Short Communication

IgA-coated particles of *Hepatitis A virus* are translocalized antivectorially from the apical to the basolateral site of polarized epithelial cells via the polymeric immunoglobulin receptor

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Although *Hepatitis A virus* (HAV) is transmitted by the faecal–oral route, its target for replication is the liver. Little is known of its interactions with cells of the gastrointestinal tract, and it is not known by which mechanisms HAV crosses the intestinal epithelium. In this study, it is shown that HAV associated with IgA is translocated from the apical to the basolateral compartment of polarized epithelial cells via the polymeric immunoglobulin receptor by IgA-mediated reverse transcytosis. The relevance of this mechanism, by which HAV–IgA complexes may overcome the intestinal barrier and contribute to infections of the liver, results from the fact that HAV–IgA complexes are infectious for hepatocytes and that significant amounts of intestinal HAV–IgA are present during acute infections, which are also partly transmitted. Besides supporting the primary infection, this mechanism may play a role in relapsing infections by establishing an enterohepatic cycle for HAV.

Hepatitis A virus (HAV), a picornavirus, is spread by the faecal–oral route, but the site of virus replication is the liver (reviewed by Cuthbert, 2001). The events that occur during the passage of HAV across the intestinal epithelium into blood, in which the virus reaches the liver, are not understood.

Only weak evidence has been provided for replication of HAV in intestinal cells of animal models (Karayiannis *et al.*, 1986; Asher *et al.*, 1995; Hornei *et al.*, 2001), and Blank *et al.* (2000) demonstrated, by the observation of vectorial apical (lumenal) entry and release of HAV from Caco-2 cells, that infection of polarized intestinal cells by HAV does not result in penetration of the intestinal epithelium.

An alternative mechanism is transcytosis of HAV across the epithelial barrier of the intestinal tract without infection of this area. However, transcytosis of HAV virions by intestinal epithelial cells could not be detected (Blank *et al.*, 2000) and transcytosis by M cells (Silvey *et al.*, 2001; Ouzilou *et al.*, 2002) has never been demonstrated.

One additional possibility for HAV to overcome the intestinal barrier is carrier-mediated transport. In an earlier study, we demonstrated that HAV–anti-HAV IgA complexes (HAV–IgA) are infectious for human hepatocytes by IgA-mediated endocytosis via the IgA-specific hepatocellular asialoglycoprotein receptor (ASGPR) (Dotzauer *et al.*, 2000). This IgA carrier mechanism may contribute to the efficiency of HAV infections and may even enable prolonged or relapsing courses of disease (Glikson *et al.*, 1992), which occur in the presence of otherwise neutralizing antibodies. We speculated that, seemingly contrary to the view of IgA function in mucosal defence (reviewed by Rojas & Apodaca, 2002), intestinal HAV–IgA may enter the bloodstream by existing transport activities of intestinal cells specific for IgA, allowing transepithelial passage of HAV–IgA complexes.

IgA transport across the intestinal epithelial layer is carried out by the epithelial polymeric immunoglobulin receptor (pIgR) (Mostov, 1994) and, in the following, we will show that HAV–IgA translocation from the intestinal lumen into blood is possible via this receptor.

In order to examine transepithelial passage of HAV–IgA, we used Madin–Darby canine kidney (MDCK) cells transfected stably with the IgA-specific pIgR. In contrast to the Caco-2 cell model (Blank *et al.*, 2000), MDCK cells are not permissive for HAV infection (Dotzauer *et al.*, 1994) and, therefore, results possibly confounded by HAV replication are excluded. MDCK–pIgR cells develop a strong barrier to diffusion of macromolecules and are a well-characterized model of pIgR-mediated IgA transcytosis in polarized epithelium (Mostov & Deitcher, 1986). The customary route of IgA transepithelial transport by the pIgR is from the basolateral (lamina propria) to the apical (intestinal lumen)

cell surface. Basal-to-apical transcytosis of antigen–IgA complexes was also demonstrated, a mechanism contributing to the elimination of pathogens (Kaetzel et al., 1991; Gan et al., 1997). However, as pIgR missorting to the apical membrane occurs (Mostov & Deitcher, 1986) and IgA not released from the pIgR at the apical site can be transcytosed back and released basolaterally (Breitfeld et al., 1989), we supposed that reverse transcytosis of antigen–IgA complexes from the apical to the basolateral cell surface is possible, a pathway accessible to intestinal HAV–IgA.

