The role of the neonatal FC receptor in the uncoating of echoviruses and coxsackievirus A9

The aim of this study was to determine the role of the human neonatal receptor for the Fc fragment of IgG (hFcRn) as a common uncoating cellular receptor for echoviruses and coxsackievirus A9 during infection of human rhabdomyosarcoma (RD) cells.

Materials and methods. The protective effect of the human serum albumin, purified from globulins, (HSA-GF) and antibodies to hFcRn was studied in RD cells infected with several strains and clones of species B enteroviruses possessing different receptor specificity (echoviruses 3, 9, 11, 30 and coxsackieviruses A9, B4, B5).

Results. It was shown that HSA-GF at concentrations of 4% or less protected RD cells from infection with echoviruses 3, 9, 11 and coxsackievirus A9. The antibodies to hFcRn at concentrations of 2.5 μg/mL or less demonstrated the similar spectrum of protective activity in RD cells against infection with echoviruses 3, 9, 11, 30 and coxsackievirus A9. The protective effect of HSA-GF or the antibodies to hFcRn was not observed in RD cells infected with coxsackieviruses B4 and B5 that need coxsackievirus-adenovirus receptor for uncoating.

Discussion. The usage of the previously characterized echovirus 11 clonal variants with different receptor specificity allowed us to define the function of hFcRn as a canyon-binding uncoating receptor in RD cells. The kinetics and magnitude of the observed protective effects correlated with receptor specificity of the enteroviruses used in this work supporting the two-step interaction of DAF-dependent echoviruses with the cellular receptors.

Conclusions. In this study, the function of hFcRn was defined in RD cells as a canyon-binding and uncoating receptor for echoviruses and coxsackievirus A9. The two-step interaction of DAF-dependent echoviruses during entry into the cells was confirmed: initially with the binding receptor DAF and subsequently with the uncoating receptor hFcRn.

Keywords: echovirus; coxsackievirus; uncoating cellular receptor; FcRn; DAF; albumin.

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Introduction

According to its modern classification, all types of echo-
viruses and coxsackievirus A9 (CV A9) belong to the order
Picornavirales, family Picornaviridae, genus Enterovirus,
species Enterovirus B [1]. Certain members of the genus En-
terovirus (coxsackieviruses B3 [2 – 4] and A21 [5]) engage
the two-step interaction with different cellular receptors dur-
ing virus entry into host cells. At the first step, binding re-
ceptors (attachment receptors) provide the interaction of the
viruses with the cellular plasma membrane, the clustering of
the virus-receptor complexes, the activation of transmem-
brane signal transduction and, in polarized cells, a lateral
movement of the complexes along the apical plasma mem-
brane towards the tight junctions. At the second step, the
interaction of the viruses with uncoating receptors converts
160S virions into 135S A-particles by the irreversible pro-
cess that results in the loss of the capsid protein VP4 and
restructuring of the capsid protein VP1 [6]. The binding site
for the uncoating receptors is located in the canyon area, a
depression that surrounds an eminence at each of the five-
fold symmetry axes of the virion. Subsequent event of un-
coating (release) of viral genomic RNA generates empty
80S capsids. If the uncoating receptor is expressed at the
cellular plasma membrane, then it may function as a binding
and uncoating receptor until A-particle forms.

Echoviruses may use several types of molecules as bind-
ning receptors: the complement decay accelerating factor
(DAF, UniProt: P08174) [7, 8], heparan sulfate proteogly-
cans (HSPG, UniProt: P98160) [9 – 11], integrins a2b1 and
aVB3 [12, 13]. The variants of echoviruses, which have been
described, are those which have interacted with DAF only,
with HSPG only, with both DAF and HSPG, or with nei-
ther of these two binding receptors [9]. Subtype variants of
echovirus 9 (E9) may differ in their tropism to integrin aVB3
[14]. The tropism of the echoviruses to the binding receptors
DAF or HSPG may be altered by single amino acid sub-
stitutions in the capsid proteins [11, 15, 16]. A selection of
subtype variants with an altered tropism to binding receptors
promotes the adaptation of echoviruses to various types of
cells.

The binding receptors for CV A9 are also represented by a
set of molecules. The integrin aVB3 is the binding receptor
for CV A9 in RD and GMK cells [17]. Subtype variants of
CV A9 were described that bind to integrin aVB6 in A549
cells [18] and variants that bind to HSPG in GMK cells [11].
In SW480 cell line the molecular chaperone HSPA5 (Uni-
Prot: P11021) with beta-2-microglobulin (B2M, UniProt:
P61769) provided internalization of CV A9 that did not re-
quire the integrin aVB6 [19].

