The role of immunoglobulin A in prolonged and relapsing hepatitis A virus infections

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Hepatitis A virus (HAV) infections result in different courses of the disease, varying between normal, prolonged and relapsing. However, the reason for these heterogeneous clinical appearances is not understood. As HAV–anti-HAV IgA immunocomplexes (HAV–IgA) infect hepatocytes, IgA was postulated as a carrier supporting hepatotropic transport of HAV, and it was speculated that this carrier mechanism contributes to the various clinical outcomes. In this study, the IgA-carrier mechanism was investigated in a mouse model. We show that HAV–IgA immunocomplexes efficiently reached the liver not only in HAV-seronegative mice, but also, and this is in contrast to free-HAV particles, in immunized HAV-seropositive animals. This IgA-mediated transport of HAV to the liver in the presence of immunity depended on the stage of development of the immune response. We conclude that over a period of several weeks after infection, anti-HAV IgA is able to promote an enterohepatic cycling of HAV, resulting in continuous endogenous reinfections of the liver. Our experiments indicate that highly avid IgG antibodies, which are present at later times of the infection, can terminate the reinfections. However, the endogenous reinfections in the presence of a developing neutralizing immunity might contribute to prolonged as well as to relapsing courses of HAV infections. Furthermore, the results show that serum IgA may act as an infection protracting factor.

INTRODUCTION

Hepatitis A virus (HAV) is an enterically transmitted human picornavirus that selectively infects the liver. A liver-specific viral receptor has not been identified so far. The acute icteric course of the disease (Fig. 1) varies from common, over approximately 10 % prolonged cases to relapsing courses in up to 20 % of the patients (Glikson et al., 1992; Havens, 1944; Vallbracht et al., 1985; Yotsuyanagi et al., 1996). The common course lasts for approximately 2 months. The prolonged course is characterized by the presence of symptoms over a period of several months. During a relapse the normal course repeats itself at intervals of 1–3 months, and normally two relapsing phases occur. The reasons for these different manifestations are unknown. The immune response against HAV (Fig. 1) is characterized by a slowly developing IgG response, and a strong, long-lasting IgA response (Naudet, 1988; Sikuler et al., 1983), in which the majority of the HAV-specific IgA remains in blood as serum IgA (sIgA) (Stapleton et al., 1991). This sIgA is cleared from blood by the liver and partially secreted into bile. The liver-specific asialoglycoprotein receptor (ASGPR) plays a major role in this clearance of sIgA as well as of IgA–antigen complexes. Besides protective functions of sIgA in general, infection of hepatocytes by HAV mediated by HAV-specific IgA via the ASGPR could be demonstrated (Dotzauer et al., 2000). The anti-HAV IgM response parallels that of IgA, but declines rapidly and disappears in the course of 4 months after exposure. The role of IgM in the protection against HAV infections seems to be limited, as neutralizing activity is barely detectable (Flehmig et al., 1984; Zahn et al., 1984). It is remarkable that the prolonged and relapsing cases occur while the neutralizing IgG response is developing or almost fully developed, and while activated HAV-specific cytotoxic T-cells are present. A persistent infection is assumed for the prolonged and relapsing courses (Glikson et al., 1992), but
this is not plausible as there is no evidence that HAV infections become chronic, and there is no explanation either for such a persistence of the virus or its final disappearance.

An alternative explanation for a protracted course of the disease is an endogenous reinfection of the liver. This interpretation is supported by the prolonged presence of HAV-RNA detected in the blood by RT-PCR of hepatitis A patients (Normann et al., 2004). Still, reinfections seem to be implausible as HAV-neutralizing IgG antibodies are already present at later times in the course of prolonged cases and at the time of the relapses (Flehmig et al., 1984).