In order to have separate access to basolateral and apical cell surfaces necessary for studying this possibility of HAV–IgA transcellular transport, MDCK–pIgR cells were cultivated on porous-membrane filter chambers (0·45 µm pore size; Millicell HA, Millipore) inserted into six-well plates with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (FCS). Integrity and polarization of the cell monolayer were tested by measuring the transmembrane electrical resistance with a Volt–Ohm meter (Millicell-ERS; Millipore). As confirmed by impermeability to IgG conjugated with peroxidase, readings of $>240\ \Omega\ cm^{-2}$ were required for cell use. This was achieved after 5–6 days in culture and was retained for at least 26 h, a time course relevant to the experiments.

HAV–IgA complexes were prepared by incubating HAV ($10^5\ TCID_{50}\ ml^{-1}$), which was obtained by triple freeze–thaw cycles and removal of cellular debris from FRhK-4 (fetal rhesus monkey kidney) cells infected with a tissue culture-adapted variant of strain HM175 (Dotzauer et al., 2000), with 20 µg monoclonal mouse anti-HAV IgA antibody 1.193 ml^{-1} (Ping & Lemon, 1992) for 2 h at room temperature in DMEM. As this antibody, which contains monomeric and pIgR-affine polymeric IgA molecules in nearly equal amounts (Dotzauer et al., 2000), neutralizes HAV upon infection of FRhK-4 cells, complex formation was assayed by infection inhibition on FRhK-4 cells (Dotzauer et al., 2000). Neutralization to a $TCID_{50}$ titre of $<10^2\ ml^{-1}$ indicated that >99 % of the virus was associated with IgA.

After rinsing the cells with PBS, 500 µl HAV–IgA was added to the apical surface of MDCK–pIgR cells. After 2 h at 37 °C for adsorbtion, DMEM/1 % FCS was added to both sides of the filter and cells were incubated at 37 °C for 24 h. Samples of the basolateral and apical media were analysed for intact HAV–IgA. For this purpose, the complexes were separated (from 500 µl samples) by using biotin-labelled goat anti-mouse IgA (Kirkegaard and Perry Laboratories) bound onto streptavidin-coated magnetic beads (Dynabeads M-280; Dynal) (60 µl, incubation at room temperature for 1 h), which were prepared by incubation of 50 µg beads with 0·5 µg antibody for 1 h at room temperature, and tested for HAV by RT-PCR amplification of the viral 2C region. PCR products were visualized by dot-blot hybridization (Dotzauer et al., 1994) (upper half of Fig. 1, dot-blot analysis). Quantification of HAV in the samples was performed by using a RealArt HAV LC RT-PCR kit (Artus) and a LightCycler instrument (Roche Diagnostics) (lower half of Fig. 1, real-time RT-PCR analysis).

