1   The association of human astrovirus with extracellular vesicles facilitates cell

2   infection and protects the virus from neutralizing antibodies

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Abstract

Viral gastroenteritis has a global distribution and represents a high risk for vulnerable population and children under 5 years because of acute diarrhea, fever and dehydration. Human astroviruses (HAstV) have been identified as the third most important cause of viral gastroenteritis in pediatric and immunocompromised patients. Furthermore, HAstV has been reported in biopsies taken from patients with encephalitis, meningitis and acute respiratory infection, yet it is not clear how the virus reaches these organs. In this work we tested the possibility that the released astrovirus particles could be associated with extracellular vesicles. Comparison between vesicles purified from astrovirus- and mock-infected cells showed that infection with HAstV Yuc8 enhances production of vesicles larger than 150 nm. These vesicles contain CD63 and Alix, two markers of vesicular structures. Some of the extracellular virus was found associated with vesicular membranes, and this association facilitates cell infection in the absence of trypsin activation and protects virions from neutralizing antibodies. Our findings suggest a new pathway for HAstV spread and might represent an explanation for the extraintestinal presence of some astrovirus strains.

Importance.

Astroviruses are an important cause of diarrhea in children; recently some reports have found these viruses in extra-intestinal organs, including the central nervous system, causing unexpected clinical disease. In this work we found that human astrovirus strain Yuc8 associates with extracellular vesicles, possibly during or after their cell egress. The association with vesicles seems to increase astrovirus infectivity in less susceptible cells, and renders virus particles insensitive to neutralization by antibodies. These data suggest that extracellular vesicles could represent a novel pathway for astrovirus to disseminate outside the gastrointestinal tract.

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Introduction

Astroviruses are considered the third most important cause of viral gastroenteritis in children, as well as in the young of many animal species (1, 2). Moreover, in some mammalian species astroviruses have been associated with different neurological disorders and have been found in biopsies of patients with encephalitis, meningitis or acute respiratory infections (1, 2). Given that mammalian astroviruses are considered intestinal viruses, the central question is: "How could astroviruses get into the central nervous system and respiratory tract?" (3).

From the structural point of view, astroviruses are small non-enveloped viruses, forming the family *Astroviridae*. They contain a single-stranded, positive sense RNA (ssRNA+) genome whose length ranges, in the case of mammalian astroviruses, from 6.1 to 6.8 kb. The astrovirus genome is organized into three open reading frames, named ORF1a, ORF1b and ORF2, which encode non-structural (ORF1a and ORF1b) and structural (ORF2) viral proteins (4, 5). Astrovirus cell entry is not completely understood, and the virus cell surface receptor is unknown, although the fact that the susceptibility of different cell lines to infection with astrovirus depends on the viral serotype (6-8), suggests that there could be more than one receptor. Astrovirus enters into cells by clathrin-mediated endocytosis and it seems that entry process follows a classical route into late endosomes (4, 9). During maturation, the astrovirus particles are subjected to distinct proteolytic processes. First, the capsid protein VP90 of the newly assembled astrovirus particles is cleaved intracellularly by caspases to give immature virions composed by the viral protein VP70. This cleavage is associated with the release of the viral particles from the infected cell (10, 11). Then, once in the extracellular medium, the virion is processed by trypsin-like extracellular proteases to render infectious, mature virions, composed by the final protein products VP27 and VP34 (12, 13).

One of the less characterized phases of the astrovirus replication cycle, is cell egress. It has been proposed that astrovirus release is a non-lytic process, during which the extracellular virions appear to be associated with membranous structures (11, 14). In this regard, it is of interest that the cell exit of different viruses has been associated with extracellular vesicles (EV) (15-17). EV are a heterogeneous group

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83 of small vesicles with a lipidic bilayer, ranging from 50 nm to 1,000 nm of diameter

84 (18). These vesicles are secreted by different types of cells and can be isolated from

85 conditioned media of cultured cells, as well as from virtually any type of body fluid,

86 including blood, urine, ascites, bronchoalveolar lavage, saliva and cerebrospinal

87 fluid (19, 20). There are different types of EV, with exosomes being the better

88 characterized, having a diameter of around 50 to 150 nm, and also well studied

89 microvesicles with diameter around 50 to 1,000 nm. Exosomes originate from the

90 endosomal compartment by fusion of multivesicular bodies with the plasma

91 membrane, while microvesicles originate from the plasma membrane by outward

92 budding (21).

93 Viral infections affect cell physiology, as well as many cellular processes,

94 including protein synthesis and degradation (22, 23), intracellular trafficking and

95 vesicle secretion (16, 24, 25). In the last few years the evidence regarding the

96 interaction between EV and different types of viruses (26-28) has accumulated.

97 Particularly, several positive-sense ssRNA viruses, like hepatitis C virus (HCV) and

98 hepatitis E virus (HEV), have been found to associate with EV or to use the

99 mechanism of EV biogenesis as an egress pathway (29-32). In addition, DNA

100 viruses like HSV-1 (33) and JC polyomavirus (34) also have been observed

101 interacting or being released with EV.

102 Given the possibility that EV could be involved in the human astrovirus

103 (HAstV) cell egress, we tested the possibility that astrovirus particles could be

104 released in association with this type of vesicles. To characterize the possible

105 interaction between EV and the virus, Caco-2 cells were infected with the Yuc8 strain

106 of HAstV and EV were purified from the cell culture media by differential

107 centrifugation coupled to polyethylene glycol 6000 (PEG) precipitation and affinity

108 magnetic sorting. Our results suggest that astrovirus infection stimulates the

109 secretion of EV and astrovirus particles seem to associate with EV. These vesicle-

110 associated viruses acquire the ability to infect cells in the absence of trypsin

111 activation. Also, viral particles associated with EV were refractory to the effect of

112 neutralizing antibodies, suggesting that EV are able to protect the virions from this

113 interaction.

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Materials and methods

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Cell lines, virus, reagents and antibodies

Human colon adenocarcinoma cells (Caco-2), and rhesus monkey epithelial cells (MA104), were obtained from American type culture collection (ATCC, Manassas, VA, USA). Dulbecco modified Eagle medium - high glucose (DMEM) was purchased from Sigma Aldrich (San Luis, MI, USA), while Advanced-DMEM (A-DMEM), fetal bovine serum (FBS) and trypsin were from Gibco (Thermo Fisher Scientific, USA). Triton X-100 was acquired from Boehringer Mannheim, (Germany), whereas Polyethylene glycol 6000, soybean trypsin inhibitor and Minimum Essential Medium (MEM) were acquired from, Sigma-Aldrich (San Luis, MI, USA). Formaldehyde was obtained from J.T. Baker, (USA), and MagCapture^{TM} exosome isolation kit PS was from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Human astrovirus serotype 8, strain Yuc8 was originally isolated in our laboratory (35). Polyclonal rabbit antibody specific for Yuc8 virus (anti-Yuc8) was prepared in our laboratory (11). Rabbit polyclonal antibodies specific for anti-CD63 and anti-Alix were acquired from Santa Cruz (Santa Cruz Biotechnology, CA, USA), and Aviva Systems Biology (Aviva Systems Biology, CA, USA) respectively, while monoclonal antibody specific to anti- protein disulfide isomerase (PDI, clone 1D3) was obtained from Enzo Life Sciences, Inc (C. Mexico, Mexico). Anti-rabbit peroxidase conjugated antibody was from KPL (MD USA), and protein A, peroxidase conjugate was from Sigma Aldrich (Sigma Aldrich).

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Cell culture and viral propagation

Caco-2 cells were cultured in DMEM supplemented with non-essential amino acids and 15% heat-inactivated FBS, in a 10% CO_2 atmosphere at 37°C (14). MA104 cells were grown in A-DMEM, supplemented with 5% FBS, at 37°C in a 5% CO_2 atmosphere (36).

A working stock of human astrovirus serotype 8, strain Yuc8 (35), was prepared as previously described (37). The virus was activated just prior to infection

145 with 200 µg/mL of trypsin, for 1 hour at 37 °C, followed by inactivation with 200 µg/mL

146 of soybean trypsin inhibitor. Before infection, Caco-2 cell monolayers were washed

147 with MEM and incubated with activated virus for 1 hour at 37 °C. Then, cell

148 monolayers were washed twice with MEM, to remove non-adsorbed virus. Finally,

149 MEM was added to the cells, and infection was left to proceed for 48 hours at 37 °C.

150   Astrovirus particles were purified essentially as described previously (38).

151 Briefly, Caco-2 cells were infected with HAstV serotype 8 (Yuc8) at an MOI of 5 as

152 described above and the infection was left to proceed for 48 hours. After this time

153 cells were detached, and frozen and thawed three times. Then, cellular lysate was

154 clarified by centrifugation at 2,000 g for 10 minutes and then passed through a 0.45

155 µm filter (Milipore). Filtered supernatants were pelleted at 60,000 g for 16 hours at

156 4 °C in a SW28 Ti rotor (Beckman), and the resulting pellet was resuspended in TNE

157 buffer (50 mM Tris-HCl [pH 7.4], 0.1 M NaCl, 10 mM EDTA). This suspension was

158 adjusted to 0.5% v/v with octyl glucoside in TNE buffer and incubated for 30 minutes

159 on ice. Finally, virus was pelleted through a 30% w/v sucrose cushion in TNE buffer

160 for two hours at 200,000 g in a SW55 Ti Beckman rotor. The pelleted viral particles

161 were resuspended in TNE buffer.