Previously, we showed anti-vectorial transcytosis of HAV in association with HAV-specific IgA across polarized epithelial cells via the IgA-specific polymeric immunoglobulin receptor (pIgR) (Dotzauer et al., 2005). Therefore, IgA is able to support translocalization of the virus from the intestinal tract into the submucosa. As significant amounts of intestinal HAV–IgA are present in the course of the disease (Locarnini et al., 1980) and as IgA-mediated transcytosis may allow HAV to enter the bloodstream again (Dotzauer et al., 2005) and reach the liver for selective infection of hepatocytes via the ASGPR (Dotzauer et al., 2000), we hypothesized an endogenous enterohepatic cycling of HAV (Dotzauer et al., 2005). This IgA-carrier mechanism would result in a continuous reinfection of the liver that would look like a persistent infection. These reinfections by HAV–IgA complexes might also occur in the presence of anti-HAV IgG by protection of HAV from these neutralizing antibodies. Finally, HAV may be eliminated through interruption of the cycling by large amounts and/or high-avidity IgG antibodies and the action of cytotoxic T-lymphocytes (CTL) (Vallbracht et al., 1989). We also postulated a contribution of the cycling of HAV–IgA complexes to the variable outcome of HAV infections (Dotzauer et al., 2000, 2005).

The IgA-carrier hypothesis for HAV infections is so far not supported by experimental data. In vivo studies of HAV infections are hampered by the lack of animal models besides primates. As mice, which although not suitable for studying HAV infections in general, are the standard model for the investigation of immunological mechanisms and operations and as cultivated mouse hepatocytes, which are not infectable by free-HAV virions, can be infected by HAV–IgA complexes, we used mice as an animal model in this study to test our hypothesis.

Our data show that HAV–IgA is efficiently transported to the liver, even in the presence of immunity. This process is strongly reduced in the presence of large amounts of highly avid anti-HAV IgG. Thus, these in vivo investigations support the assumption that endogenous reinfections of the liver by HAV–IgA occur in the presence of immunity. Reinfection is dependent on the developing stage of the IgG immune response, and may cause prolonged and relapsing courses of the disease by establishing a transient persistence of the infection.

RESULTS

We initially tested whether HAV–IgA immunocomplexes are actually delivered efficiently to the liver, and by quantification of HAV-RNA by RT-PCR we found that HAV–IgA complexes efficiently reach the liver after intraperitoneal (6.8 × 10⁷ IU HAV-RNA in 1 ml inoculum) and intravenous (6.8 × 10⁷ IU HAV-RNA in 0.2 ml inoculum) application, respectively. Free-HAV particles reach the liver in significantly lower amounts than HAV–IgA complexes, and HAV–IgG immunocomplexes do not localize to the liver. These results are not shown, but for comparison see the results with non-immunized animals shown in Fig. 2(b). HAV-RNA was not detected in blood of any animal, which proves that HAV detection in the liver did not result from contamination with blood still containing virus.

The subsequent experiments were designed to first examine whether a fully developed anti-HAV immune response is able to interfere with the delivery of HAV–IgA complexes to the liver, and then to test whether hepatotropic delivery of HAV–IgA complexes depends on the stage of development of the anti-HAV immune response. We expected that the first set of experiments would show that continuous IgA-assisted endogenous reinfections of the liver can be terminated by the fully developed anti-HAV IgG response, thus giving an explanation for the final
elimination of HAV. The second set of experiments was expected to show that prolonged and relapsing courses of the disease could in the presence of low amounts and/or low-avidity IgG be caused by endogenous reinfections of the liver by HAV–IgA, resulting in a protracted infection.

**Immunity against HAV interferes with IgA-mediated transport of HAV to the liver**

As HAV complexed with IgA targets the liver efficiently, we assumed that enterohepatic cycling of HAV by HAV–IgA complexes might occur, resulting in endogenous reinfections of the liver. However, HAV infections do not result in long-term persistence of the virus and thus the postulated continuous endogenous reinfections must be terminated. Therefore, we addressed the question whether large amounts and/or highly avid anti-HAV IgG antibodies in blood are able to interfere with the IgA-mediated transport process for HAV to the liver.