All known uncoating receptors for the members of the
genus Enterovirus belong to the immunoglobulin-like do-
main superfamily: 1) the poliovirus receptor (PVR, UniProt:
P15151) – for the three types of poliovirus; 2) the coxsackie-
virus and adenovirus receptor (CAR, UniProt: P78310) –
for the six types of coxsackieviruses B; 3) the intercellular
adhesion molecule 1 (ICAM-1, UniProt: P05362) – the re-
ceptor for all types of the major group in the Rhinovirus A
species, all types in Rhinovirus B species and five types of
coxsackieviruses A: CVA13, CVA15, CVA18, CVA20 and
CVA21 [20, 21].

A glycoprotein with a molecular mass of 44 kDa (gp44)
was isolated and previously characterized as a putative
cell receptor for echoroviruses [22]. Monoclonal antibodies
(MAB) to gp44 protected human cell line P2002 from infec-
tion with virtually all echoroviruses and CV A9 [23]. However,
no reports about continued studies of gp44 were found in the
open access data retrieval systems.

Ward et al. [24] revealed the protective effect of the MAB
to B2M in RD cells against infection with a wide spectrum
of echoroviruses and CV A9. It was shown that the addition
of bovine serum albumin (BSA) or human serum albumin
(HSA) to the cultural medium inhibited infection of RD cells
with echovirus 7, suppressing the formation of A-particles,
but did not prevent the virus from binding to the cells [25].
Furthermore, the inactivation mechanism of the E7 and E12
viruses by BSA purified from globulins and fatty acids, was
attributable to the transformation of virions into A-particles
due to the extraction of a hydrophobic pocket factor from vi-
rons [26]. The mechanism of the protective effect caused by
globulin free HSA remained unknown, although its blocking
effect was supposed either on echovirus virions or on a cell
receptor secondary to DAF.

The human neonatal receptor for FC fragment of IgG
(hFcRn) is a transmembrane heterodimer consisting of the
alpha-chain called FCGRT (UniProt: P55899) and the non-
covalently bound protein B2M [27]. The FCGRT molecule
belongs to the immunoglobulin-like domain superfam-
ily (UniProt: P55899). In cells of human origin, it has a mo-
olecular mass of 45 kDa [28] close to that of the gp44 de-
scribed earlier [22]. On the cell surface hFcRn functions as
the albumin receptor, inside the cells – as IgG receptor and
a transporter protein, which protects IgG and albumin from
proteolytic degradation in lysosomes by recirculation of the
bound ligand to the plasma membrane or by its transcytosis
in polarized cells [29].

The aim of this study was to determine the role of hFcRn
as a common uncoating receptor for echoviruses and cox-
sackievirus A9 in RD cells. The experimental part of the
work was designed to fulfill the following tasks: 1) to verify the absence of virus inactivation by globulin free HSA while using cloned variants of echovirus 11 with different tropism to the binding receptor DAF; 2) to test the spectrum of globulin free HSA protective activity in RD cells infected with the species B enteroviruses possessing various receptor specificity; 3) to study the protective activity of polyclonal antibodies (PAb) and MAb to hFcRn against infection of RD cells with the species B enteroviruses possessing various receptor specificity.

Materials and methods

Cell cultures. The continuous cell line of human rhabdomyosarcoma (RD) was received from the Federal Budgetary Institution of Healthcare “The Center for Hygiene and Epidemiology in Sverdlovsk Region” of the Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing (Yekaterinburg, RF). Confluent monolayers of RD cells were grown in 96-well plates for cell cultures (“Corning”, USA) in a humidified atmosphere of 5% CO2 incubator at 37°C. The growth medium Eagle’s MEM (“PanEco”, RF) was supplemented with 10% fetal calf serum (“Biosera”, France). For subculturing, cells were detached in Hanks salt solution with trypsin and EDTA 0.25% (“PanEco”, RF). The serum-free medium 199 (“PanEco”), RF was used as the maintenance medium during virus reproduction.