162

163 **Viral infectivity assay**

164   Viral titers were determined by immuno-peroxidase staining to detect

165 infectious focus forming units (FFU) as described previously (11, 39). In brief, Caco-

166 2 cells were cultured to confluence in 96 wells plates and washed with serum-free

167 MEM before infection. Viral samples were activated with trypsin (200 µg/mL), for 1

168 hour at 37 °C, soybean trypsin inhibitor was added (200 µg/mL), and serial fold

169 dilutions of activated viral samples were performed. Diluted samples were added to

170 each well and let to adsorb for 1 hour at 37 °C. After adsorption period, the virus

171 inoculum in each well was removed, cells were washed twice with MEM and infection

172 was left to proceed in fresh MEM for 18 hours at 37°C. Cells were fixed for 20

173 minutes with 2% formaldehyde in phosphate-buffered saline (PBS), then they were

174 washed three times with PBS and permeabilized by a 15 minutes incubation with

175 0.2% Triton-X100 solution in PBS. Finally, cells were washed again three times with

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176   PBS and incubated with a polyclonal rabbit anti-Yuc8 overnight at 4°C. Next day

177   cells were washed out three times with PBS and incubated with peroxidase

178   conjugated protein A for 2 hours at 37°C. After washing protein A, infected cells were

179   revealed by carbazole precipitation and FFU were counted.

180

181   **Kinetics of viral release**

182   Caco-2 cells were grown to confluence in 24 wells plates. Cell monolayers

183   were washed twice with MEM and infected with activated HAstV Yuc8 strain (at an

184   MOI of 5). Supernatants were harvested at three hours intervals starting at 12 hours

185   post infection (hpi) until 24 hpi, and centrifuged for 5 minutes at 500 g to separate

186   cellular debris. At the same time, MEM was added to cellular monolayers and cells

187   were lysed by two cycles of freeze-thaw. Infectious viral particles associated to cells

188   and present in supernatants were determined by an immune-peroxidase assay as

189   described above. Before trypsin activation, samples were incubated for 30 minutes

190   at 37°C with MEM or with 0.1% Triton X-100 diluted in MEM.

191

192   **Purification of extracellular vesicles**

193   Caco-2 cells, grown to confluence in 150 cm^2 flasks, were washed twice with

194   MEM and infected with trypsin activated HAstV Yuc8 at an MOI of 5. As a control,

195   cells were mock infected using an identical protocol without virus. Supernatants were

196   harvested at 18 hpi and processed by differential centrifugation essentially as

197   described before (40, 41). Briefly, supernatants were centrifuged at 500 g for five

198   minutes to obtain pellet 1 (P1), and the supernatant was again centrifuged at 2,000

199   g for 30 minutes, obtaining the pellet 2 (P2). The remaining supernatant was

200   centrifuged at 20,000 g for one hour, producing pellet 3. Finally, the last supernatant

201   was mixed with an equal volume of a solution of 16% polyethylene glycol 6000 (PEG),

202   1 M sodium chloride and left overnight at 4°C. The mixture was then centrifugated

203   at 10,000 g for one hour, yielding pellet 4. As proposed by a theoretical analysis of

204   sedimentation (42), the purified fraction in pellet 3 was considered to contain large

205   extracellular vesicles (LEV), while the fraction of pellet 4 contains small extracellular

206   vesicles (SEV). All centrifugations were performed at 4 °C and all pellets were

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207   resuspended in sterile PBS. Virus titer in purified fractions was determined by

208   immune-peroxidase assay, with and without TX-100 treatment as described above

209   for supernatants in the assays of viral release kinetics.

210   For some experiments, in order to remove possible contaminants (i.e., free

211   contaminating virions or protein aggregates) from purified vesicles in LEV or SEV

212   fractions (pellets 3 and 4, respectively), the vesicle fractions were additionally

213   purified using the MagCapture^{TM} exosome isolation kit PS, according to the

214   manufacturer protocol.

215

Immunodetection of cellular and viral proteins

217   The fractions purified by differential centrifugation from supernatants of

218   infected and mock-infected Caco-2 cells were mixed with Laemmli sample buffer (50

219   mM Tris, pH 7.5, 2% SDS, 2% β-mercaptoethanol, 10 mM EDTA and 0.1%

220   bromophenol blue), boiled for 5 min and the proteins were separated by sodium

221   dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were

222   transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA). Membranes

223   were blocked with 5% non-fat dried milk in PBS. The proteins of interest were

224   detected with specific primary antibodies followed by incubation with secondary

225   peroxidase-conjugated reagents. Primary antibodies were incubated with

226   membranes overnight at 4°C, washed three times with PBS 0.1% Tween (PBS-T)

227   and incubation continued with peroxidase conjugated secondary antibody or protein

228   A for 90 min, at room temperature. After these incubations the membranes were

229   washed again with PBS-T and proteins were visualized by Western Lightning

230   Chemiluminescence Reagent Plus (Perkin Elmer).

231

Infectivity associated to the extracellular vesicles

233   Fractions were purified from supernatant of astrovirus infected Caco-2 cells

234   by differential centrifugation coupled with isolation with magnetic beads

235   (MagCapture^{TM} exosome isolation kit PS), as described above. Vesicle containing

236   fractions were diluted in MEM and added to Caco-2 and MA104 cells, grown to

237   confluence in 96 wells plates, washed twice with MEM before addition. The fractions

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238   were subjected to the following treatments before adsorption: with 0.1% Triton X-
239   100 for 30 minutes at 37°C; or were preincubated with an anti-Yuc8 neutralizing
240   antibody for 1 hour at 37°C; or by an incubation with 0.1% Triton X-100 for 30
241   minutes at 37°C, followed by neutralization with anti-Yuc8 antibody (1 hour at 37°C).
242   Control, non-treated samples were incubated in the same conditions but using an
243   equivalent volume of FBS-free MEM instead of Triton X-100 and/or anti-Yuc8
244   antibody. Vesicles were left to adsorb to cells during 2 hours at 37°C, then cells were
245   washed, fresh medium was added and the infection was left to proceed for 18 hours.

246   To test the capacity of purified EV to promote infection with externally bound
247   virus particles, LEV and SEV vesicles were purified from non-infected Caco-2 cells
248   as described above, including the isolation step with the MagCapture™ exosome
249   isolation kit PS. These vesicles were then incubated for 1 hour at 37 °C with a known
250   amount of non-activated purified HAstV Yuc8. After incubation, the vesicles were
251   treated as described above (0.1% Triton X-100 for 30 minutes at 37°C, anti-Yuc8
252   neutralizing antibody for 1 hour at 37°C or 0.1 % Triton X-100 followed by
253   neutralization with anti-Yuc8 antibody). After the treatments, vesicles with virus were
254   added to Caco-2 or MA104 cells grown in 96 wells, and incubated during 2 hours at
255   37°C. After this time cells were washed, and infection was left to proceed for 18
256   hours. As control, an equal amount of the identical purified trypsin activated or non-
257   activated astrovirus was used in the same conditions without EV incubation. Infected
258   cells were counted in selected area, of two wells per sample using 20X lens. Images
259   were acquired with 10X lens in a Nikon Diaphot 300 microscope.

260

261   **Transmission Electron Microscopy**

262   LEV and SEV vesicular fractions were purified from infected Caco-2 cells as
263   described above, using differential centrifugation coupled to isolation with the
264   MagCapture™ exosome isolation kit PS. Purified fractions and non-activated purified
265   virus particles were bound on carbon vaporized copper grids covered with formvar
266   and negatively stained with 3% uranyl acetate. Images were acquired using a Zeiss
267   Libra 120 electron microscope operating at 80 KV coupled with a GATAN Multiscan
268   600HP 794 CCD camera.

