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Immunogenicity of a chimeric hepatitis A virus (HAV) carrying the HIV gp41 epitope 2F5

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Abstract

Its stable particle structure combined with its high immunogenicity makes the hepatitis A virus (HAV) a perfect carrier to expose foreign epitopes to the host immune system. In an earlier report [Beneduce, F., Kusov, Y., Klinger, M., Gauss-Müller, V., Morace, G., 2002. Chimeric hepatitis A virus particles presenting a foreign epitope (HIV gp41) at their surface. Antiviral Res. 55, 369–377] chimeric virus-like particles (HAV-gp41) were described that carried at their surface the dominant gp41 epitope 2F5 (2F5e) of the human immunodeficiency virus HIV-1. Extending this work, we now report that chimeric virus HAV-gp41 replicates in HAV-susceptible cells as well as in non-human primates. Infected marmosets developed both an anti-HAV and anti-2F5 epitope immune response. Furthermore, an HIV-neutralizing antibody response was elicited in guinea pigs immunized with HAV-gp41 chimeric particles. The results demonstrate that the replication-competent chimeric HAV-gp41 can serve as either a live or a subunit vaccine for eliciting of antibodies directed against a foreign antigenic epitope.

Keywords: Anti-HIV; Vaccine; Virus replication; Marmoset; Guinea pig

1. Introduction

Acquired immunodeficiency syndrome (AIDS) caused by the human immunodeficiency viruses (HIV-1 and -2) is a paramount threat to the public health, in spite of more than 20 years efforts to prevent its spread. Since 1983 when HIV was first isolated, more than 60 million people have been infected worldwide. Various strategies have been probed to prevent or limit viral replication and to delay disease progression, yet a safe and lasting protective regimen remains elusive (Markel, 2005). Eliciting a neutralizing antibody response against HIV by immunization is a major goal in HIV vaccinology. Based on the potent neutralizing ability of the monoclonal antibody 2F5 (mAb 2F5), the respective linear epitope ELDKWAS (2F5e) that is highly conserved and located in the membrane proximal part of the glycoprotein (gp) 41 ectodomain, is considered a dominant target for neutralization and vaccine development (Muster et al., 1994). 2F5e and other epitopes seem to be implicated in the cell fusion machinery and binding to these sequences can abolish viral spreading (Finnegan et al., 2002). The presentation of this epitope to the host immune system has already been explored (Muster et al., 1994; Eckhart et al., 1996; Marusic et al., 2001).

Hepatitis A virus (HAV) is a picornavirus that is unique in several aspects. By yet unknown molecular mechanisms, its replication in cell culture is slow, leaving the host cell metabolism mostly unaffected. In immature viral particles, the major viral structural protein VP1 contains the C-terminal extension 2A that is essential for the initial step in viral particle assembly (Probst et al., 1999). 2A is removed from morphogenic precursor particles and is present in low amounts in mature virions excreted by the infected individual (Anderson and Ross, 1990). Genetic analyses indicated that HAV 2A is exposed on the immature particle's surface (Beneduce et al., 2002) and that its C-terminal part is dispensable for viral replication (Harmon et al., 1995; Cohen et al., 2002). Recently, we presented evidence

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that foreign sequences can be inserted into domain 2A rendering immature particles an epitope carrier (Beneduce et al., 2002; G. Morace and Y.Y. Kusov, unpublished observations). Transmitted via the fecal-oral route and possibly amplified in the intestinal tract with the induction of a specific IgA response (Sikuler et al., 1983; Gauss-Müller and Deinhardt, 1988; Asher et al., 1995), the virus is transported to the liver, which is its predominant replication site (Sompayrac and Hepatitis, 2002). Based on the observation that HAV-IgA complexes are infectious to liver cells, the IgA-mediated reverse transcytosis has been proposed as a liver specific transport mechanism (Dotzauer et al., 2000, 2005).