Viruses. Strains and clones of species B enteroviruses used in this work are listed in Table 1. Hemagglutinating (Daf+) clone 431-1 of E11, which uses DAF as the primary cellular receptor, and nonhemagglutinating (Daf−) clone 431-6 of E11, which uses another unidentified cellular receptor, were selected and described earlier [16]. All other strains listed in Table 1 were obtained at the Laboratory of Enteric Viral Infections of Yekaterinburg Research Institute of Viral Infections as clinical isolates on RD cells cultures from cerebrospinal fluid samples of patients with enteroviral meningitis. Genotyping of the clinical isolates was performed according to methods described earlier [30, 31], using two fragments in the structural part of the enteroviruses’ genome, coding capsid proteins VP1 and VP4-VP2.

Albumins and antibodies to hFcRn. The following commercially available biological products were used in cell protection experiments: 1) Human serum albumin essentially globulin free (A8763, Merck/Sigma-Aldrich, Germany) (HSA-GF) was obtained in lyophilized form; 2) Rabbit IgG isotype PAb, directed against recombinant human FCGRT and B2M heterodimer protein, purified by affinity chromatography (CT009-T08, Sino Biological, China) (hFcRn-PAb) were obtained as a sterile filtered solution in PBS without preservative.

Testing of the human serum albumins for virus inactivating capability. To test the virus inactivating capability of HSA-GF in solution, 100 μl of the virus containing fluid (VCF) with 200 TCID50 of the appropriate virus was added to 100 μl of 8% (w/v) solution of the albumin in medium 199 to obtain a 4% final concentration and incubated for 60 min at 37°C. Control samples of VCF were incubated for 60 min at 37°C with 100 μl of medium 199 without albumin. After incubation, the remaining viral infectious titers were determined on RD cells in 96-well plates by the end-point dilution method (8 replicates per dilution) and calculated according to the Spearman-Karber method.

Cell protection assay with human serum albumin in RD cells. Cells protection assays with HSA-GF were conducted on RD cells in 96-well plates with the appropriate viruses. In preliminary experiments, it was shown that HSA-GF at final concentrations from 0.25 to 4% (w/v) did not exert cytotoxic effect on the cells during a 5 day observation period. To reveal the dependence of the protective effect on the concentration of HSA-GF, equal infectious doses of the viruses (100 TCID50 per well) were tested with varied concentrations of the albumin solution in medium 199, prepared by two-fold dilutions. After removing the growth medium, cell monolayers were washed once with medium 199, and 100 μL of the albumin solution per well was added. Cells were incubated for 60 min at 37°C and subsequently infected with 100 TCID50 of the appropriate virus in 100 μl of the VCF (multiplicity of infection (MOI) was 0.001 TCID50/cell). To observe the viral cytopathic effect (CPE) in the control wells, 100 μl of medium 199 was added to the control wells instead of the albumin solution. Control wells used for monitoring intact cell monolayers were supplied with 200 μl of medium 199. Each final concentration of HSA-GF (4%, 2%, 1%, 0.5% and 0.25%) was tested in 8 replicates with each virus in two independent experiments. CPE was recorded daily by examination of the monolayers with an inverted microscope. The final assessment of CPE was done on the fifth day, following a fixation of the cells in 96% ethanol and staining with 0.5% crystal violet. The degree of CPE was assessed as the percentage of cells with typical for echoviruses signs of cytopathology in compliance with the conventional scale: “−” (0%); “+” (<25%); “++” (25% to <50%); “+++” (50% to <75%) and “++++” (75% to 100%). Following this, the mean values with standard deviations were calculated and statistically analyzed.

Cell protection assay with hFcRn-PAb in RD cells. Cells protection assays with hFcRn-PAb were performed on RD cell monolayers in 96-well plates. Preliminary experiments