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Nanoparticle Tracking Analysis

Nanoparticle tracking analysis (NTA), was conducted using a NanoSight NS300 (Malvern Instruments Ltd., Worcestershire, UK) to assess the hydrodynamic diameter of non-activated virus particles and vesicles purified by differential centrifugation from infected and non-infected cells supernatants. Purified fractions were analyzed after diluting the samples in sterile and microfiltered PBS (1:100-200 in the case of vesicles and 1:1000 in case of purified virus particles). For each condition 5 videos of one-minute length each, were recorded sizing 20-40 particles/frame and analyzed using the NanoSight NTA 3.1 software (43). This technique uses dynamic light scattering to measure the diffusion coefficient of particles moving under Brownian motion and converts it to hydrodynamic diameter using the Stokes-Einstein equation (44). A blank of sterile filtered PBS was used for particle calculations in every measurement, and after each measurement the flushing lines were thoroughly washed three times to prevent contamination.

Statistical Analysis

Statistical analysis of the obtained results was performed using the GraphPad prism 5.0 software (GraphPad Software, Inc.), with an interval of confidence of 95%.

Results

HAstV Yuc8 titer in detergent-treated media increases with time of infection

It has been previously observed that a fraction of the astrovirus particles produced in infected cells floats to low-density fractions when separated by density centrifugation, suggesting that they interact with membranous structures (14). To characterize the possible association of astrovirus particles with membranes in the cell culture media, we evaluated the kinetics of astrovirus release in Caco-2 cells. Media from astrovirus infected cells were collected at different time points after infection (from 12 to 24 hpi), and the virus titer was determined. The infectivity of the virus in the supernatant was activated with trypsin after the samples were treated or not with detergent (Triton X-100). Under these conditions, if astrovirus particles were

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300   associated with vesicles or membranes, the detergent treatment would release the

301   virions, leading to an increase in viral titer as compared to the titer of samples not

302   treated with detergent. We observed the virus present in the supernatant starting at

303   the first time point analyzed (12 hpi), without any significant change in the titer after

304   Triton X-100 treatment ($p>0.05$) (Fig. 1), similar to previously published results (10).

305   The titer of virus present in the media not treated with detergent showed little

306   increase from 15 to 24 h post infection, however, the viral titer increased

307   considerably in the supernatant after detergent treatment, reaching almost twice as

308   much infectivity compared to non-treated samples, at later time points (Fig. 1). No

309   significant cellular damage was detected at the different time points (being under

310   10% of total LDH in both mock and Yuc8 infected cells), as determined by an LDH

311   assay (results not shown). These results suggest that astrovirus particles are

312   released from infected cells before appreciable cell lysis, and that they could be

313   associated with detergent soluble structures in the extracellular medium. In all the

314   subsequent experiments shown here, media were harvested at 18 hpi.

315

316   **Astrovirus infection increases the secretion of extracellular vesicles**

317   **from Caco-2 cells**

318   To characterize the effect of astrovirus infection on the production of EV in

319   Caco-2 cells, supernatants from infected or mock-infected cells cultured in serum-

320   free MEM were harvested at 18 hpi and processed by differential centrifugation.

321   Initially, detached cells were pelleted at 500 g for 5 min, getting pellet 1 (P1). Pellet

322   2 (P2) was obtained by centrifugation of the remaining supernatant at 2,000 g for 30

323   min. We expected this fraction to contain very large vesicles and some cell debris

324   and organelles. Pellet 3 was obtained by centrifugation of the remaining supernatant

325   at 20,000 g for 1 h, to collect large extracellular vesicles (LEV), theoretically

326   calculated to be over 122 nm (42). Finally, pellet 4 was obtained by overnight

327   precipitation of remaining vesicles and particles in the remaining supernatant by 8%

328   PEG 6000 and 0.5 M NaCl, followed by centrifugation at 10,000 g for 1 h, producing

329   small extracellular vesicles fraction (SEV), calculated to be under 170 nm (41, 42).

330   As consequence, we expect some size overlapping between LEV and SEV fractions.

331   An equal portion of each pellet fraction was analyzed after SDS-PAGE. By

332   silver staining of the gel, it was clear that the amount of total proteins present in each

333   fraction was increased in astrovirus-infected cells (Fig. 2A). The presence of different

334   cellular markers in the pelleted fractions was analyzed by immunoblotting; EV

335   specific markers tested were CD63 and ALIX, while endoplasmic reticulum

336   associated PDI protein was used as non-EV associated protein control. In fractions

337   P1 and P2, which probably contain cells, cell debris and large vesicles, all proteins

338   markers were observed, and their presence also has increased after astrovirus

339   infection. Interestingly, the LEV and SEV fractions purified from astrovirus-infected

340   Caco-2 cells showed a higher content of EV specific proteins (ALIX and CD63) as

341   compared to mock-infected cells (Fig. 2B), presumably representing larger amounts

342   of EVs. (Fig. 2B).

343   To quantitate the concentration and size of the purified vesicles more

344   precisely, the pellet 3 (LEV fraction) and the fraction purified after PEG 6000

345   precipitation (SEV fraction) were analyzed by nanoparticle tracking analysis (Fig. 3).

346   There was a clear and significative increase in the vesicle number in the LEV fraction

347   from Yuc8-infected cells (p<0.05), compared to that of mock-infected cells (Fig. 3A

348   and 3C). In the case of the SEV fraction obtained by PEG 6000 precipitation, there

349   was only a small, not significant increase (p>0.05) on the number of vesicles present

350   in preparations obtained from either infected cells or mock-infected cells (Fig. 3B

351   and 3C). These results suggest that astrovirus infection might stimulate the

352   production of EV, particularly those present in the LEV fraction.

353

354   **Astrovirus particles seem to associate with extracellular vesicles**

355   Given the presence of vesicles with different sizes in the cell culture medium,

356   we analyzed whether astrovirus particles were associated with a particular fraction

357   and if an increased infectivity could be observed after treating the different fractions

358   with Triton X-100 before activation of the virus with trypsin. We found that different

359   amounts of infectious viral particles were present in fractions P2, LEV, and SEV;

360   treatment with Triton X-100 before trypsin activation significantly increased virus titer

361   in fractions P2 (p<0.05) and LEV (p<0.01), but not in fraction SEV (Fig. 4A). These

362   observations suggest that a portion of the astrovirus particles could be present inside

363   vesicles or, alternatively, that groups of viral particles could be associated with EV

364   from the outside, and consequently membrane solubilization releases individual

365   particles, increasing virus titer.

366   To determine if there is a direct association between virions and vesicles in

367   the LEV fraction, we analyzed by transmission electron microscopy (TEM) this

368   fraction purified from astrovirus infected Caco2 cells. The LEV fraction was chosen

369   since the largest increase in virus infectivity when the trypsin activation was done

370   after the Triton X-100 treatment was observed in this fraction. By TEM we found

371   virus-like particles, associated with what appeared to be vesicles (Fig. 4B, pointed

372   by arrows). The electro-dense virus-like particles observed in this micrograph, are

373   similar in form and size (30 nm) to purified astrovirus particles (Fig. 4C), suggesting

374   that they represent bona-fide virus particles associated with membranes. Such virus-

375   like particles were not observed in vesicles present in LEV fraction purified from

376   mock-infected cells (data not shown). Since the infectivity of astroviruses requires

377   activation by proteolytic processing of the VP70 protein precursor, we analyzed by

378   western blot the virus protein composition of the LEV-associated virions. We

379   observed that the virus particles are mainly composed by the VP70 protein (70 KDa)

380   with no evidence of neither VP90 precursor protein, nor any activated viral proteins

381   of 34, 27 or 25 KDa proteins (Fig. 4D).

382

383   **Vesicle-associated astrovirus particles are infectious without**

384   **proteolytic treatment and are protected from antibody neutralization**

385   EVs have an intrinsic capacity to fuse with other cells, and thus to transfer

386   proteins, genetic material, and even viral particles to other recipient cells (16, 21, 24,

387   45). Using this mechanism, different types of viruses are able to infect otherwise

388   refractory cells. Such is the case of human immunodeficiency virus 1 (HIV-1) (46,

389   47) and herpes simplex virus 1 (HSV-1) (33). The association with vesicles has also

390   been shown to confer some viruses with resistance to neutralization with specific

391   antibodies [hepatitis A virus (HAV), or HSV-1] (33, 48). To test whether vesicle-

392   associated astrovirus strain Yuc8 is able to infect other cell lines, vesicles present in

393   the LEV and SEV fractions purified from infected Caco-2 cells were added to Caco-

394   2 and MA104 cells. Caco-2 cells were used as fully permissive cell line, while MA104

395   cells are at least 100 times less permissive to astrovirus Yuc8 infection (8, 49).

396   Before adding to the cell monolayers, the samples were either incubated with 0.1%

397   Triton X-100 for 1 h to disrupt possible membranes; or incubated with polyclonal

398   neutralizing polyclonal antibodies to Yuc8 to neutralize the infectivity of accessible

399   viral particles; or incubated with detergent followed by neutralization with the

400   neutralizing polyclonal antibodies in order to neutralize all viral particles present. As

401   control, fractions were only incubated in MEM.

