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Echovirus 6 strains derived from a clinical isolate show differences in haemagglutination ability and cell entry pathway

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Abstract

Two echovirus 6 (EV6) strains were isolated from a clinical sample after successive sub-cultures in PLC (human hepatocellular carcinoma) and HeLa (human cervical adenocarcinoma) cells. The first strain retained its haemagglutinating capacity (HAEV6) while the second became non-haemagglutinating (NHAEV6). Virus binding assay showed that HAEV6 was capable of binding to DAF-expressing cells but not NHAEV6 confirming the role of DAF in EV6 haemagglutination. The lack of competition between the two viral strains during coinfections suggested that each strain used a different cell entry pathway. We provide evidence showing that HAEV6 used preferentially the lipid raft-dependent caveolae pathway, whereas NHAEV6 followed the clathrin-mediated pathway. Comparison of the sequences of HAEV6 and NHAEV6 revealed five amino acid changes in the VP1, VP2 and VP3 capsid proteins distributed in domains which are known to be highly immunogenic or suggested to be involved in receptor binding, virion stability and pathogenicity.
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1. Introduction

Echovirus 6 (EV6) is a human pathogen of the *Picornaviridae* family and the genus *Enterovirus*, responsible for outbreaks of aseptic meningitis and various infections in neonates and young children (Abe et al., 2000; Chomel et al., 2003; Joo et al., 2005; Ventura et al., 2001). EV6 chronic infections have also been described and thought to be implicated in the development of motor neuron diseases (Berger et al., 2000). The virus has an icosahedral capsid made up of 4 structural proteins (VP1, VP2, VP3 and VP4) with 60 copies each enclosing a single-stranded sense-messenger polyadenylated RNA genome of approximately 7400 nucleotides. Similar to other RNA viruses, enteroviruses have the capacity to evolve rapidly, as the low fidelity viral RNA-dependent RNA polymerase induces mutations during virus replication in both the structural and non-structural proteins (Bailly et al., 2000). An enterovirus population is therefore a collection of similar but genetically different viruses called quasispecies (Piirainen et al., 1998). This viral genomic heterogeneity could be an advantage to the viral population, as it allows for rapid adaptation to environmental changes that present varying types and degrees of selective pressure (Vignuzzi et al., 2005).

In recent years, the early events involved in picornaviruses infections have been intensively studied. Two different mechanisms have been proposed for the viral genome entry into the cytoplasm of host cells. In the first mechanism, similar to polioviruses, the genome delivery into the cytoplasm occurs by direct penetration across the plasma membrane. This process is initiated by the binding of the virus capsid to its cognate cell

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surface receptor, the poliovirus receptor (CD155) (Racaniello, 2001). In the second mechanism, the virus is invaginated into the cell by endocytosis via cellular compartments, such as clathrin-coated vesicles or caveolae (Joki-Korpela et al., 2001; Marjomaki et al., 2002).

Many animal viruses are known to use the receptor-mediated endocytosis pathway for host cell entry. The endocytosis pathway could be either mediated by clathrin lattices or caveolae, which involve specialised cholesterol and sphingolipid domains called lipid rafts (Meier and Greber, 2004). The use of pharmacological inhibitors or specific antibodies against endocytotic components, dominant-negative mutants or small interfering RNAs have been used to characterise the cell entry pathways of some picornaviruses. These studies have identified the clathrin-dependent pathway for human parechovirus 1, human rhinovirus serotype 2 and coxsackievirus B3 and the involvement of lipid rafts for echovirus 1, echovirus 11, coxsackievirus A9 and coxsackievirus B4 (Stuart et al., 2002; Triantafilou and Triantafilou, 2003, 2004). The cell entry pathway of EV6 has thus far, not yet being characterised.

Some EV6 strains have the ability to haemagglutinate human erythrocytes and, this feature is related to the attachment of the virus to the decay accelerating factor (DAF), a 70 kDa glycosylphosphatidylinositol (GPI)-anchored glycoprotein on the cell membrane and which play a role in the regulation of complement activation (Powell et al., 1999). DAF also mediates the cell surface attachment and infection of several echovirus such as the serotypes 6, 7, 13, 21, 29 and 33, which are all haemagglutinating viruses (Powell et al., 1998). Like other glycosylphosphatidylinositol (GPI)-anchored proteins, DAF are found in lipid rafts cholesterol-rich ordered domains within the plasma membrane at the apical surface of the epithelial cells (Coyne and Bergelson, 2006; Simons and Ikonen, 1997).