| Types, strains and clones of enteroviruses used in the experiments |
|---------------------------------|------------------|-----------------|-----------------|-----------------|-----------------|
| **Enterovirus type (Abbreviation)** | **Strain (clone) ID number** | **Daf phenotype** | **GenBank Accession number** |
| Echovirus 11 (E11)          | (431-1)          | Daf+             | JF925116         |
| Echovirus 3 (E3)           | (431-6)          | Daf−             | JF925117         |
| Echovirus 9 (E9)           | 206              | Daf+             | MK962649, MK962655 |
| Echovirus 30 (E30)         | 8100             | Daf−             | MK962651, MK962657 |
| Coxsackievirus B4 (CVB4)   | 7500             | Daf−             | MK962648, MK962654 |
| Coxsackievirus B5 (CVB5)   | 1000             | Daf−             | MK962653, MK962659 |
| Coxsackievirus A9 (CVA9)   | 3122             | Daf−             | MK962652, MK962658 |
|                             | 3000             | Daf−             | MK962650, MK962656 |
have shown that the hFcRn-PAb were not cytotoxic for the cells at final concentrations ranging from 0.15 to 10 ug/mL during 5 days of observation period. Equal infectious doses of the viruses (100 TCID₅₀ per well) were tested with varied concentrations of the antibodies in medium 199. After removing the growth medium, cell monolayers were washed once with medium 199, then 50 uL per well of the antibody solution with varied concentration was added and cells were incubated for 1 h at 37°C. Subsequently 50 uL of the VCF per well was added with 100 TCID₅₀ of the appropriate virus (MOI 0.001 TCID₅₀/cell). Each final concentration of the antibodies (10.0, 5.0, 2.5, 1.25, 0.6 and 0.3 ug/mL) was tested in 8 replicates with each virus, in two independent experiments. Control wells used to observe viral CPE contained 50 uL of the VCF and 50 uL of medium 199 without antibodies. Control wells used to verify the absence of antibodies’ cytotoxicity contained 50 uL of medium 199 instead of VCF and 50 uL of the antibody solution per well. Control wells used for monitoring intact cell monolayers contained 100 uL of medium 199. Controls for the absence of nonspecific virus neutralization by hFcRn-PAb (10.0 ug/mL) were performed according to the experimental scheme described.

Statistical analysis. Viral infectious titers were calculated using Spearman-Karber procedure with the estimates of the total analytical error [32]. Statistically significant differences between the experimental groups were determined by one-way ANOVA [33] or by using nonparametric Mann–Whitney U test. Differences were considered statistically significant when the P values were less than 0.05.

Results

The absence of the virus inactivating capability of the HSA-GF solution. Since it was known that the solution of BSA, which was purified from globulins and fatty acids, inactivated echoviruses 7 and 12 [26], we have verified the absence of the virus inactivating capability for the globulin-free HSA solution. Experimental conditions were close to that of cell protection assays: stocks of cloned E11 variants (100 TCID₅₀ per 100 uL) were incubated in the solution of HSA-GF with 4% final concentration at 37°C for 1 h, a time interval long enough to infect RD cells. No virus inactivation in HSA-GF solution was detected for both E11 clones.

Protective effect of the albumin in RD cells infected with various types of species B enteroviruses. The concentration-dependent protective effect of HSA-GF was studied using several time intervals post infection (p.i.) of RD cells, with an equal infectious dose (100 TCID₅₀ per well, MOI 0.001 TCID₅₀/cell) of echoviruses 3 (E3), 9 (E9), 11 (E11), coxsackieviruses A9 (CVA9), and B5 (CVB5). Results are shown in Table 2.

The comparison of the protective effect of HSA-GF in RD cells against two closely related clones of E11 have shown a more pronounced inhibitory effect of the albumin in the case of infection with the daf⁺ clone 431-6, which did not interact with the binding receptor DAF, contrary to the DAF-dependent daf⁺ clone 431-1. Provided that there was an absence of the albumin in the maintenance medium, 100% CPE developed after 48 h p.i. (down to 40.6±4.6%). A maximum duration of the protective effect in the case of the daf⁺ clone 431-1 was observed at a 4% concentration of HSA-GF after 96 h p.i. (CPE 50.0%), whereas a maximum duration of the protective effect against the daf⁻ clone 431-6 at 4% HSA-GF was observed even after 120 h p.i. (CPE 12.5±4.7%). In addition to this, the daf⁺ clone 431-1 demonstrated a more rapid increase of CPE over time at 1% and 2% concentrations of HSA-GF in contrast to the daf⁻ clone 431-6.

A comparison of the protective effect of HSA-GF in RD cells infected with E3 and E9 revealed faster kinetics of CPE accumulation (lower protection) in the case of the Daf⁻ strain of E3 in contrast to the Daf⁺ strain of E9. Provided there was an absence of the albumin in the maintenance medium, a 100% CPE developed after 48 h p.i. with each of the E3 and E9 strains. In the case of the Daf⁺ E3 strain, the minimal concentration of HSA-GF, which had caused a statistically significant decrease in CPE after 48 h p.i. (down to 37.5±4.7%), was equal to 1.0%. In the case of the Daf⁻ E9 strain, the minimal concentration of HSA-GF, which had caused a statistically significant decrease in CPE after 48 h p.i. (down to 18.8±4.1%), was four times lower: 0.25%. A maximum duration of the protective effect in the case of the Daf⁺ E3 strain was observed at 4% concentration of HSA-GF after 120 h p.i. (CPE 62.5%±6.4%), whereas the maximum duration of the protective effect against the Daf⁻ E9 strain was observed after 120 h p.i. at 4%, 2% and 1% concentration of HSA-GF (after 120 h p.i. at 1% HSA-GF CPE was 6.3%±4.1%).