402   The results of these assays show that the LEV and SEV vesicle-associated

403   astrovirus viral particles were able to infect both Caco-2 and MA104 cells (Fig. 5A

404   and B), while viral infection was completely abolished after membrane solubilization

405   with Triton X-100, suggesting that membranes or vesicles are indispensable for

406   infection, since astrovirus particles in these assays were not proteolytically activated.

407   Preincubation of both types of vesicles with anti-Yuc8 neutralizing polyclonal

408   antibody left a fraction of virus particles infectious, suggesting that some of these

409   viruses (10-20%) were protected from the neutralization by the antibodies, possibly

410   by being inside the vesicles (Fig. 5A, B and C). Accordingly, pretreatment of the

411   vesicle fractions with detergent, allowed complete antibody neutralization of the virus

412   particles (Fig. 5A and B), supporting the hypothesis that vesicles in these fractions

413   are important to allow viral infection and to shield viral particles from neutralizing

414   antibodies.

415   Protected viral particles were observed in both, LEV and SEV fractions, and

416   they were able to infect a similar number of both Caco-2 and MA104 cells (Fig. 5C).

417   When the infectivity was compared between Caco-2 and MA104 cells, Caco-2 cells

418   showed more infected cells by LEV fraction than MA104 cells, while infection

419   associated with SEV fraction was similar between both cell lines (Fig. 5C). In Caco-

420   2 cells there were more infected cells after infection with vesicles from LEV fraction,

421   as compared to SEV fraction, while in MA104 cells infectivity of these two fractions

422   was similar (Fig. 5C).

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Association of non-activated astrovirus Yuc8 with purified EVs enhances viral infectivity

To further evaluate the possibility that the association of the virus with membranous structures promotes virus infectivity without the need of trypsin treatment, non-activated purified Yuc8 particles were incubated for 1 h at 37 °C with LEV and SEV fractions obtained from supernatants of mock-infected Caco-2 cells. After virus-EV incubation, the virus-vesicle mixture was subjected to the same treatments described in the previous experiment: neutralization with polyclonal anti-Yuc8 antibody, membrane solubilization by incubation with 0.1% Triton X-100, or detergent treatment followed by neutralization. Untreated virus-vesicle samples, in MEM, were used as control. After treatment, the samples were added to Caco-2 and MA104 monolayers and infection was left to proceed as described. The non-activated astrovirus particles that were incubated with both types of purified vesicles (LEV and SEV fractions) acquired the capacity to infect both Caco-2 and MA104 cells (Fig. 6A and B). Detergent treatment of the samples before addition to the cells abolished the infectivity in both cell lines, again confirming the contribution of membrane vesicles to viral infectivity of particles non-activated by trypsin, possibly by direct interaction between virus and vesicles (Fig. 6A and B). Pre-incubation with neutralizing antibodies abolished infectivity, suggesting that all viral particles were accessible to the antibodies. Of note, no infection was detected when either cell line was incubated with the same amount of non-activated virus, in the absence of vesicles, unless the virus was activated by treatment with trypsin (Fig. 6C). As expected, the combined treatment of detergent and neutralizing antibodies also abolished the infection (Fig. 6A and B). These results suggest that free viral particles could associate with vesicles, and this interaction facilitates their cell entry and infectivity, even if the virus is not activated. When the infectivity of the vesicle-associated non-activated astrovirus particles was compared in Caco-2 and MA104 cells, both LEV and SEV fractions showed similar capacity to promote infection in both cell lines (Fig. 6C). Of interest, non-activated astrovirus particles incubated with purified EV, infected MA104 cells more efficiently (>200%) than the same amount of free virus activated with trypsin (Fig. 6C), while in Caco-2 cells (astrovirus fully

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455 permissive cell line), the vesicle-associated particles had a 17% average infectivity

456 of the free, trypsin activated virus (Fig. 6C).

457

458 **Discussion**

459   Astrovirus cell release has been reported to be a non-lytic process promoted

460 by caspase processing of the viral capsid-precursor protein VP90 to VP70 (10, 11).

461 It is a gradual process in which the majority of the new particles (about 90% of the

462 total progeny) remain inside infected cells, and only 10% are released to the

463 extracellular media (10). In this study, we found that a portion of the new progeny

464 was present in the cell supernatant as early as 12 hpi and the amount of released

465 virus increased with time. Interestingly, starting at 15 hpi, a significant portion of the

466 released virus particles were not susceptible to trypsin treatment, and required to be

467 solubilized from their association with membranous structures by detergent

468 treatment, to become accessible to the protease. The amount of virus protected from

469 trypsin increased with time. This observation suggests that there could be more

470 potentially infectious virus particles in the extracellular media than originally thought

471 (10), most probably explained by the association of viruses with EVs.

472   Using differential centrifugation, we purified several fractions of EV from the

473 media of astrovirus Yuc8 infected Caco-2 cells, and two of these fractions were

474 characterized in more detail: the LEV fraction, obtained after cellular debris depletion

475 and by centrifugation for 1 h at 20,000 g; and the SEV fraction, purified after PEG

476 6000 based precipitation and centrifugation at 10,000 g (40, 42). Both fractions

477 contained markers of extracellular vesicles, CD63 and Alix. Since CD63 is an

478 specific marker of exosomes (50, 51), and ALIX has been reported to be involved in

479 both microvesicle and exosome biogenesis (45), it is possible that both of these

480 vesicles, which overlap in size, are present in both fractions. Analysis of the

481 concentration and size of the vesicles in LEV and SEV fractions by nanoparticle

482 tracking showed partial overlap in size, however the LEV fractions showed a

483 significative increase in the vesicle number when infected versus mock-infected

484 conditions were compared. A similar increase in EV secretion after infection was

16

485   also reported with other viruses, like HIV-1 (52, 53), HSV-1 (33, 54), rotavirus (27)

486   and tick-borne Langat virus (55).

487   Different viruses have been found to be able to interact directly with EV.

488   Among these, hepatitis A virus (HAV) (48), HCV (29), HSV-1 (33), and JC

489   polyomavirus (34). Analysis of the LEV fraction, purified from astrovirus-infected

490   cells, showed electrodense astrovirus-like particles associated with vesicles of about

491   200 nm, resembling the appearance of extracellular vesicles (56). The membranous

492   structures observed by TEM seem to associate with more than one viral particle.

493   This observation opens up the possibility that during astrovirus infection, EV could

494   participate both as virion carriers, protecting the virions, as well as a form of

495   concentrating viral particles, forming the so called collective infectious units (CIU),

496   capable to gather together several infectious particles. Similar observations have

497   been made recently for rotavirus and norovirus, where several viral particles were

498   reported to be associated with vesicles (27, 57).

499   It has been described previously that the association with vesicles could

500   protect some viruses from neutralization by antibodies, for example hepatitis A, B

501   and C viruses (29, 48, 58), and HSV-1 (33), among others. In this work we observed

502   that a portion of the astrovirus particles present in LEV and SEV fractions remained

503   infectious even after incubation with an anti-Yuc8 neutralizing antibody, suggesting

504   that a portion of the isolated astrovirus particles were inaccessible to the neutralizing

505   antibodies. The presence of vesicles was crucial for the infectivity of these non-

506   activated viral particles, since the solubilization of membranes with detergent

507   abrogated all infectivity. To confirm that vesicles are important in non-activated

508   astrovirus infectivity, LEV and SEV vesicles were purified from non-infected Caco-2

509   cells, and then incubated with non-trypsin activated purified astrovirus particles. Our

510   results showed that the purified virus, was able to interact with these vesicles, and

511   acquired the capacity to enter and infect the cells without protease activation. It is

512   not clear if these interactions between astrovirus particle and EVs interaction are

513   specific or not, but the virus particles in this mix acquired the capacity to infect even

514   low susceptibility cells like MA104. The infectivity was abolished by solubilization of

515   the vesicles with detergent, or by incubation with neutralizing antibodies, suggesting

17

516   that the interaction between viral particles and EV somehow facilitates the interaction

517   between the virus and the cell surface.

518   Since extracellular vesicles could facilitate the internalization of the virus

519   apparently through a viral receptor independent pathway, the viral particles

520   associated with vesicles could be internalized by a mechanism triggered by vesicles

521   themselves (59). The incubation of non-activated purified astrovirus with LEV or SEV

522   fractions leads to similar level of infection in both Caco-2 and MA104 cells, while

523   non-activated purified astrovirus particles were not able to infect these cell lines.