For this purpose, C3H mice were immunized against HAV as described in Methods. Six months after the primary injection the mean anti-HAV titre was 1435 milli (m) IU ml⁻¹. As HAV has one main immunodominant epitope, the anti-HAV titre corresponds with the neutralization titre. As shown in Fig. 2(a), HAV infection (10⁶ TCID₅₀ ml⁻¹) of FRhK-4 cells was inhibited by the mouse immune serum (50 mIU ml⁻¹) for 3 h and FRhK-4 cells were inoculated for 2 h at 34 °C. After incubation for 5 days, HAV-RNA was determined by quantitative RT-PCR. For comparison the neutralization by mAb anti-HAV IgG 7E7 is shown. The data represent means of at least two replicates and experiments were carried out at least twice.

![Fig. 2.](image)

**Fig. 2.** Immunity against HAV interfered with IgA-mediated transport and infectivity. (a) HAV infection of FRhK-4 cells was inhibited by the murine serum taken 6 months after immunization against HAV. HAV (10⁶ TCID₅₀ ml⁻¹) was treated with murine anti-HAV immune serum (50 mIU ml⁻¹) for 3 h and FRhK-4 cells were inoculated for 2 h at 34 °C. After incubation for 5 days, HAV-RNA was determined by quantitative RT-PCR. For comparison the neutralization by mAb anti-HAV IgG 7E7 is shown. The data represent means of at least two replicates and experiments were carried out at least twice. (b) Immunity against HAV prevented transport of HAV to the liver, but HAV–IgA was still able to reach the liver, although with reduced efficiency. Ten mice immunized against HAV were inoculated with HAV–IgA 6 months after the primary injection for immunization or were given injections of free-HAV virions (HAV) in amounts equivalent to those in the complex inoculum, respectively. As controls, 10 animals mock-immunized with PBS were inoculated with HAV–IgA complexes or were inoculated with free-virus particles. Four days later, the livers were tested for the presence of HAV-RNA by means of quantitative RT-PCR. (c) Large amounts of/or highly avid HAV-specific IgG competitively inhibited the IgA function in the HAV–IgA immunocomplexes. NCTC 1469 cells were inoculated for 2 h at 34 °C with HAV–anti-HAV IgA complex preparations and HAV–IgA complexes treated either with serum of immunized mice or mAb anti-HAV 7E7. After inoculation, HAV-RNA was determined by quantitative RT-PCR. The data represent means of at least two replicates and experiments were carried out at least twice.
HAV virions to the liver (Fig. 2b). This demonstrates the strong neutralizing potential of the immune response against HAV 6 months after immunization. After HAV–IgA inoculation a strong neutralizing effect was also observed, but HAV still reached the liver in significant amounts in spite of the existing immune response (Fig. 2b).

To find out whether the neutralizing effect observed after treatment of seropositive mice with HAV–IgA complexes was due to neutralization of the IgA function in the HAV–IgA immunocomplexes by serum IgG, HAV–IgA complexes were incubated with the immune serum from the immunized mice, and then cultured mouse hepatocytes were inoculated to test the complexes for binding to the IgA-specific ASGPR. HAV-RNA was determined 2 h after inoculation by quantitative RT-PCR. While HAV–IgA complexes strongly bound to the cells, this specific effect could be neutralized by the immune serum and to the same extent by a mAb anti-HAV IgG (Fig. 2c). Thus, the results from this in vitro approach correlate to those from the animal experiment and explain the reduction of IgA-mediated transport of HAV to the liver in seropositive mice by formation of neutralizing HAV–IgG complexes.

In addition, preparation of HAV–IgA complexes was performed in the presence of the mouse immune serum. In this case also, no significant binding of HAV to the murine hepatocytes could be observed.

These results show that large amounts of or highly avid anti-HAV IgG do not only prevent HAV–IgA complex formation, but also interfere with the IgA already bound to HAV, thereby neutralizing infection of hepatocytes by HAV–IgA complexes, and this demonstrates that a fully developed immune response is able to terminate the postulated HAV–IgA-mediated endogenous reinfection cascades.