Thus, the pairwise comparison revealed the more pronounced protective effects of HSA-GF against infection with the daf⁻ clone 431-6 and the Daf⁺ strain of E9, than the protective effect observed with the daf⁺ clone 431-1 and the Daf⁺ strain of E3.

Unlike echoviruses and coxsackieviruses B used in this work, the CVA9 strain demonstrated delayed kinetics of CPE accumulation: 100% CPE in the absence of the albumin was observed only after 72 h p.i.. The minimal concentration of HSA-GF, which had caused a statistically significant decrease in CPE after 72 h p.i. (down to 43.8±4.1%), was equal to 0.25%. After 120 h p.i., the protective effect was observed with HSA-GF concentrations of 2% (CPE 65.6±4.6%) and 4% (CPE 94±4.6%).

The CVB5 strain demonstrated a 100% CPE, both in the absence of and in the presence of HSA-GF after 48 h p.i., with all concentrations of the albumin up to 2%. A decrease of CPE with 4% HSA-GF after 48 h p.i. was not statistically significant in comparison to the control wells without the albumin.

Protective effect of antibodies to hFcRn in RD cells infected with various types of species B enteroviruses. In preliminary experiments, no virus neutralizing activity was observed with hFcRn-PAb (10.0 ug/mL), incubated for 1 h at 37°C with 100 TCID₅₀ of the appropriate viruses before infection of RD cells (data not shown). The concentration-dependent protective effect of the hFcRn-PAb was studied at several time intervals p.i. of RD cells with an equal infectious dose (100 TCID₅₀ per well, MOI 0.001 TCID₅₀/cell) of viruses E3, E9, E11, E30, CVA9, CVB4 and CVB5. Results are shown in Table 3.

A comparison of the protective effect of the hFcRn-PAb in RD cells against two closely related clones of E11 (daf⁺ clone 431-1 and daf⁻ clone 431-6) revealed the same minimal concentration of the PAb (2.5 ug/mL) that caused the absence of CPE for 120 h p.i. However, at a lower
concentration of the PAb (1.25 ug/mL) kinetics of CPE accumulation over time (after 72, 96 and 120 h p.i.) was faster (lower protection), in the case of the DAF-dependent daf+ clone 431-1 contrary to the daf– clone 431-6, which did not interact with DAF.

Minimal concentrations of the hFcRn-PAb that caused the absence of CPE for 120 h p.i. in RD cells infected with E3, E9 and E30 strains equaled 5.0, 2.5 and 1.25 ug/mL respectively. A more pronounced protective effect of the hFcRn-PAb was observed in the case of the Daf– strains of E9 and E30 in comparison to the Daf+ strain of E3.

The CV A9 strain demonstrated delayed kinetics of CPE accumulation compared to those of echoviruses or coxsackieviruses B used in this work. A 100% CPE in the absence of the hFcRn-PAb was observed only after 72 h p.i. The minimal concentration of the PAb which caused the absence of CPE for 120 h p.i. in cells infected with CV A9 was equal to 0.6 ug/mL.

All concentrations of the hFcRn-PAb up to 10.0 ug/mL exerted no protective effect in RD cells infected with the CVB4 strain or the CVB5 strain.

Discussion

Species B enteroviruses demonstrate a high intratypic variability concerning the interaction with binding receptors, such as DAF [2, 34], HSPG [11], integrins αVβ3 and αVβ6 [18]. However, the interaction with the known uncoating receptors for enteroviruses, such as CAR, ICAM-1 and PVR is highly conservative within subspecies groups of genus Enterovirus.

In this study, we have demonstrated that HSA-GF at physiological concentrations protected RD cells from infection with several types of echoviruses (E3, E9, E11) and CV A9 independently of the used binding receptors. Furthermore, infection of RD cells with CVB5, which uses CAR as uncoating receptor, was not inhibited by HSA-GF. The