524   These results suggest that the astrovirus proteolytic processing by trypsin

525   (activation), is important for virus-cell adhesion and/or entry, but probably not for the

526   decapsidation process. The ability of astrovirus particles associated with vesicles to

527   infect not only susceptible Caco-2 cells, but also the poorly susceptible MA104 cell

528   line (Fig 6C), suggest that the vesicle-associated virus particles could bypass certain

529   blocks in astrovirus tropism, probably the specific virus-receptor interaction,

530   potentially increasing their pathogenicity. This observation also suggest that

531   extracellular vesicles could help astrovirus to disseminate outside the

532   gastrointestinal tract like it has been reported before for HAstV serotype 4 and the

533   novel astroviruses strains MLB and VA (2), possibly by allowing astroviruses to avoid

534   the immune response and cellular barriers until they get into permissive cells far

535   away from their common environment (gastrointestinal tract).

536   Our observations suggest the possibility that EV could be acting as platforms

537   to create collective infectious units (60, 61), rendering virus particles insensitive to

538   neutralization with antibodies and promoting their internalization in a non-receptor

539   dependent manner. The mechanisms by which EV promote viral internalization in

540   new cells remain unclear, as well as the contribution of EV to the whole astrovirus

541   infectivity.

542

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713

Figure legends

714   **Figure legends**

715   **Figure 1. Kinetics of astrovirus release from infected Caco-2 cells.** Caco-

716   2 cells were infected with astrovirus Yuc8 at MOI of 5 and supernatants were

717   collected at different time points post infection (from 12 to 24 hours). Viral titer was

718   determined with (grey bars) and without (white bars) 0.1% Triton X-100 treatment

719   before trypsin activation. The results represent the mean focus forming units (FFU)

720   per milliliter (mL) ± standard error of the mean of three independent experiments

721   done in duplicate. Statistical analysis was done with two-way ANOVA, p value

722   *<0.05; ** p<0.01, *** p<0.001.

723

724   **Figure 2. Identification of vesicular markers in vesicles purified from**

725   **Caco-2 cells.** Caco-2 cells were infected or mock-infected with astrovirus strain

726 Yuc8 (MOI 5) and 18 hours post infection the supernatant was collected. Different

727 fractions were purified by differential centrifugation; pellet 1 was obtained by

728 centrifugation at 500 g (P1), pellet 2 obtained by centrifugation at 2,000 g (P2),

729 fraction containing large extracellular vesicles (LEV) was obtained after 20,000 g

730 centrifugation and final small extracellular vesicles (SEV) fraction was obtained by

731 precipitation with 8% polyethylene glycol 6000, 0.5M NaCl. (A) The same volume of

732 each pelleted fraction was separated by SDS-PAGE and proteins were detected by

733 silver staining. (B) Samples were resolved on SDS-PAGE and analyzed by western

734 blotting, using antibodies specific for CD63 and Alix as vesicle markers and protein

735 disulfide isomerase (PDI) as endoplasmic reticulum protein to assess preparation

736 contamination. Immunoblots are representative of five independent experiments.

737 **Figure 3. Astrovirus Yuc8 infection stimulates secretion of extracellular**

738 **vesicles in Caco-2 cells.** Confluently grown Caco-2 cells were infected with

739 astrovirus Yuc8 (MOI 5) or mock infected. Supernatants were harvested 18 hours

740 post infection and processed by differential centrifugation. Fractions obtained after

741 pelleting at 20,000 g, corresponding to large extracellular vesicles (LEV) (A) and

742 obtained after PEG 6000 precipitation, small extracellular vesicles (SEV) (B), were

743 resuspended in PBS and used for nanoparticle tracking analysis in the NanoSight

744 NS300. In each experiment five videos were recorded and used for analysis.

745 Distribution of particle-vesicle size (hydrodynamic diameter in nm) and concentration

746 (particles/mL) from 3 to 5 independent experiments are shown. Vesicles purified

747 from mock infected cells are represented by blue line, Yuc8 purified vesicles are

748 represented by red line. (C) Comparison of the mean number of particles present in

749 LEV and SEV fractions shown in A and B. All results are expressed as the mean of

750 the whole concentration of particles ± standard error of the mean of three

751 independent experiments. Statistical analysis was done using two-way ANOVA *

752 $p < 0.05$.

753 **Figure 4. Astrovirus particles associate with large extracellular vesicles.**

754 Confluently grown Caco-2 cells were infected with astrovirus Yuc8 (MOI 5) or mock

755 infected. Supernatants were harvested 18 hours post infection and processed by

756 differential centrifugation. Fraction pellet 2 was obtained by centrifugation at 2,000 g

757 (P2), fraction containing large extracellular vesicles (LEV) was obtained after

758 centrifugation at 20,000 g and final small extracellular vesicles (SEV) fraction was

759 obtained by precipitation with PEG 6000, and NaCl after centrifugation at 10,000 g.

760 All fractions were resuspended in same volume (100 µL) of sterile PBS. (A) Purified

761 fractions were trypsin activated, with or without previous incubation with detergent,

762 and titered in Caco-2 cells. Viral content was expressed as total focus forming units.

763 Grey bars represent samples treated with 0.1% Triton X-100 prior trypsin activation;

764 white bars (MEM), represent samples activated with trypsin without Triton X-100

765 treatment. The mean of viral particles in each sample ± standard error of the mean

766 of three independent experiments are shown. (B) Large extracellular vesicles

767 purified from Yuc8 infected Caco-2 cells as described in A were further clarified by

768 additional isolation using MagCapture^{TM} Exosome Isolation kit PS. One drop of

769 sample was fixed onto carbon vaporized coper grids and negative stained with uranyl

770 acetate. Samples were observed in EFTEM ZEISS Libra 120 electron microscope.

771 Electrodense particles of 30 nm, possibly viral particles, are pointed by black arrows.

772 (C) Purified Yuc8 virions were bound to carbon vaporized coper grids and stained

773 as described in B. Electrodense particles, which resemble astrovirus particles are

774 pointed by arrows. Size bars are shown. (D) Immunoblotting of LEV fraction. Sample

775 used in A, purified from Caco-2 infected cells as described in A, was not-treated

776 (lane 1) or treated (lane 2) with trypsin, and separated in SDS-PAGE, transferred to

777 nitrocellulose membrane and viral proteins were detected using anti-astrovirus

778 polyclonal antibody. Viral proteins are pointed on right hand side, while molecular

779 weight in kilodaltons is shown on left hand side. Images are representative of three

780 independent experiments yielding similar results. Statistical analysis was done with

781 two-way ANOVA, p value *<0.05; ** p<0.01, *** p<0.001.

782 **Figure 5. Vesicle associated astrovirus is infective in Caco-2 and MA104**

783 **cells.** Large extracellular vesicles (LEV) and small extracellular vesicles (SEV) were

784 purified by differential centrifugation and MagCapture^{TM} exosome isolation kit PS

785 from supernatants of Caco-2 cells infected with astrovirus (strain Yuc8). Samples

786 were treated with medium (MEM); 0.1% Triton X-100; anti-Yuc8 (1:1500); or 0.1%

787 Triton X-100 followed by anti-Yuc8 (1:1500) before addition to (A) Caco-2 or (B)

788   MA104 cells. Treated samples were let to adsorb for two hours, after which time

789   unbound vesicles were washed and infection was left to proceed for 18 hours.

790   Infected cells were detected by immuno-peroxidase staining (darker cells). Cells

791   were observed in a Nikon Diaphot 300 microscope with 10X magnification and they

792   are representative of three independent experiments done in three wells each. (C)

793   Focus forming units (FFU) of each sample were counted in 3 wells of three

794   independent experiments. Bars represent the viral focus forming units (FFU) in each

795   sample ± standard error of the mean. Samples where 0 FFU were observed (Triton

796   X-100 and Triton X-100 plus anti-Yuc8 treatments) are not graphically represented.

797   Statistical analysis was done with two-way ANOVA, p value *<0.05; ** p<0.01, ***

798   p<0.001.

799

800   **Figure 6. The interaction of non-activated astrovirus Yuc8 with vesicles**

801   **enhances their infectivity**

802   Large extracellular vesicles (LEV) and small extracellular vesicles (SEV) were

803   purified by differential centrifugation and MagCapture^{TM} exosome isolation kit PS

804   from supernatants of non-infected Caco-2 cells. Purified vesicles were incubated

805   with purified non-activated astrovirus Yuc8 particles for 1 hour at 37°C. Vesicle-virus

806   mixtures were treated in 4 different conditions: medium (MEM); 0.1% Triton X-100;

807   anti-Yuc8 (1:1500); and 0.1% Triton X-100 followed by anti-Yuc8 (1:1500). Treated

808   fractions were then let to adsorb in (A) Caco-2 or (B) MA104 cells for two hours, after

809   which time the unbound vesicles and viral particles were washed out. After 18 hpi

810   infected cells were detected by immuno-peroxidase staining (darker cells). Images

811   magnified with a 10X lens were observed in a Nikon Diaphot 300 microscope and

812   they are representative of three independent experiments done in triplicate. (C)

813   Infected cells observed in experimetns described in A and B were counted in three

814   wells of three independent experiments and compared with the same amount of

815   trypsin activated (Yuc8 Act) and non-activated (Yuc8 NA) astrovirus probed without

816   pre-incubation with EV in Caco-2 and MA104 cells. Graphics shown the amount of

817   infected cell in each sample expressed as focus forming units (FFU). Bars represent

818   the mean FFU ± standard error of the mean. Statistical analysis was done with two-

819   way ANOVA, p value *<0.05; ** p<0.01, *** p<0.001.

