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Serum-derived IgG from coxsackievirus A6-infected patients can enhance the infection of peripheral blood mononuclear cells with coxsackievirus A6

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ABSTRACT

Coxsackievirus A6 (CV-A6) has recently emerged as an enterovirus causing Hand Foot and Mouth Disease with severe complications. The pathogenic mechanisms of CV-A6- associated Hand foot and Mouth disease are largely unknown. In this study, it was investigated whether serum and IgG from patients with CV-A6 infection can enhance the infection of PBMC with the virus. Serum samples were obtained from five children with CV-A6 infection confirmed by RT-PCR and seven controls. IgG was isolated from serum by using affinity chromatography columns. CV-A6 was incubated with serum or IgG from controls and patients then the mixtures were added to PBMC cultures. The levels of IFNα in supernatants were measured by ELISA, and the levels of intracellular viral RNA were measured by RT-qPCR. It has been observed that there is an anti-CV-A6 enhancing activity in serum and serum-derived immunoglobulin G of children with CV-A6 infection but not in those of uninfected controls. Whether this activity has implications in the pathogenesis of CV-A6 associated diseases should be investigated.

1. Introduction

Coxsackievirus A6 (CV-A6), a single-stranded RNA virus, belongs to *Picornaviridae* family (Genus Enterovirus). This enterically transmitted virus has emerged as a pathogen responsible for Hand, foot and mouth disease (HFMD) [1]. HFMD is a common infectious disease in children characterized by fever, vesicular rashes on hand, feet, buttock and ulcers in the oral mucosa. Even though HFMD is a self-limiting disease, a small proportion of children may experience severe complications such as meningitis, encephalitis, acute flaccid paralysis and neuro-respiratory syndrome.

Until 2008, most of the HFMD outbreaks were mainly due to two enterovirus serotypes namely enterovirus 71 (EV-71) and coxsackievirus A16 (CV-A16). However, since 2008 coxsackievirusA6 (CV-A6) has been found associated with epidemics and outbreaks of HFMD in Europe, in Asia-Pacific region and in India [2–5].

Outbreak data revealed that CV-A6 could affect broader demographic regions and different age groups (both children and adults), resulting in most severe forms of disease compared to classic HFMD. Primary multiplication of enteroviruses takes place in the respiratory or gastrointestinal tract, followed by the appearance of the infectious virus in the blood, facilitating further spread to secondary target organs. Though CV-A6 causes different clinical manifestations varying from mild asymptomatic infection to severe encephalitis to date. There has been no study unravelling the pathogenic mechanisms of CV-A6-associated disease.

Serum-dependent enhancement of virus infection was reported in infection by other members of the *Picornaviridae* family such as EV 71, poliovirus, foot-and-mouth disease virus [6]. Our team has identified that coxsackievirus B4 (Genus Enterovirus), can infect monocytes by the antibody-dependent mechanism through interactions between the virus, antiviral antibodies, and specific receptors that result in increased IFN-α production [7,8].

In this study, it was investigated whether serum from CV-A6-infected children can enhance the infection of PBMC with CV-A6.

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2. Materials and methods

Cells: Peripheral blood mononuclear cells (PBMC) were isolated from buffy coat cell pellet provided by The French Blood Establishment (EFS) Lille. Mononuclear cells have been separated on density gradients of Ficoll-Hypaque. Briefly the buffy coat was directly layered on top of Ficoll-Paque™ (GE Healthcare) (ratio 1:1), and centrifuged at 500 g for 30 min at 20 °C. PBMC layer was collected and washed twice with RPMI-1640 medium. The cells were then suspended in RPMI medium supplemented with 10% heat-inactivated fetal calf serum, 1% non-essential amino acid, 1% penicillin-streptomycin and 1% of L-glutamine. The cells were counted, and the cell number was adjusted to 5×10^6 cells/mL. **Vero cells**: (ATCC® CCL81™) were grown in Dulbecco's modified eagle's medium (DMEM; Gibco BRL, Invitrogen, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (FCS, Sigma St Louis, MO, USA), 2–6 mM L-Glutamine (Gibco BRL), 50 mg/mL streptomycin, and 50 IU/mL penicillin (Bio Whittaker).

Virus: CV-A6 isolated from a clinical sample was propagated in Vero cells (ATCC® CCL81™). The infectious titers of culture supernatants were assessed using the end-point dilution assay, and the Reed-Muench statistical method was used to determine the tissue culture 50% infectious dose ($TCID_{50}$). The virus isolate was aliquoted and stored at −80 °C.

Serum samples: Obtained from children with CV-A6 infection (Hand Foot and mouth Disease/encephalitis/meningitis/myocarditis) confirmed by reverse transcriptase - polymerase chain reaction. Serum samples from age-matched control children with clinical symptoms of dengue-like illness or influenza-like illness received at Manipal Centre for Virus Research, Manipal, India were included. The consent of parents was obtained, and the study was approved by Institutional Ethical committee.

Microneutralization Assay: Fifty microliters of sample dilutions (2 fold serial dilutions) and 50 μL of virus stock containing 200 $TCID_{50}$ CV-A6 were mixed and incubated in a microtiter plate with Vero cells at 37 °C for seven days. The cell control and virus control were run simultaneously. The neutralizing antibody titres were expressed as the reciprocal of highest dilution of serum that completely inhibited the cytopathic effect of the virus.