| Type clone / strain | Time post infection (hours) | Concentration of the albumin HSA-GF (%) |
|--------------------|---------------------------|----------------------------------------|
|                    | 0.0 | 0.25 | 0.5 | 1.0 | 2.0 | 4.0 |
| E11 431-1          | 24  | 12.5±4.7 | 12.5±4.7 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
|                    | 48  | 100.0±0.0 | 100.0±0.0 | 81.3±4.1* | 12.5±4.7* | 0.0±0.0* | 0.0±0.0* |
|                    | 72  | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 |
|                    | 96  | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 50.0±0.0* |
| E11 431-6          | 120 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 90.6±4.6 |
|                    | 24  | 18.8±4.1 | 18.8±4.1 | 0.0±0.0* | 0.0±0.0* | 0.0±0.0* | 0.0±0.0* |
|                    | 48  | 100.0±0.0 | 81.3±4.1* | 40.6±4.6* | 0.0±0.0* | 0.0±0.0* | 0.0±0.0* |
|                    | 72  | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 90.6±4.6 |
|                    | 96  | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 6,3±4.1* |
| E3 206             | 120 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 12.5±4.7* |
|                    | 24  | 40.6±4.6 | 12.5±4.7* | 0.0±0.0* | 0.0±0.0* | 0.0±0.0* | 0.0±0.0* |
|                    | 48  | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 37.5±4.7* | 6,3±4.1* | 0.0±0.0* |
|                    | 72  | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 62.5±4.7* |
|                    | 96  | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 62.5±4.7* |
| E9 8100            | 120 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 62.5±4.7* |
|                    | 24  | 18.8±4.1 | 6,3±4.1 | 0.0±0.0* | 0.0±0.0* | 0.0±0.0* | 0.0±0.0* |
|                    | 48  | 93.8±4.1 | 18.8±4.1* | 12.5±4.7* | 0.0±0.0* | 0.0±0.0* | 0.0±0.0* |
|                    | 72  | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 |
|                    | 96  | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 |
| CV A9 3000         | 120 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 |
|                    | 24  | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
|                    | 48  | 37.5±4.7 | 0.0±0.0* | 0.0±0.0* | 0.0±0.0* | 0.0±0.0* | 0.0±0.0* |
|                    | 72  | 100.0±0.0 | 43.8±4.1* | 12.5±4.7* | 0.0±0.0* | 0.0±0.0* | 0.0±0.0* |
|                    | 96  | 100.0±0.0 | 100.0±0.0 | 65.6±4.6* | 31.3±4.1* | 0.0±0.0* | 0.0±0.0* |
| CVB5 3122          | 120 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 87.5±4.7 |
|                    | 24  | 12.5±4.7 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
|                    | 48  | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 |
|                    | 72  | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 |
|                    | 96  | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 |
|                    | 120 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 |

Note. * – Average percent of CPE for 8 replicates in each of the 2 experiments ± standard error of mean value; * – Statistical significance of the difference between the mean CPE value in a table cell and a cell at the same line for 0% HSA-GF (Mann–Whitney U test).
same spectrum of protective activity was demonstrated for hFcRn-PAb, which protected RD cells from infection with echoviruses (E3, E9, E11, E30) and CV A9, but not from infection with CVB4 and CVB5. Since the binding of the albumin is the physiological function of hFcRn, the common mechanism of the protective effect exerted by the albumin and hFcRn-PAb was associated with their binding to hFcRn which interfered with the interaction of this receptor with echoviruses and CV A9.

The spectrum of the protective activity observed for HSA-GF and hFcRn-PAb in RD cells corresponded to the spectrum of the protective activity against enteroviruses de-