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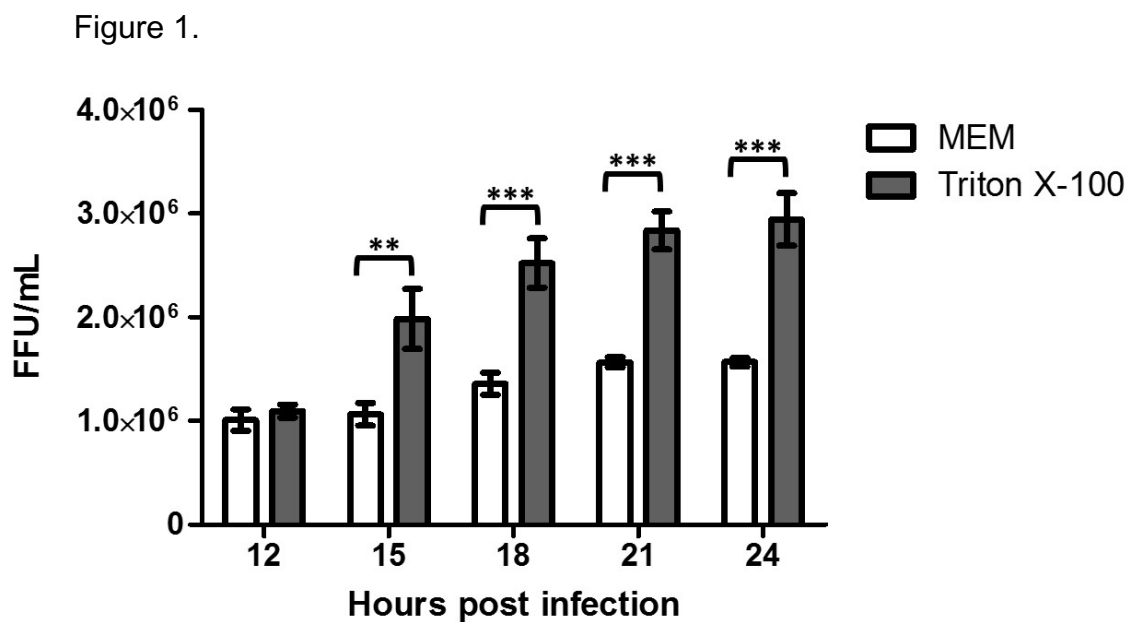
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849 **Figures**

850

851 Figure 1.

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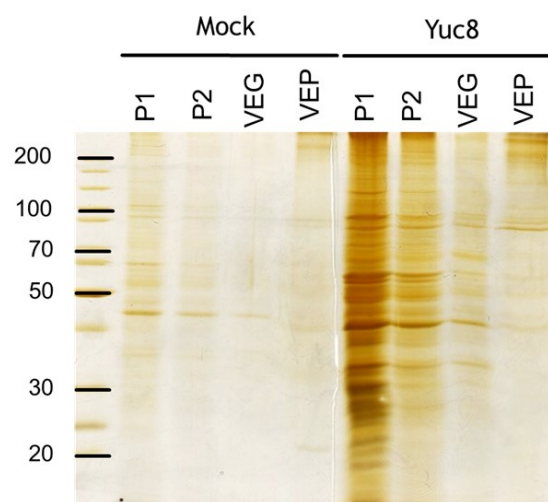
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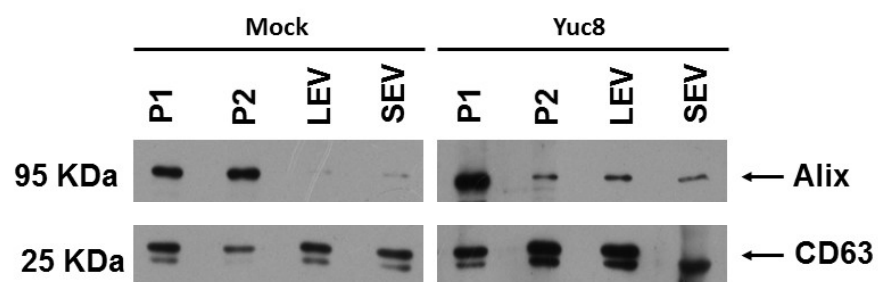
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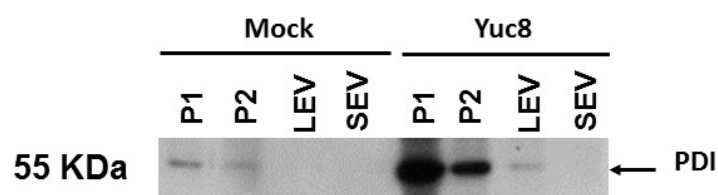
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869   Figure 2.

870   A.

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872   B.

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882 Figure 3.

883 A.
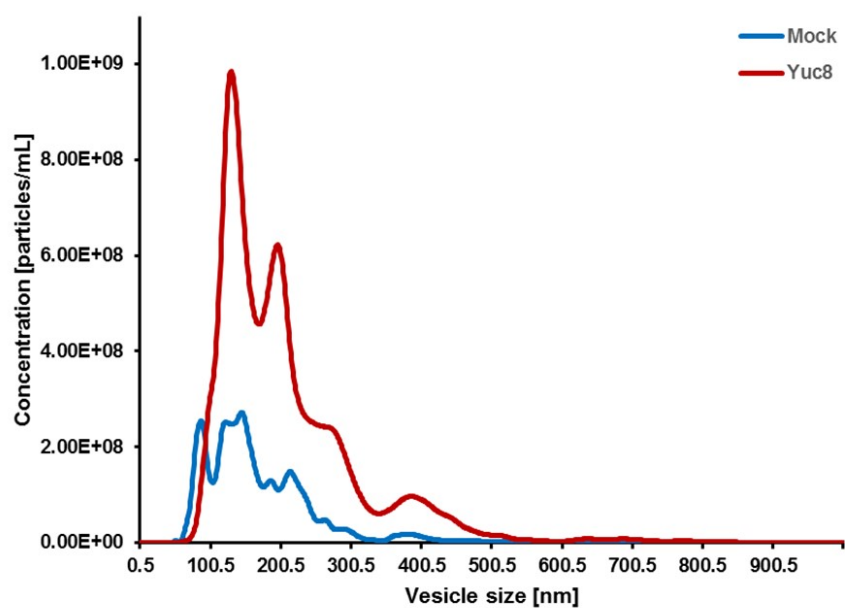
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885 B.
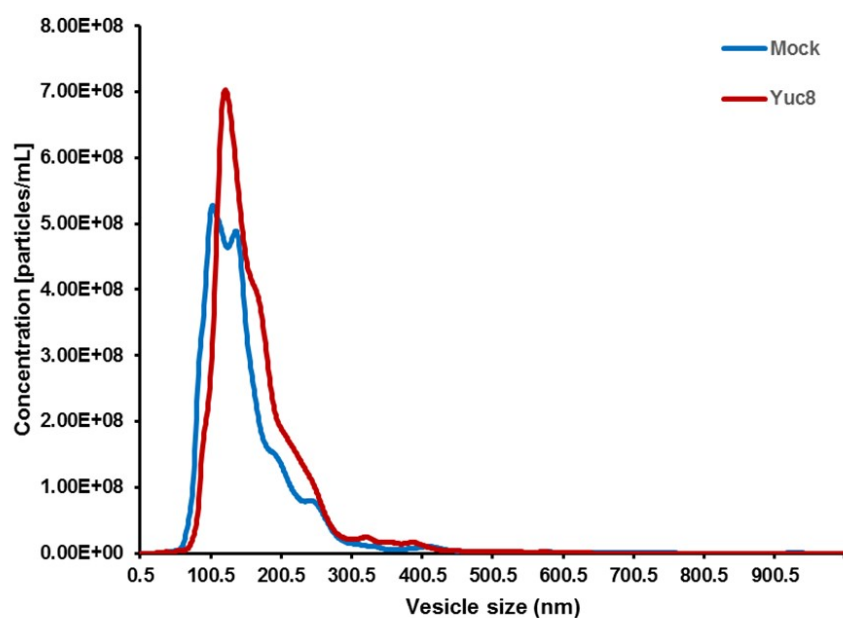
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891   C.
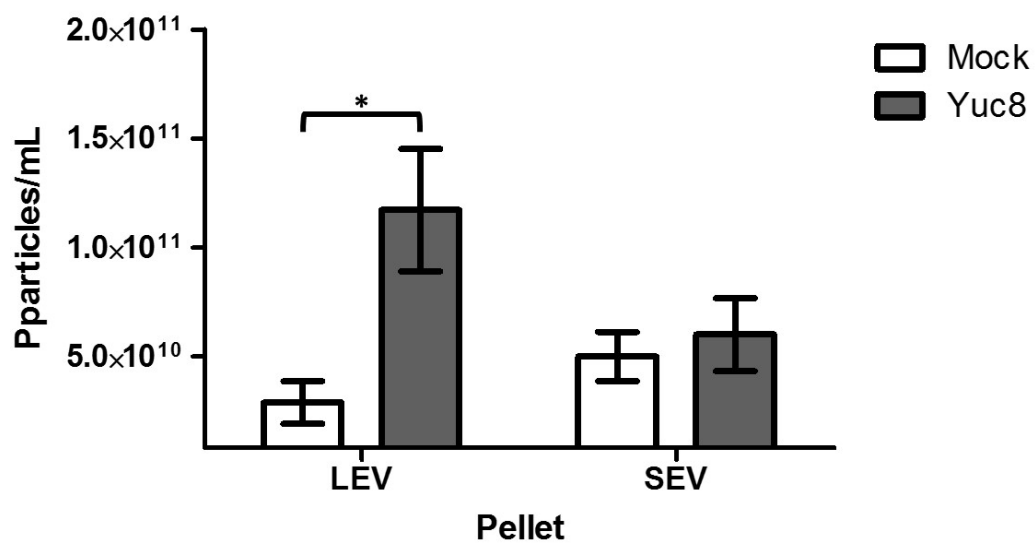
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912   Figure 4.