Since more than 10 years an inactivated and highly immunogenic vaccine is successfully used to prevent the hepatitis A infection in endemic areas and viral spreading in sporadic outbreaks (Andre et al., 2002). Following the successful precedence of the oral poliovirus vaccine, various attenuated HAV strains were assessed as live and orally administered vaccines. The latter kind of vaccine could combine low cost, effective immunogenicity and mucosal immunity (Zhao et al., 2000; Purcell et al., 2002). These particular properties of anti-HAV prophylactic measures persuaded us to assess HAV as a live and subunit carrier of foreign antigens. We were aiming at a prophylactic approach that might induce anti-HIV mucosal immunity (Matoba et al., 2004; Markel, 2005).

Exploiting the excellent HAV vaccine properties and extending our earlier work (Beneduce et al., 2002), we show that the chimeric virus HAV-gp41 replicates in cell culture and in marmosets inducing both an anti-HAV and anti-gp41 immune response. In addition to the live vaccine approach using these non-human primates, the immune response against chimeric virus-like particles was assessed in guinea pigs. These animals elicited a neutralizing anti-HIV response determined by a reduction of the HIV reverse transcriptase activity. Our data suggest that virus-like chimeric HAV particles might be well suited as a subunit vaccine vehicle for the presentation of foreign epitopes. Furthermore, the chimeric virus HAV-gp41 might be a promising live vaccine candidate for the protection against both HAV and HIV infections.

2. Materials and methods

2.1. Viruses and cells

Human hepatoma Huh-7 cells were cultured in Dulbecco's-modified minimal essential medium (DMEM, Invitrogen) supplemented with 10% (growth medium) or 2% (maintenance medium) fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Huh-T7 cells, a derivative of Huh-7 stably expressing T7 RNA polymerase (Schultz et al., 1996), were additionally supplemented with G-418 sulphate (Sigma) (400 μg/ml). Human cutaneous T cell lymphoma (HUT78) cells were grown in RPMI 1640 (Invitrogen) supplemented as above. The cell culture medium was changed every 3–4 days.

2.2. Construction of pHAV-gp41 and production of chimeric virus

The sense primer 5′-aaa gaa ctg aga tta gaa gtt GAG CTC GAT AAA TGG GCA AGT gaa ttg tca aat gaa gta ctt-3′ and the complementary anti-sense primer were used to insert the 2F5e sequence (ELDKWAS) into domain 2A of HAV strain HM175, 18f (Accession number M59808) using the QuikChange mutagenesis kit of Stratagene as described previously (Beneduce et al., 2002). The foreign sequence with an additional restriction site (SacI) is shown in uppercase and underlined, respectively. The mutated subgenomic fragment (AccI–PflMI) was sequenced before it was transferred into pT7-18f (Kusov and Gauss-Müller, 1999), resulting in pHAV-gp41. For the production of infectious virus, pHAV-gp41 was transfected into a 70% confluent monolayer of Huh-T7 cells using LipofectAmin according to the Manufacturer's protocol (Invitrogen). Transfected cells were incubated for 20 days at 37 °C in G418 sulphate containing maintenance medium, before a soluble cell extract was prepared and the HAV and 2F5e antigenicity was detected (see below). The rescued virus, called HAV-gp41, was passaged twice in Huh-7 cells prior to extraction of viral RNA and its RT-PCR amplification with appropriate primers. The amplification product was sequenced to confirm the chimeric nature of the viral genome. To determine the specific infectivity of the chimeric genome, various amounts (from 1.0 to 0.125 μg/well with step 2 dilution) of pHAV-gp41 and of the parental plasmid were transfected into Huh-T7 cells grown to 50% confluency in six-well plates. After 15 days of incubation at 37 °C, a soluble extract was prepared with phosphate-buffered saline containing 0.05% Tween-20 (PBS-Tw). HAV and gp41 antigenicity was detected by the 7E7- and the 2F5-ELISA (Beneduce et al., 2002), respectively. Briefly, the anti-gp41 human monoclonal antibody (mAb 2F5, a kind gift of Dr. G. Stiegler, Vienna; Purtscher et al., 1994) was used at a concentration of 1 μg/ml in 50 mM carbonate buffer, pH 9.6 for coating the wells of a 96-well immunoplate (100 μl/well). After overnight incubation at room temperature (RT) and washing with TBS-T (50 mM Tris, pH 8.0, 150 mM NaCl and 0.5% Triton X-100), the immunoplate was blocked at RT for 1 h with 1% bovine serum albumin (BSA) in TBS-T (BSA-TBS-T, 200 μl), washed (3 × 200 μl) and incubated at 37 °C for 1.5 h with either 100 μl of 10% marmosets feces or appropriately diluted lysates of HAV-gp41-infected cells. Finally, the washed immunoplate was incubated with 7E7 anti-HAV monoclonal antibody conjugated to horse radish peroxidase (HRPO, 1:20,000, Mediagnost, Tübingen). After staining with 3,3′,5,5′-tetramethylbenzidine (TMB, Sigma), the plate was read at 450 nm.

