments, Winooski, VT). All ODs were transformed and expressed as binding units according to the following formula:

$$\text{Binding units (BU)} = \left(\frac{\text{OD}_{\text{Sample}}}{\text{OD}_{\text{Cutoff}}}\right) \times 100$$

where ODSample is the OD reading of the current sample and ODCutoff is the cutoff value. The cutoff value for anti-peptide ELISA was calculated as mean normal sera OD plus 5 standard deviations. All normal sera were obtained from healthy individuals and were selected to be negative for IgG and IgM antibodies in Serion classic enterovirus ELISA.