913   A.
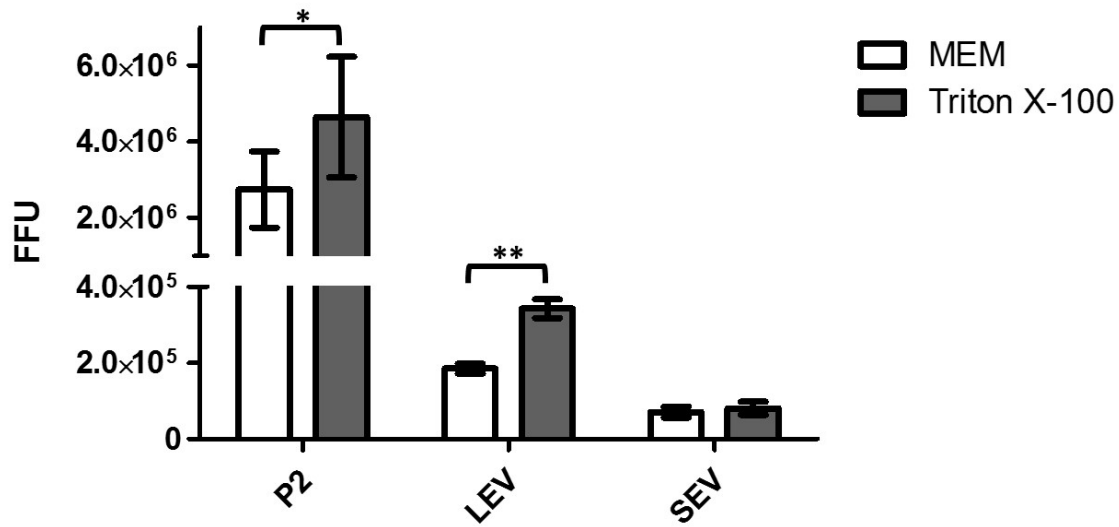
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915   B.
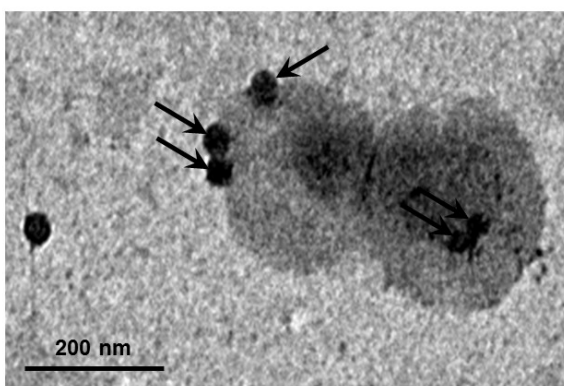
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925   C.

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200 nm
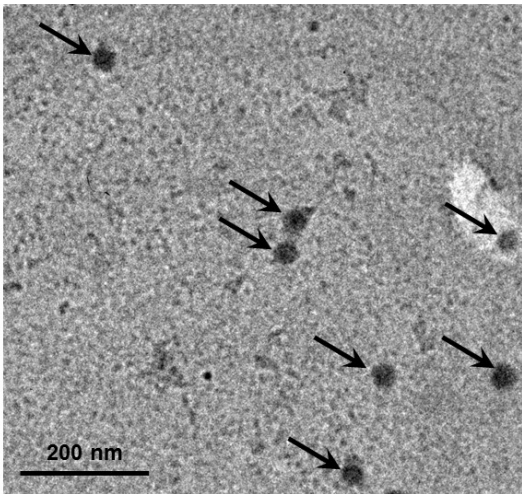
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932   D.