Extraction of IgG from serum: Protein G HP Spintrap (GE healthcare UK limited) was used to isolate IgG from the serum samples. IgG-enriched fraction and IgG-depleted fractions were processed separately.

Serum-dependent enhancement assay: Serum/eluted IgG was diluted with RPMI-1640 media in the ratio 1/10, 1/100 and 1/1000. Twenty-five microliters of medium or diluted serum/eluted IgG was incubated for 2 h at 37 °C with 25 μl of CV-A6 suspension (10^5 $TCID_{50}$). Post-incubation, the mixtures were inoculated into PBMC cultures in microplates (5×10^5 cells/well). After 48 h of incubation, supernatants were collected for INFα detection by ELISA. After collection of the supernatant, the wells containing the cells were washed four times with cold 1× PBS (4 °C) by centrifuging at 1500 rpm for 10 min. The cells were removed easily by adding TRI Reagent® (Sigma- Aldrich), collected in a total volume of 900 μL tri-reagent (Sigma) in 1.5 ml tubes and subjected to RNA extraction.

ELISA (sandwich ELISA): IFN-α was measured by using pan-specific INF-α ELISA kit (Mabtech® Human IFN-α ELISA ^{PRO} kit) according to manufacturer's instructions.

RNA Extraction: The extraction of total RNA was performed using the Tri-reagent method.

Quantitative Real-Time Reverse transcriptase PCR (rRT-PCR): Affinityscript® QPCR cDNA Synthesis (Agilent Technologies, USA) kit was used for the synthesis of cDNA from RNA. The reaction mix, with a total volume of 50 μL, included master mix, water, forward primer at a concentration of 10 mM/L and RT stratastript enzyme mix (Stratagene, USA). The reaction was performed in the Perkin Elmer 2400 thermocycler with the following program: 25 °C for 5 min, 42 °C for 15 min, and 95 °C for 5 min.

Pan enterovirus quantitative PCR was performed with the kit Brilliant® II QPCR (Agilent Technologies, USA) on the Mx3000p® (Stratagene, USA). The reaction mixture, with a total volume of 20 μL, contained 2× Brilliant Master mix, nuclease-free water, probe (2 mM/L), forward primer, reverse primer (each primer concentration 10 mM/L) and cDNA. The amplification program was set for 10 min at 95 °C and then 40 cycles of heating (30 s at 95 °C) and annealing (1 min at 60 °C). The primer sequences targeting the highly conserved 5′nontranslated region of enteroviruses were: forward primer CCC ATG TGA CGG CTA ATC ATT ACC ATA GTC AGC AGC, reverse primer ATT ACC ATA GTC AGC AGC, probe FAM-CGA AAC CTA CTT TCC TGG GTG GTG TTT-ROX. The standard for quantification of RNA was enterovirus 71 (VIRCELL, Granada, Spain) with 5-point range: 1.26×10^4; 1.26×10^3; 126; 12.6 and 1.26 copies/mL.

Statistical analysis: performed with GraphPad Prism® V6.0 version. Mann–Whitney *U* Test was used when appropriate. The *p*-value < 0.05 was considered statistically significant.

3. Result

3.1. Children with CV-A6 infection

Serum was collected from 5 children (less than ten year-old) with CV-A6 infection confirmed by detection of enteroviral RNA by reverse-transcriptase real-time PCR (RT-PCR) in cerebrospinal fluid and/or throat swab and/or vesicle swab. Based on clinical symptoms associated with CV-A6 infection, these patients were further classified as acute encephalitis syndrome (n = 2), meningoencephalitis (n = 1), myocarditis (n = 1) and HFMD (n = 1) (Table 1). The titres of neutralizing anti-CV-A6 antibodies in serum were < 1:4 as displayed by micro neutralization assay. The serum samples were collected after the onset of the disease as soon as one day in the case of patients number 3, and 4–6 days in the case of other patients.

Serum samples were obtained from age-matched controls without symptoms of infection (n = 1) or with symptoms of dengue-like illness (n = 3) or influenza-like illness (n = 3). The titres of neutralizing anti-CVA6 antibodies in serum from 5 subjects were < 1:4 and from 2 others were 1:32 and 1:128 as displayed by micro neutralization assay.

3.2. Serum from children with CV-A6 infection can enhance the CVA6-induced production of INFα by PBMC

The levels of INF-α in supernatants of PBMC culture inoculated with CV-A6 mixed with medium was lower than 20 pg/ml. When CV-A6 was mixed with various dilutions of serum or serum-derived IgG from

Table 1
Demographic and clinical details of Coxsackie virus-A6 infected children (n = 5).

No	Age(Y)	Sex	Clinical symptoms	Syndrome	Outcome
1	6.5	F	Fever, headache, vomiting, Seizure	Acute Encephalitis	Death
2	4	M	Fever,coryza,myalgia and altered sensorium	Acute Encephalitis	Death
3	8	M	Fever and Skin Lesions	Hand Foot Mouth Disease	Recovery
4	2	M	Hepatomegaly, Myocarditis, Vomiting Diarrhea, Mechanical Ventilation	Myocarditis	Recovery
5	4	M	Fever,coryza,myalgia and altered sensorium	Meningoencephalitis	Recovery