We found that, under these experimental conditions, a significant proportion (~ 20 %) of the HAV–IgA complexes added to the apical compartment of the cells were translocalized into the basolateral medium as intact immune complexes (Fig. 1, lane 1). The mouse hepatocyte NCTC 1469 cell line, which is only susceptible to HAV infection by
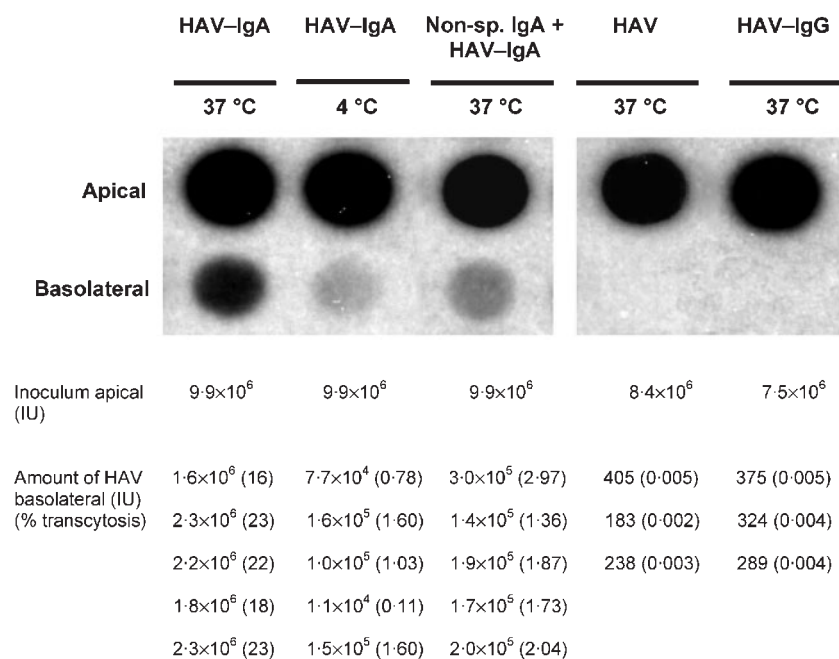

	HAV–IgA	HAV–IgA	Non-sp. IgA + HAV–IgA	HAV	HAV–IgG
	37 °C	4 °C	37 °C	37 °C	37 °C
Apical					
Basolateral					
Inoculum apical (IU)	$9·9×10^6$	$9·9×10^6$	$9·9×10^6$	$8·4×10^6$	$7·5×10^6$
Amount of HAV basolateral (IU) (% transcytosis)	$1·6×10^6$ (16)	$7·7×10^4$ (0·78)	$3·0×10^5$ (2·97)	405 (0·005)	375 (0·005)
	$2·3×10^6$ (23)	$1·6×10^5$ (1·60)	$1·4×10^5$ (1·36)	183 (0·002)	324 (0·004)
	$2·2×10^6$ (22)	$1·0×10^5$ (1·03)	$1·9×10^5$ (1·87)	238 (0·003)	289 (0·004)
	$1·8×10^6$ (18)	$1·1×10^4$ (0·11)	$1·7×10^5$ (1·73)		
	$2·3×10^6$ (23)	$1·5×10^5$ (1·60)	$2·0×10^5$ (2·04)		

Fig. 1. RT-PCR/dot-blot analysis (one representative experiment is shown) and real-time RT-PCR quantification of HAV virion RNA (expressed in IU; 1 IU is equivalent to 10 HAV genomes) associated with anti-HAV IgA in apical and basolateral media 24 h after apical incubation of polarized MDCK–pIgR cells that were cultivated on porous membranes. Besides incubation at 37 °C, incubation at 4 °C and preincubation with non-specific (non-sp.) IgA were included as controls (five independent experiments). As additional controls, HAV and HAV−anti-HAV IgG were used as inocula, added apically instead of HAV−anti-HAV IgA (three independent experiments).

HAV–IgA via ASGPR, allows differentiation between free and IgA-bound HAV (Dotzauer *et al.*, 2000). However, infection experiments using this cell line are not possible due to low HAV–IgA amounts in the basolateral medium. Nevertheless, detection of HAV not bound to IgA can be excluded as, with the magnetic immunoseparation method applied, free HAV particles are not detectable.