**Interference of anti-HAV IgG with IgA-mediated HAV transport to the liver depends on the developmental stage of the IgG response**

To investigate whether the ability of anti-HAV serum to neutralize infection of the liver by HAV–IgA immunocomplexes depends on the amount and the avidity of the developing IgG response, mice were immunized against HAV as described in Methods. Fourteen of these mice were intraperitoneally inoculated with HAV–IgA immunocomplexes 3 or 6 months after the first injection for immunization.

Three months after immunization including one boost, the HAV–IgA complexes injected reached the liver efficiently (Fig. 3), although not to the same extent as in seronegative animals [mock (PBS)-immunized mice]. A significant reduction in the amount of HAV–IgA complexes that reached the liver was observed 6 months after immunization including two boosts. Furthermore, the variability in the amount of HAV reaching the liver in mice not immunized and in mice 3 months after immunization was not observed 6 months after immunization. This indicates that before the immune reaction becomes effective, transport of HAV–IgA in blood to the liver strongly depends on individual physiological conditions of the mice.

In Fig. 3, the anti-HAV titres at the different time points are also shown. The mean anti-HAV antibody titre was 984 mIU ml\(^{-1}\) 3 months and 1170 mIU ml\(^{-1}\) 6 months after the first injection for immunization. This means that after 3 months there was already a distinctive immune response against the virus. This is remarkable because at this time point HAV–IgA complexes still reached the liver in notable amounts, and it also indicates that only at later times in the course of the immune response the avidity of the serum anti-HAV IgG antibodies was strong enough to interfere notably with the IgA in the virus–IgA complexes. This is in accordance with the hypothesis that the increasing amount and/or avidity of the IgG antibodies results in an increasing replacement of IgA in the complexes with HAV.

The results show that there is a long period of time after an HAV infection during which neutralizing anti-HAV antibodies may not be able to prevent infections of the liver by HAV complexed with IgA.
DISCUSSION

The processes resulting in prolonged and relapsing courses of hepatitis A, in which the disease lasts for a long time or recurs after initial resolution of the symptoms, are not known. They occur in the presence of immunity (Fig. 1), and a persistent infection of unknown cause and resolution is assumed. Especially for relapsing forms, but also for prolonged courses, endogenous reinfection(s), which would allow the virus to establish an apparent persistence, are an explanation, but this seems impossible in the presence of immunity.

We postulated that anti-HAV IgA plays a role as carrier supporting delivery of HAV to the liver even in the presence of neutralizing IgG. To prove this hypothesis we performed animal experiments with mice, and it was possible to investigate the passage of HAV in blood to the liver in these animals.

In HAV-seronegative mice HAV ligated to IgA accumulated in the liver with a significantly better delivery than free HAV, which also reached the liver despite the ubiquitous expression of the HAV-attachment protein TIM1/HAVcr-1 (Feigelstock et al., 1998).

This indicates that HAV–IgA complexes increase the efficiency of HAV infections in general by cooperating with free-virus particles in infections of the liver. The greater variability in the amount of HAV-RNA detected in individual mice of the HAV–IgA treatment group as compared with the HAV-treated animals suggests that the

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**Fig. 4.** Enterohepatic cycling of HAV assisted by anti-HAV IgA during different stages of infection. (a) At an early stage of the infection progeny virions, which are released from replication sites in the liver into bile, associate with anti-HAV IgA also released into bile and intestinal tract by liver functions. Besides faecal excretion, part of these complexes reinfect the liver by intestinal transcytosis via the polymeric immunoglobulin receptor (pIgR) combined with hepatocellular endocytosis via the asialoglycoprotein receptor (ASGPR). This cycling of HAV is not influenced by the anti-HAV IgG response, which is normally detectable 3 weeks p.i. for the first time and reaches its peak titre 5 months p.i. (b) During development of the IgG response low avidity IgG antibodies are produced, which weakly compete with the IgA for binding to HAV, and although reduced, endogenous reinfection of the liver at new sites by HAV–IgA is maintained. At sites where viral replication is already established, activated CTL infiltrate the liver and lyse the cells infected. (c) Large amounts of highly avid IgG from the fully developed immune response block the IgA in the HAV–antibody complexes, resulting in inhibition of IgA-mediated HAV reinfections of the liver and termination of virus transmission. Remaining replication foci are eliminated by CTL. [Modified from reference Dotzauer et al. (2005), with permission.]
transport of HAV–IgA is affected to a higher degree by individual factors than the delivery of free HAV is.