### Table 3

| Type clone / strain | Time post infection (hours) | Concentration of the polyclonal antibodies (ug/mL) |
|---------------------|----------------------------|---------------------------------------------------|
|                     | 0.0 | 0.6 | 1.25 | 2.5 | 5.0 | 10.0 |
|                     | 24  | 25.0±0.0 | 0.0±0.0* | 0.0±0.0* | 0.0±0.0* | 0.0±0.0* |
| E11 431-1           | 48  | 100.0±0.0 | 25.0±0.0* | 12.5±4.7* | 0.0±0.0* | 0.0±0.0* |
|                     | 72  | 100.0±0.0 | 75.0±0.0* | 46.9±3.1* | 0.0±0.0* | 0.0±0.0* |
|                     | 96  | 100.0±0.0 | 87.5±4.7 | 50.0±0.0* | 0.0±0.0* | 0.0±0.0* |
|                     | 120 | 100.0±0.0 | 100.0±0.0 | 62.5±4.7* | 0.0±0.0* | 0.0±0.0* |
| E11 431-6           | 24  | 12.5±4.7 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
|                     | 48  | 100.0±0.0 | 18.8±4.1* | 0.0±0.0* | 0.0±0.0* | 0.0±0.0* |
|                     | 72  | 100.0±0.0 | 78.1±3.1* | 12.5±4.7* | 0.0±0.0* | 0.0±0.0* |
|                     | 96  | 100.0±0.0 | 75.0±0.0* | 25.0±0.0* | 0.0±0.0* | 0.0±0.0* |
| E3 206              | 24  | 50.0±0.0 | 0.0±0.0* | 0.0±0.0* | 0.0±0.0* | 0.0±0.0* |
|                     | 48  | 100.0±0.0 | 25.0±0.0* | 0.0±0.0* | 0.0±0.0* | 0.0±0.0* |
| E9 8100             | 72  | 100.0±0.0 | 100.0±0.0 | 68.8±4.1* | 12.5±4.7* | 0.0±0.0* |
|                     | 96  | 100.0±0.0 | 100.0±0.0 | 75.0±0.0* | 21.9±3.1* | 0.0±0.0* |
|                     | 120 | 100.0±0.0 | 100.0±0.0 | 75.0±0.0* | 25.0±0.0* | 0.0±0.0* |
| E30 7500            | 24  | 12.5±4.7 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
|                     | 48  | 100.0±0.0 | 43.8±4.1* | 0.0±0.0* | 0.0±0.0* | 0.0±0.0* |
| E30 7500            | 72  | 96.9±3.1 | 12.5±4.7* | 0.0±0.0* | 0.0±0.0* | 0.0±0.0* |
|                     | 96  | 100.0±0.0 | 100.0±0.0 | 68.8±4.1* | 25.0±0.0* | 0.0±0.0* |
|                     | 120 | 100.0±0.0 | 100.0±0.0 | 46.9±3.1* | 0.0±0.0* | 0.0±0.0* |
| E30 7500            | 24  | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| E30 7500            | 48  | 100.0±0.0 | 0.0±0.0* | 0.0±0.0* | 0.0±0.0* | 0.0±0.0* |
| E30 7500            | 72  | 100.0±0.0 | 0.0±0.0* | 0.0±0.0* | 0.0±0.0* | 0.0±0.0* |
|                     | 96  | 100.0±0.0 | 0.0±0.0* | 0.0±0.0* | 0.0±0.0* | 0.0±0.0* |
|                     | 120 | 100.0±0.0 | 21.9±3.1* | 0.0±0.0* | 0.0±0.0* | 0.0±0.0* |
|                     | 24  | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| CVA9 3000           | 48  | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 |
|                     | 72  | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 |
|                     | 96  | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 |
|                     | 120 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 |
| CVA9 3000           | 24  | 25.0±0.0 | 25.0±0.0 | 25.0±0.0 | 25.0±0.0 | 25.0±0.0 |
| CVA9 3000           | 48  | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 |
| CVA9 3000           | 72  | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 |
| CVA9 3000           | 96  | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 |
| CVA9 3000           | 120 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 |

**Note.** * – Average percent of CPE for 8 replicates in each of the 2 experiments ± standard error of mean value; * – Statistical significance of the difference between the mean CPE value in a table cell and a cell at the same line for 0.0 ug/mL of polyclonal antibodies (Mann–Whitney U test).
scribed earlier for the MAb to gp44 in P2002 cells of human origin [23]. Since the MAb to gp44 inhibited infection with virtually all types of echoviruses, and assuming that gp44 is identical to FCGRT (alpha-chain of hFcRn), our results can be extrapolated for the entire group of echoviruses.

The role of hFcRn as a “primary”, “pan-echovirus” receptor was recently shown by other methods and in other cell lines [35]. Direct interaction of E11 and E30 virions with the recombinant molecule constructed from the extracellular domain of hFcRn and B2M (rFcRn-B2M) has been demonstrated by coprecipitation in vitro and subsequent immunoblotting. Aside from this, rFcRn-B2M possessed a virus-neutralizing activity.