To confirm that the apical-to-basolateral translocalization of HAV–IgA observed was dependent on the pIgR pathway and, therefore, that reverse transcytosis occurred, several controls were included. After incubation at 4 °C instead of at 37 °C, only a small amount (~1 %) of the complexes could be detected basolaterally (Fig. 1, lane 2). This is indicative of the involvement of an active-transport mechanism and additionally shows that diffusion did not take place. Preincubation of the apical surface for 2 h with 100 µg non-specific IgA ml^{-1} (polyclonal mouse anti-trinitrophenyl IgA antibody MOPC 315; Sigma) competitively inhibited translocation of the HAV–IgA complexes by approximately 90 % (~2 % could be detected basolaterally) (Fig. 1, lane 3). This demonstrates that the transcellular transport of the complexes was IgA-specific.

Virus alone in amounts equivalent to those in the immunocomplex inoculum added to the apical surface of the cells did not translocalize into the basolateral medium (<0·005 % was detected basolaterally) (Fig. 1, lane 4). These medium samples were tested for HAV by RT-PCR after RNA extraction with a QIAamp viral RNA kit (Qiagen). Also, HAV–anti-HAV IgG, which was prepared as described for IgA-complex formation by using the monoclonal anti-HAV IgG antibody 7E7 (Mediagnost), did not traverse the MDCK–pIgR epithelial cell sheets (<0·005 % was detected basolaterally) (Fig. 1, lane 5). The samples were analysed as described for IgA complexes, using anti-mouse IgG for magnetic separation. These controls, again, showed that HAV–IgA translocation from the apical to the basolateral compartment was dependent on IgA, that no diffusion occurred and that MDCK cells translocate neither HAV nor HAV–IgG complexes from the apical to the basolateral site. Similarly, MDCK cells without the gene encoding the pIgR did not translocate HAV–IgA from the apical into the basolateral compartment. In three independent experiments, apical inoculation with HAV–IgA containing 9×10^6 IU viral RNA (1 IU is equivalent to 10 HAV genomes), as determined by real-time RT-PCR (Heitmann *et al.*, 2005), resulted in a mean basolateral HAV RNA amount of 396 IU (435, 407 and 346 IU), which corresponds to 0·004 % transcytosis. This indicates that the reverse transport of HAV–IgA across MDCK–pIgR cells was dependent on the pIgR.

These experiments show that free HAV is not translocated by transcytosis from the apical cell surface to the basolateral compartment of MDCK and MDCK–pIgR cells, which are model epithelial cell lines. This is in accordance with findings using Caco-2 cells (Blank *et al.*, 2000) that transcytosis of HAV across the epithelial barrier of the intestinal tract by epithelial cells does not occur. However, HAV–anti-HAV IgA complexes can be translocated by reverse transcytosis from apical to basolateral compartments by epithelial cells via the pIgR. This seems contrary to the view of IgA function in mucosal defence. Polymeric IgA synthesized in the lamina propria binds to the pIgR, which is localized on the basolateral cell surface. After transcytosis to the apical surface, the extracellular, IgA-binding portion of the pIgR [secretory component (SC)] is cleaved off and released into the secretions of the small intestines, where the SC remains associated with IgA (Mostov, 1994). These IgA–SC molecules (secretory IgA) cannot rebind to intact pIgR molecules present on the apical surface of the cells, which ensures that the IgA–pIgR pathway is directed vectorially towards the intestinal lumen.

In this context, it is an important finding that, after infection with HAV, the IgA response is extraordinarily fast, strong and, after reaching its peak titre 50 days post-infection (p.i.), it is long-lasting for up to 5 years (Lofgren *et al.*, 1980; Sikuler *et al.*, 1983; Naudet, 1988). The majority of the IgA is not secreted by the IgA–pIgR pathway (Stapleton *et al.*, 1991), but a significant fraction of this serum IgA is released into the intestinal tract via bile by liver functions (Mestecky *et al.*, 1991; Shimada *et al.*, 1999). Progeny HAV, also released from the liver to the gastrointestinal tract via bile, can bind to these IgA molecules, which are not associated with the pIgR SC. The presence of significant amounts of intestinal HAV–IgA is demonstrated by the finding that HAV is partly transmitted as HAV–IgA complexes (Locarnini *et al.*, 1980; Karayiannis *et al.*, 1988).