2.3. Preparation of 160S and 70S HAV-gp41 particles

Huh-7 cells were infected with the HAV-gp41 at a multiplicity of infection (moi) of approximately 0.1 50% cell culture infectious dose per cell ($TCID_{50}$/cell = 0.1). Two weeks later the infected cells were extracted by a freeze/thaw procedure, the cell debris was pelleted by centrifugation for 5 min at 13 krcf, and the supernatant was collected. To enhance virus extraction, the cell

debris was incubated for 5 min at 37 °C with TN buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl) supplemented with 0.1% sodium laurylsarcosinate (SLS), 0.05% Tween 20, 0.05% Triton X-100, 4 mM EDTA, and a proteinase inhibitor cocktail (Sigma, 1:200) supplemented with 4 mM Pefabloc (Roche). After centrifugation (13 krcf, 5 min), the supernatants were pooled and extracted with an equal volume of Freon-113. The aqueous phase was loaded onto a linear sucrose gradient (5–45%). After centrifugation at 6 °C for 4 h at 176.9 krcf, the HAV-positive fractions of 160S and 70S were separately pooled. The 160S viral particles were used to infect 2 marmosets. The protein concentration of the 160S fraction was 3.8 μg/ml, as determined by the BCA protein assay (Pierce, IL). For the immunization of guinea pigs, 70S particles that were positive for both antigens (HAV and gp41) were diluted 1 to 10 with TN buffer and pelleted by centrifugation at 158 krcf for 3 h at 4 °C. The pellet was resuspended in 2 ml of TN buffer and the protein concentration determined to be 10.2 μg/ml. HAV antigenic titer of both materials was >1:128, as determined by the 7E7 ELISA.

2.4. Inoculation of marmosets

Animals were housed, maintained, and cared in compliance with relevant guidelines and requirements. Prior to inoculation, the suspension of 160S HAV-gp41 particles was incubated for 16 h at 4 °C with kanamycin sulfate (100 μg/ml). Two marmosets (Callitrix jacchus) seronegative for anti-HAV were given 0.3 ml (per os) and 0.7 ml (per femoral vein) of the particle preparation. Two other marmosets were used as control and given 1 ml PBS. HAV infection was previously shown in marmosets (Zamjatina et al., 1990). The feces were collected daily, if available, and stored at −80 °C. Stool suspension (10% extract in PBS) was prepared as described (Zamjatina et al., 1990). Blood was taken from the femoral vein one day before inoculation, twice within the first 2 weeks and once per week later. Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and isocitric and dehydrogenase (ICD) were analyzed in marmosets' sera as described previously (Zamjatina et al., 1990). For intravenous inoculation and blood sampling, the animals were immobilized by ketamine anestesia (Ketamine-Calypsol, Hungary).

2.5. Propagation of virus shed in feces

10% extracts of marmosets' feces collected at various time points post inoculation (see Fig. 1) were diluted 1 to 100 using OptiMEM1 (Invitrogen) and inoculated (1 ml) into Huh-7 cells grown in 25 cm^2 flasks. After 3 h of adsorption, the inoculum was replaced by 5 ml of growth medium. After 8 or 20 days of incubation at 37 °C, cells were scraped in PBS-Tw and lysed by three cycles of freeze/thawing. The lysate was used for the detection of the HAV antigen, of 2F5e, of the fusion protein VP1-2A-gp41, and for RT-PCR amplification of the VP1-2A region (see below). The second passage was performed in a similar way after a 1 to 100 dilution of the cell lysates in OptiMEM1.