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| MW | 1 | 2 |

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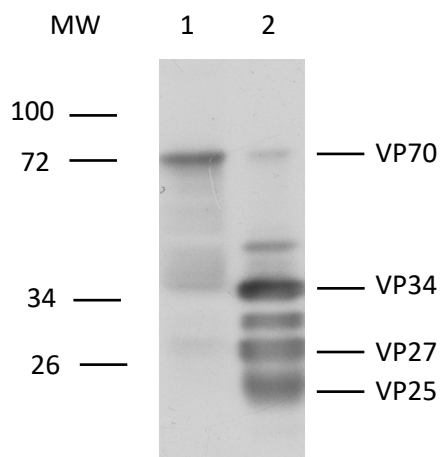
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947   A.
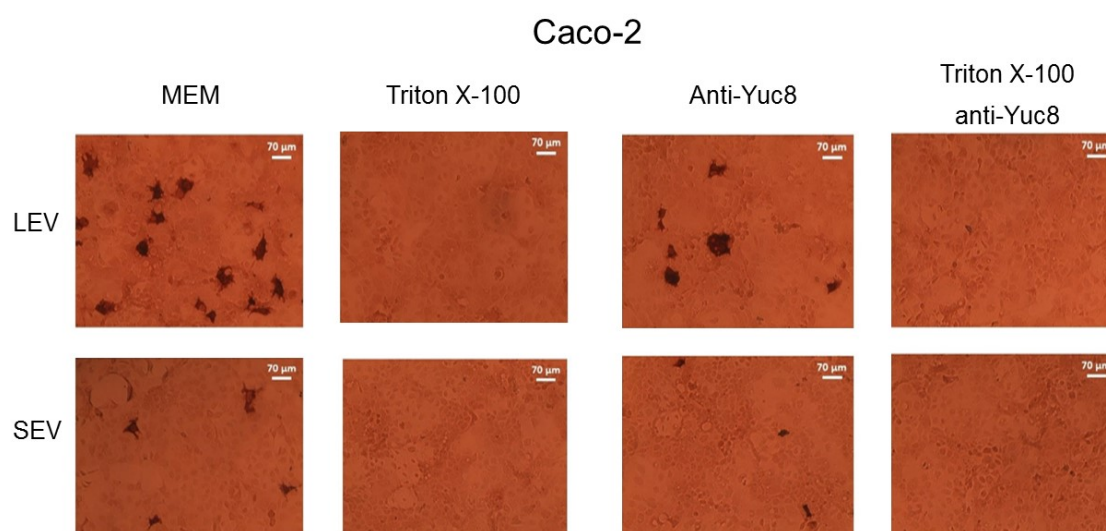
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949   B.
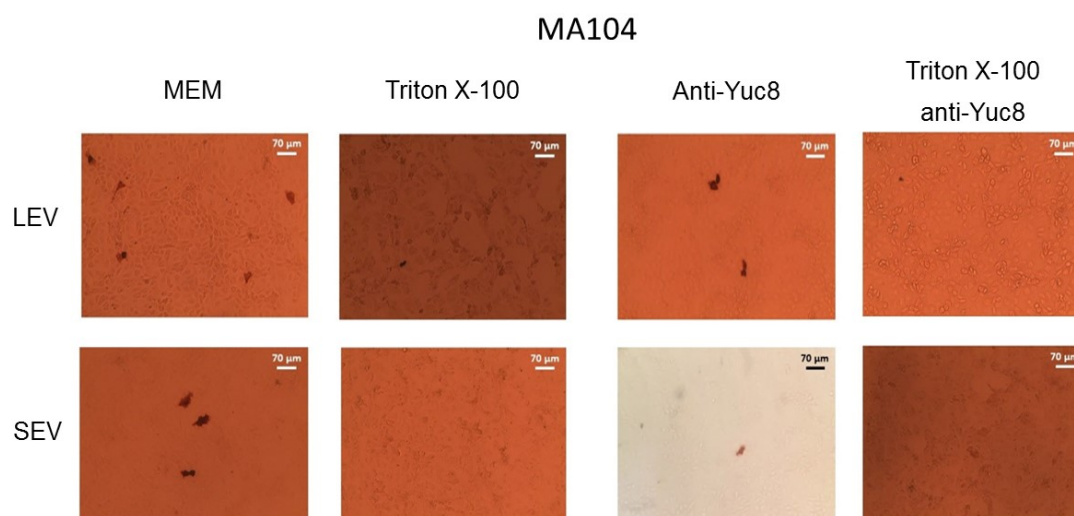
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958   C.
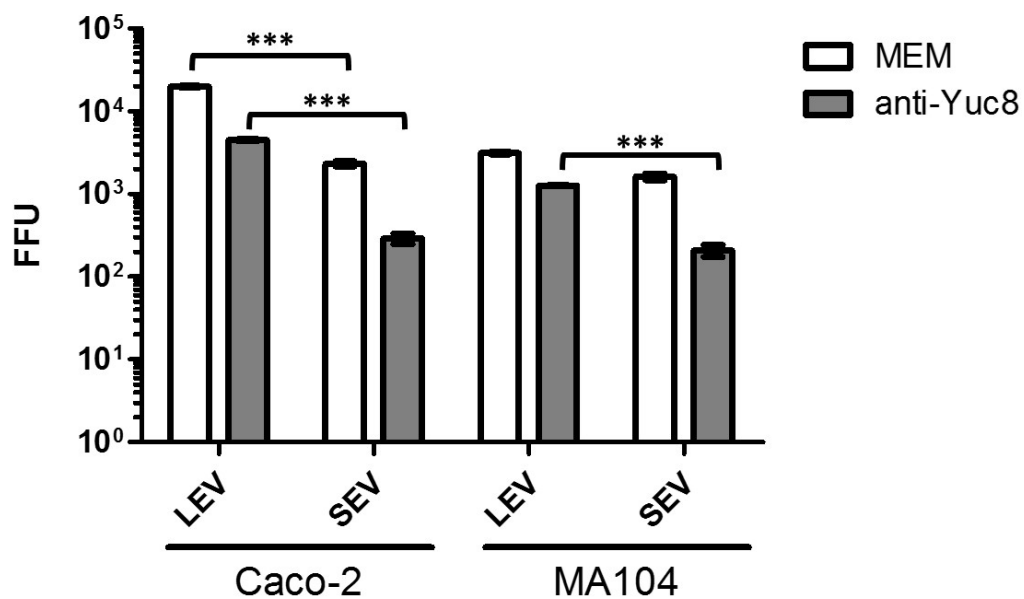
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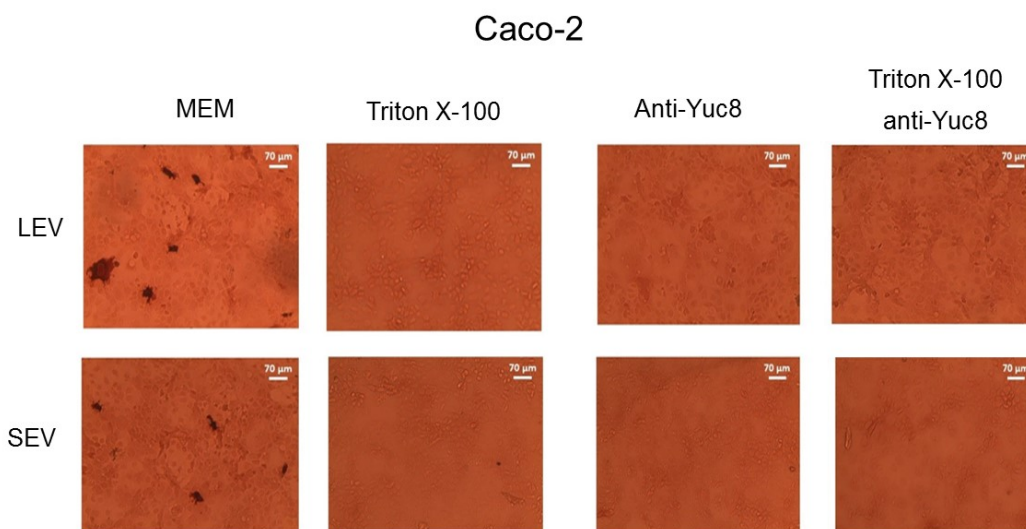
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976   Figure 6.

977   A.

Caco-2

978

979   B.

MA104
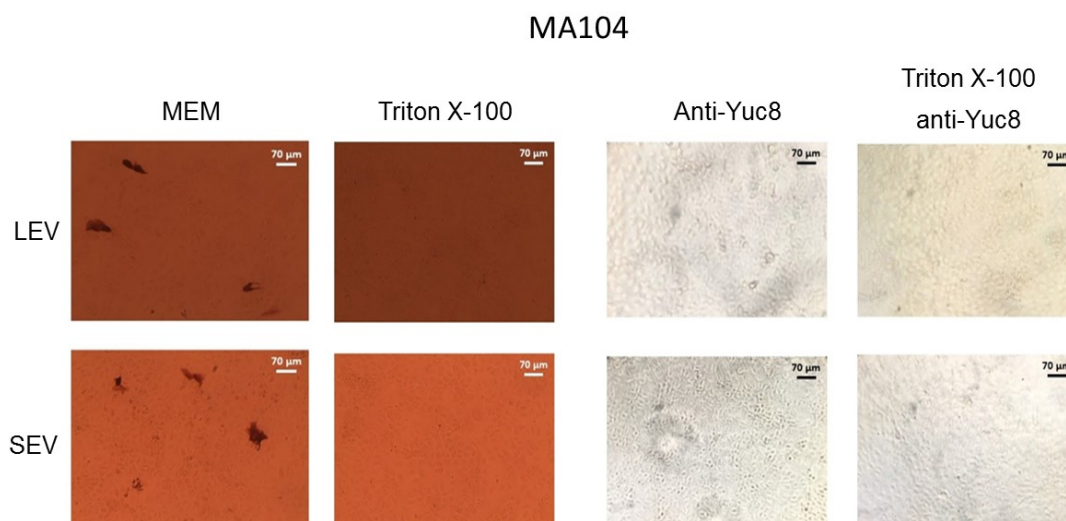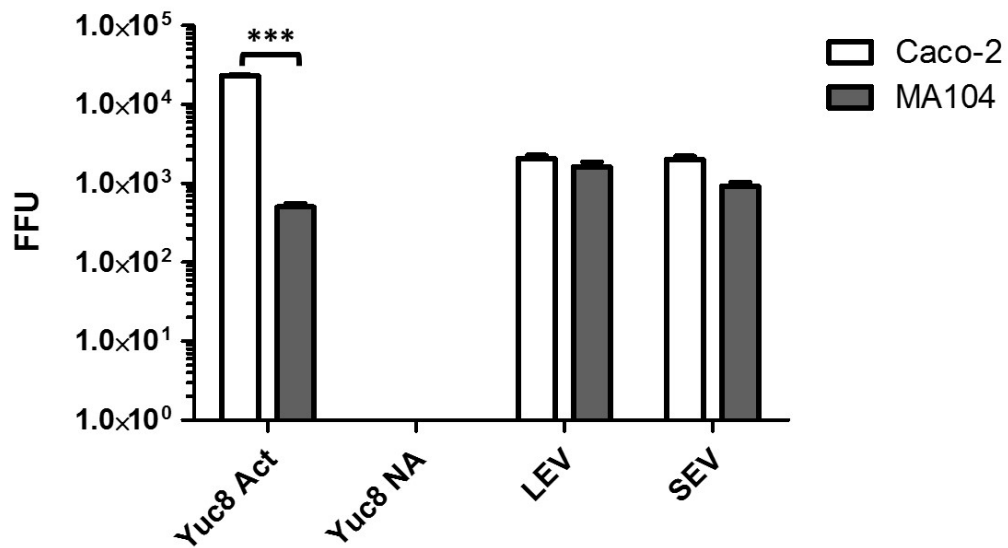

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988 C.

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