Previously, we demonstrated that HAV–IgA is astonishingly stable in acidic environments (Dotzauer *et al.*, 2000). We found that 100 % of the complexes remained stable at pH 3·5 for 3 h; 60 % remained associated after treatment with pH 2·5 for 3 h or treatment with pH 1·5 for 1 h. Therefore, after transmission via faeces, especially after transmission from person to person through close contacts, these complexes, which can pass through the harsh conditions of the stomach undamaged (Dotzauer *et al.*, 2000), may bind to missorted pIgR of intestinal cells (Breitfeld *et al.*, 1989) and IgA-mediated reverse transcytosis may allow HAV to enter the bloodstream and reach the liver, resulting in infection of the hepatocytes through uptake of the complexes via the ASGPR (Dotzauer *et al.*, 2000), which mediates uptake of both IgA (serum IgA) and IgA–SC (secretory IgA) (Tomana *et al.*, 1985, 1988) (Fig. 2a). As HAV shows only weak replication in the intestinal tract, this mechanism, which prevents transport of the IgA–SC immune complexes back to the intestinal lumen by the pIgR, would increase the amount of HAV available for infection of the liver and therefore support the infectious outcome.

However, more interestingly, an enterohepatic cycling of HAV could be established by this IgA-mediated intestinal transcytosis via pIgR combined with hepatocellular endocytosis via ASGPR, resulting in reinfection of the liver (Fig. 2b). As in the intestinal environment, association of
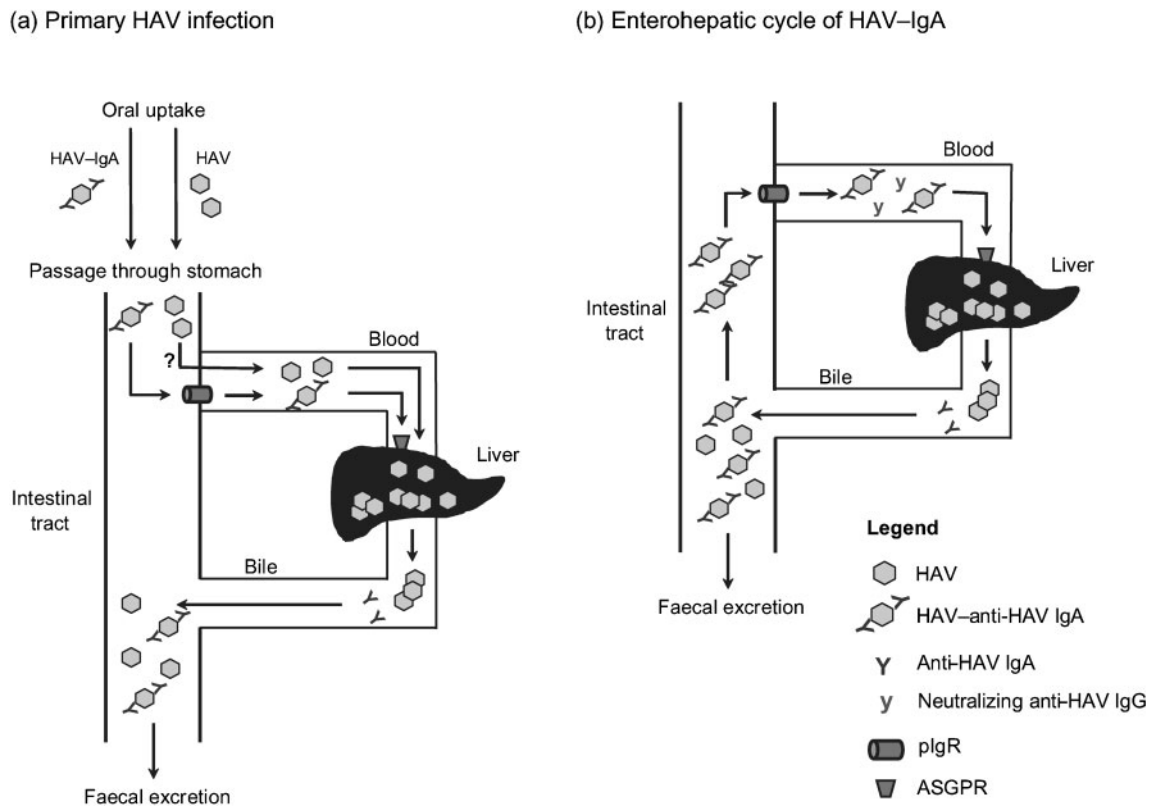
Fig. 2. Hypothesis of primary HAV infection (a) and enterohepatic cycling of HAV (b). (a) Primary HAV infection is supported by HAV–anti-HAV IgA complexes (HAV–IgA), which are already present during transmission. Progeny virus and anti-HAV IgA are secreted into the intestinal tract via bile by liver functions and may form HAV–IgA immunocomplexes. (b) Besides excretion of these complexes in faeces, the complexes could be transported back to the liver, resulting in a reinfection, by which relapses are initiated. These reinfections by HAV–IgA may occur in the presence of HAV-neutralizing IgG antibodies. HAV–IgA-mediated infections of the liver are based on our findings of IgA-mediated transcytosis of HAV via pIgR (polymeric immunoglobulin receptor) and IgA-mediated hepatocellular endocytosis of HAV via ASGPR (asialoglycoprotein receptor).