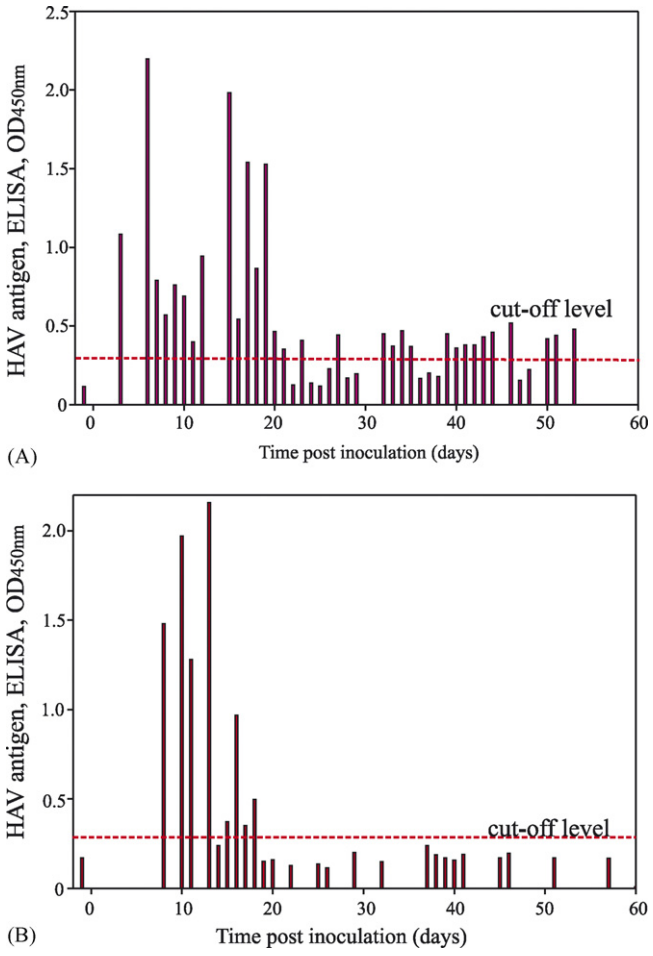


Fig. 1. Infectivity of virus fecally excreted by infected marmosets. (A and B) 7E7-ELISA antigenicity detected in lysates of Huh-7 cells that were inoculated with fecal extracts of marmoset #1 (A) and marmoset #2 (B) obtained at the indicated time points after inoculation. Infected cells were lysed 20 days post infection and the HAV antigen was determined by ELISA. The cut-off level (dashed horizontal line) represents 2.1 × *N*, where *N* is the mean value of four independent negative samples (not shown, see Bradley et al., 1977).

2.6. Immunization of guinea pigs

The animals had an initial body weight of 220–250 g. Purified 70S procapsids of HAV-gp41 (170 μl) were mixed with complete Freund's adjuvant (CFA, 170 μl, Sigma). The suspension was divided into three equal parts and one part each was injected subcutaneously and intramuscularly into two legs of four animals. Inactivated hepatitis A virus vaccine HAVRIX (one dose 1440 units/ml, GlaxoSmithKline Biologicals, Belgium) was administrated in a similar way into three other animals. Freund's adjuvant mixed with PBS in equal volumes was used in control animals. The immunization schedule consisted of three injections with 2 weeks interval. Serum was prepared from blood taken by heart-puncture on day 42.

2.7. Detection of anti-HAV

The AxSym HAVABM assay (Abbott) was used to test anti-HAV IgM antibody in marmosets' sera. The combined anti-HAV

IgM + IgG activity was tested by a in-house made competitive ELISA. Briefly, after coating with a high titer human polyclonal anti-HAV IgG and BSA-blocking, the wells of an immunoplate were incubated with approximately 1 optical unit of HAV antigen (determined by 7E7 ELISA) at 37 °C for 2.5 h. After washing, the wells were incubated (2 h at RT) with the animal sera diluted 1:100 in BSA-TBS-T. Finally, an in-house made HRPO-conjugated polyclonal anti-HAV IgG (1:500) or a HRPO-conjugated monoclonal anti-HAV (7E7-HRPO, Mediagnost) was incubated for 2 h at 37 °C prior to the colour reaction with TMB as described above.