In HAV-seropositive mice, IgA-mediated transport of HAV to the liver occurred in the presence of immunity, but this depended on the titre and avidity of IgG antibodies, which in time increasingly neutralized the IgA function in the HAV–IgA complexes.

With regard to HAV–IgA complex formation, it is of interest to know where HAV–IgA association may occur, especially in the presence of neutralizing IgG antibodies in blood. As compared to HAV–IgA complex generation in blood, neither the association in the biliary tract, into which HAV and serum IgA are released by liver functions (Brown et al., 1984; Mestekcy et al., 1991), nor in the intestinal environment is influenced by the developing and competing anti-HAV IgG immune response and here complex formation preferentially seems to occur. This is supported by the fact that notable amounts of HAV–IgA are detectable in the intestinal tract as well as in stool samples of patients (Karayiannis et al., 1988; Locarnini et al., 1980).

Based on several experimental data, we developed the following enterohepatic pathway for hepatitis A infections (Fig. 4): at an early stage of the disease, virus and anti-HAV serum IgA are released by the liver into bile and intestines. HAV–IgA complex formation occurs and a certain fraction of these complexes enter the bloodstream again by IgA-mediated reverse transcytosis via the pIgR through the intestinal epithelium (Dotzauer et al., 2005). By binding of these HAV–IgA to the hepatocellular ASGPR, reinfections at different sites of the liver occur (Dotzauer et al., 2000). In this phase of continuous reinfections of the liver, the barely neutralizing HAV-specific IgM immune response declines, whereas the IgG response develops. Free HAV, which is also able to enter the bloodstream again, is now increasingly neutralized. However, the major fraction of the circulating HAV–IgA still reaches the liver, as the not fully developed anti-HAV IgG immune response is able to compete only weakly with the IgA bound to HAV already. During this period, HAV-specific CTL are activated (Fleischer et al., 1990; Vallbracht et al., 1989), infiltrate the liver at sites where HAV replication occurs, and, after lysis of the cells infected, leave the liver back into blood (Vallbracht et al., 1986), which might result in transient resolution of the symptoms. But HAV–IgA-mediated reinfections occur continuously and at different hepatic sites. Continuous reinfiltrations of the liver by the CTL take place to fend off the new replication foci. Therefore, during this period of the disease an apparent persistent infection is established. At last, neutralization of the IgA function in the circulating HAV–IgA complexes by large amounts of the avidity-matured IgG immune response, which in humans usually reaches its peak titre 4–6 months post-infection (p.i.), occurs, possibly by competitive replacement of the IgA in the HAV–IgA complexes, resulting in termination of the IgA-mediated infection cascades. Eradication of HAV from the liver is accomplished by CTL. Depending on individual physiological conditions and multiple independent factors, such as amount of virus progeny, strength of the IgA and IgG responses or of the activity of CTL, normal, prolonged or relapsing courses, but no final viral persistence, may result.

In summary, a protracted infection of the liver in the course of prolonged or relapsing hepatitis A is explained by our hypothesis. Furthermore, as HAV is partly transmitted as HAV–IgA (Karayiannis et al., 1988; Locarnini et al., 1980), the IgA-carrier mechanism would increase the efficiency of the primary infection by compensating several restrictions of HAV reaching the liver, such as no significant replication in intestinal epithelial cells or unspecific attachment of HAV to non-liver cells. Several clinical findings are also in accordance with, and readily explained by, this hypothesis. For example, the age-dependent severity of the disease might depend on the age-dependent potential of the IgA response, which does not reach its peak until puberty. Although inactivated HAV vaccines work well, efforts are made to develop a live, attenuated vaccine. This might not be advantageous, as IgA might be induced and enhance infection.