In this report, we describe the isolation of two cell culture-derived strains from an EV6 clinical isolate, which differed in their haemagglutinating capacity. The cell entry pathways of the haemagglutinating strain (HAEV6) and the non-haemagglutinating strain (NHAEV6) were characterised in Dev cells, a human glial precursor cell line, and in A549 cells, a human lung carcinoma cell line. Sequence determination of the capsid proteins of HAEV6 and NHAEV6 showed five amino acid differences localised in the VP2, VP3 and VP1 proteins. We demonstrate that the strains used different cell entry pathways, involving lipid rafts for HAEV6 and a clathrin-dependent pathway for NHAEV6. The difference in haemagglutination phenotype and the cell entry pathways may be related to the amino acid differences in the capsid proteins of these two viruses. Our results also confirmed that a clinical isolate can be composed of a mixture of quasispecies capable of using different routes to achieve their entry into cells.

2. Materials and methods

2.1. Virus and cells

A clinical EV6 strain was isolated in human lung fibroblasts (MRC5 cell line) from a throat sample obtained from a patient who suffered from a meningitis. PLC cells (human hepatoma cell line, ATCC) and HeLa cells (Human epithelial carcinoma cell line, ATCC) used for viral sub-strains selection were cultured in Medium 199 (Cambrex) and Dulbecco's modified Eagle's medium (Cambrex), respectively, supplemented with 10% of fetal calf serum and 2% penicillin and streptomycin (Invitrogen) in a humidified atmosphere (5% CO_2) at 37 °C. Dev cells (precursors of human glial cells) and A549 cells (human lung carcinoma, ATCC) used for viral sub-strains endocytic pathway study were maintained in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal calf serum (Cambrex) (Jennings et al., 1992).

2.2. Haemagglutination test

The haemagglutination test was carried out by a microtiter method (Cova and Aymard, 1980). Serial twofold dilutions of the virus were made in 50 μl of 150 mM phosphate buffer solution, pH 8.0, to which 50 μl of 0.75% human erythrocyte O positive suspension was added. The plates were then incubated at 4 °C for 1 h. The HA titres were expressed as the reciprocal of the highest dilution showing haemagglutination.

2.3. Virus binding assay

CHO and CHO-DAF cells in 6-well plates were inoculated with 0.01 MOI of the HA or NHA viruses for 30 min at 4 °C. The cell monolayers were then washed three times with phosphate-buffered saline (PBS) before trypsinization. Virus attachment was then evaluated from 1×10^6 cells using "real-time" RT-PCR analysis described below.

2.4. Competition assay

A549 cells seeded into 6-well culture plate were co-infected by both the haemagglutinating and non-haemagglutinating EV6 strains. A constant multiplicity of infection (MOI) of 1 and 10 was used in HAEV6 infections while NHAEV6 infections were performed at increasing MOI of 0.5, 1, 5, 10, 50 and 100. After 24 h of incubation at 37 °C, a haemagglutination test was performed on each cell supernatant.

2.5. Endocytic entry pathway inhibitors

2.5.1. Drugs

Nystatin (Sigma) was used as a lipid raft-dependent endocytosis disruptor whereas chlorpromazine (Sigma) was utilized to inhibit clathrin-mediated endocytosis (Stuart et al., 2002; Sanchez-San Martin et al., 2004). Nystatin and chlorpromazine were dissolved in dimethyl sulfoxide and water, respectively.

2.5.2. Dominant-negative mutants

The plasmids pCINeo/IRES-GFP/caveolin-1 DN, GFP-EΔ 95/295 Eps15 and GFP-Dyn^{K44A} were kindly provided by Jan Eggermont (Laboratorium voor fysiologie, Leuven, Belgium), Alice Dautry-Varsat (Unité des interactions cellulaires, Institut Pasteur, Paris, France) and Mark A. McNiven

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