HAV with IgA is not influenced by the developing and competing anti-HAV IgG response, which is detectable 3 weeks p.i. for the first time and reaches its peak titre 4 months p.i., this IgA-mediated mechanism, which could protect the virus from neutralizing IgG in blood, may play a role in maintaining HAV infection of the liver for a longer time. Recently, it was shown that HAV is detectable in blood for at least 3–4 months after onset of icterus (Normann et al., 2004).

Also, enterohepatic cycling of HAV as HAV–IgA may play a role in relapsing courses (Fig. 2b). As the clinical picture, including the IgM immune response, is similar to the primary phase (Schiff, 1992), reinfections of the liver seem to occur in this case. These recurrent infections appear during reconvalescence between 30 and 90 days after the primary infection in up to 20 % of patients (Glikson et al., 1992). As it was shown for acute infections that HAV is detectable in faeces for at least 90–120 days after onset of icterus (Yotsuyanagi et al., 1996) and faecal anti-HAV IgA persists at least for 120 days after the onset of symptoms (Yoshizawa et al., 1980), HAV–IgA may be the cause of recurrent

infections, especially in the presence of significant amounts of neutralizing anti-HAV IgG. At last, eradication of HAV from the organism may result by displacement of the IgA in the HAV–IgA complexes by neutralizing high-avidity IgG of the matured IgG response.

Although the significance of these IgA carrier mechanisms, which were found in cell culture, for HAV infections in vivo remains to be shown, they deserve some consideration. Different observations in vivo can be related to an association of HAV with IgA beside prolonged and relapsing courses. Although HAV is able to infect a number of non-liver cells in cell culture (Dotzauer et al., 1994), no extrahepatic sites of HAV replication have been identified clearly in the whole organism. In association with IgA, HAV would be neutralized for infection of IgA receptor-negative cells, so that no extrahepatic site of replication could be identified. Although viral RNA is detectable in blood for several weeks in significant amounts (Normann et al., 2004), viral antigen is found only from week 3 until week 5 p.i. (viraemic phase) and in low amounts. Association of HAV with IgA would interfere with the detection of HAV antigen.

We are presently planning to investigate the IgA carrier hypothesis for HAV infections by using a mouse model.

Acknowledgements

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