Earlier we have shown that the amino acid substitutions caused by the adaptation of a cloned E11 from RD cells to the cell lines of human (HEp-2, L-41/KD84) and simian (BGM) origin occurred in the viral canyon [15]. Later 2 closely related daf+ (431-1) and daf– (431-6) clones of E11 were selected in RD cells so that they differed only in a single amino acid substitution in the binding site for DAF and had identical structure of the capsid proteins in the canyon. Anti-DAF MAbs efficiently inhibited the reproduction of the daf+ clone 431-1 in RD cells, and so this clone possessed the DAF-dependent phenotype in contrary to the daf– clone 431-6. The reproduction of both clones was inhibited by anti-B2M MAbs [16]. These findings implied the existence of a common canyon-binding receptor for the 2 clones with its functionality dependent on B2M. Since B2M constitutes a subunit of hFcRn and it was shown here that HSA-GF and hFcRn-PAB exerted a protective effect against infection of RD cells with either daf+ clone 431-1 or daf– clone 431-6, then the common canyon-binding receptor for the two clones can be identified as hFcRn. Moreover, the DAF-dependent clone 431-1 demonstrated a two-step interaction with RD cells: the first step was blocked by the anti-DAF MAb, the second step was blocked by anti-B2M MAb, HSA-GF or hFcRn-PAB.

The distinctive property of uncoating receptors is their ability to convert mature virions of enteroviruses into A-particles. Combining the evidence of a decrease in E11 infectious activity caused by direct interaction between the rFcRn-B2M and E11 [35] and the evidence of a reduced production of A-particles and blockage of E11 uncoating in RD cells by anti-B2M MAbs [36], with the results of our studies on mapping the binding site for an alternative to DAF cell receptor in the canyon area of E11 virion, allow us to propose the identity of hFcRn as a canyon-binding and uncoating receptor for E11 in RD cells.

Given the protective effect of HSA-GF and hFcRn-PAB, reported here for RD cells infected with CVA9; the protective effect of the anti-B2M MAb in RD cells against CVA9 reported earlier [24] and the protective effect of MAb to gp44 in P2002 cells infected with CVA9 [23], it can be supposed that the function of hFcRn in regards to CVA9 is similar to the function in regards to echoviruses. Additionally, Heikkila et al. [37] have shown that the RNA silencing of B2M not only effectively inhibited reproduction of CVA9 in A549 cells, but also caused an accumulation of CVA9 on the surface of the cells. This finding implied an engagement of B2M containing molecules (including hFcRn) in the internalization of CVA9 in A549 cells. Thus, the functional activity of hFcRn is the necessary condition for CVA9 infection of RD cells as well as for echoviruses.

Considering the two-step interactions of certain echovirus variants with binding and uncoating receptors, the role of hFcRn as a binding receptor may not be universal, as far as other binding receptors exist. If binding of viruses to cells occurs on the plasma membrane, then a weak (or a lack of) expression of the uncoating receptor on the surface of the cells combined with low multiplicity of infection would make cells unsusceptible to the viruses possessing a mono-receptor tropism to hFcRn. In the case of viruses, possessing a multireceptor tropism, a weak (or a lack of) surface expression of an uncoating receptor is compensated by their interaction with binding receptor(s). The two-step scheme of interactions explains how blockage of the binding receptors prevented infection of the cells with DAF-dependent or HSPPG-dependent variants of echoviruses. In accordance with the two-step scheme, we observed that protective effects of the albumin or the PAb to hFcRn was less advanced in the case of DAF-binding echoviruses compared to the echoviruses which did not bind DAF provided that interaction of the viruses with hFcRn was not blocked completely (simulating its low expression).

The identification of hFcRn as a common uncoating receptor for echoviruses and CVA9 opens new opportunities to study the relationship of hFcRn expression and function in various types of cells and tissues with the pathogenesis of diseases caused by echoviruses and CVA9. This determination of the key role that hFcRn plays in reproduction of echoviruses and CVA9 allows the usage of transgenic mice expressing hFcRn [38, 39] in experimental studies of echovirus infections in animals [35] and hence preclinical testing of antiviral drug candidates. The specificity of the protective effects of the HSA-GF and the hFcRn-PAb in RD cells allows the usage of these reagents for subspecies classification of poorly studied or emergent enteroviruses.

Conclusions

1. In this study, we defined the role of hFcRn as a canyon-binding uncoating receptor for echovirus 11 in RD cells.

2. The kinetics and magnitude of the protective effects exerted by the albumin HSA-GF or the antibodies to hFcRn were less advanced in the case of DAF-binding echoviruses compared to the echoviruses that did not bind to DAF, provided that the interaction of the viruses with hFcRn was not blocked completely. This finding supports the proposed two-step interaction of DAF-dependent echoviruses initially with the binding receptor DAF and subsequently with the uncoating receptor hFcRn.

3. The protective effects of the albumin HSA-GF and the antibodies to hFcRn reported here, can be explained by the blockage of hFcRn interaction with echoviruses and CVA9 during binding, internalization or uncoating steps of the viral infection.

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