**METHODS**

**Animals and inoculation.** Male C3H mice (Charles River Laboratories), from which the cultivated hepatocytes (NCTC 1469) used in previous experiments were derived, were used. All experiments were performed in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and approved by the local ethical committee.

For immunization, mice (6 weeks old) were inoculated intraperitoneally and subcutaneously with 0.5 ml each of HAVp (106 TCID50 ml−1 cell culture extract). The second injections were performed 4 weeks and the third injections 12 weeks after the primary injections. In control groups, the same volume of PBS was injected in an identical fashion. For test bleeds, blood was collected from the retrobulbar venous complex under CO2 anaesthesia. Anti-HAV antibody titres were determined using anti-HAV EIA (Mediagnost) according to the manufacturer’s instructions.

In order to study hepatotropic transport of HAV, the animals were intraperitoneally inoculated with 1 ml of the inocula (with an HAV equivalent of 1×106 TCID50 ml−1 or 6.8×107 IU ml−1 as quantified by real-time RT-PCR). Four days later the mice were killed and blood and different tissues were collected.

**Cells.** The murine hepatocellular cell line NCTC 1469 and fetal rhesus monkey kidney cells (FRHK-4) were maintained as continuous cultures in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1% FCS.

IgA-assisted infection with HAV was examined by inoculating NCTC 1469 cells with the inoculum in the presence of 10 mM Ca2+ (calcium chloride) for 2 h at 37 °C. After five washes, the cells were incubated at 37 °C for times indicated. HAV infections of FRHK-4 cells were carried out using DMEM and cells were inoculated for 2 h at 37 °C.

**Virus.** HAVp, a tissue culture-adapted variant of strain HM175 (Brack et al., 1998; Cromeans et al., 1987), was prepared and the TCID50 titre was determined as described previously (Dotzauer et al., 2000).

**Preparation of HAV immunocomplexes.** HAV–anti-HAV IgA complexes were prepared by incubating HAV (106 TCID50 ml−1;
corresponding to 6.8 x 10^7 IU ml^-1 as quantified by real-time RT-PCR with 20 μg mAb mouse anti-HAV IgA 1.193 ml^-1 (Ping & Lemon, 1992) for 3 h at room temperature in DMEM. Complex formation was assayed by infection inhibition on FRHK-4 cells. The neutralization assay showed a HAV titre of 2 x 10^5 TCID50 ml^-1, corresponding to more than 99% association of HAV and IgA. HAV-anti-HAV IgG complexes were prepared as described for IgA complex formation using the mAb IgG 7E7 (Mediagnost) or murine anti-HAV immune serum.

For the IgA-IgG competition experiments, HAV-IgA complex preparations were treated with IgG 7E7 (15 mIU ml^-1) or murine anti-HAV immune serum (15 mIU ml^-1) for 3 h. Alternatively, HAV (10^4 TCID50 ml^-1) was treated with IgA 1.193 in the presence of IgG 7E7 or murine anti-HAV immune serum for 3 h.

Detection and quantification of HAV-RNA. Total RNA was extracted from homogenized tissue with the RNeasy mini kit (Qiagen) and HAV-RNA was quantified with the RealArt HAV LC RT-PCR kit (Qiagen) according to the manufacturer’s instructions (Heitmann et al., 2005). 18S rRNA as well as GAPDH-RNA was used for normalization. One IU HAV-RNA corresponds to 10 genome equivalents and correlates stably with the TCID50 value.

Statistical analysis. The data were analysed using the Mann-Whitney U test. P-values of <0.05 were considered statistically significant (*) and of <0.01 highly significant (**). The results are shown as box blot diagrams in which the black lines represent the median values.

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