2.8. Detection of anti-gp41

2.8.1. ELISA

The 96-wells of an immunoplate were coated with the synthetic peptide GGGLELDKWASLW (100 μl, 1 μg/ml, kind gift of Dr. G. Stiegler) that was dissolved in carbonate buffer, pH 9.6 and incubated overnight at 4 °C. After washing with TBS-T and blocking with BSA-TBS-T (see above), 100 μl of the test serum diluted 1:50 in 1% BSA-TBS-T was incubated for 2 h at RT. Anti-human (1:1000) or anti-guinea pig (1:1000) HRPO-conjugated anti-Ig diluted in BSA-TBS-T was incubated for 1.5 h at 37 °C. The plate was washed, stained and read as above.

2.8.2. Western blot

The INNO-LIA HIV confirmation assay (Innogenetics, Belgium) based on the antibody interaction with immobilized HIV proteins (including gp41) followed by the staining with anti-human IgG conjugated to alkaline phosphatase (anti-human AP, 1:10,000, Dianova, Hamburg, Germany), was directly used to analyze the anti-gp41 activity in marmosets' sera. To increase the sensitivity of the assay, the 16-h sample incubation version was performed as recommended by the Manufacturer. To analyze the sera of immunized guinea pigs, the assay was modified by replacing the anti-human AP conjugate with that of anti-guinea pig AP (1:5000, Dako).

2.9. Detection of anti-3C

Bacterially expressed recombinant HAV 3C was purified as described (Malcolm et al., 1992) and after electrophoresis in a SDS-12% polyacrylamide gel (PAGE, 0.5 μg/lane) immobilized on non-charged modified nylon blotting membrane (INYU15010, Millipore). Strips of the immobilized protein (localized by Ponceau S staining) were incubated overnight at RT with the marmosets' sera diluted 1:20 in TBS-T (first antibody) and then for 2 h at RT with anti-human AP conjugate as second antibody. Antibody binding was determined by colorimetric staining with p-nitroblue tetrazolium chloride (0.03%) and 5-bromo-4-chloro-3-indolyl phosphate (0.015%) in 0.1 M phosphatase buffer, pH 9.8 supplemented with 1 mM $MgCl_2$.

2.10. Detection of fusion protein VP1-2A-gp41

Huh-7 cells harvested in 0.25 ml PBS-Tw 8 days post infection with fecal extracts of marmosets were lysed by freeze/thaw. Proteins were separated by SDS-15% PAGE, transferred onto the nitrocellulose blotting membrane (Protran, Schleicher & Schull), and developed with anti-VP1, anti-2A, and anti-gp41 (mAb 2F5) as described previously (Beneduce et al., 2002).

2.11. HIV neutralizing activity

To determine the anti-HIV neutralizing activity, an HIV stock (strain LAV_{LAI}) was titrated in HUT78 cells and the 50% cell culture infectious dose ($CCID_{50}$) was determined. Filter-sterilized sera (0.2 μm) of immunized animals (guinea pigs and monkeys) were diluted 1:40 in RPMI 1640 growth medium. Fifty microliters of the diluted serum was pre-incubated in quadruplicate with 100 μl HIV (100 $CCID_{50}$ per well) for 1 h at 37 °C and added to HUT78 cells (50 μl, final concentration 10^5 cells/ml). Cell morphology was controlled three times per week. The cell culture medium was exchanged with an identical concentration of the respective serum or antibody at day 4 and 7. At day 9 post infection the supernatant was collected, diluted 1:12.5 with lysis buffer (50 mM Tris, pH 7.8, 80 mM KCl, 2.5 mM DTT, 0.75 mM EDTA, 0.5% Triton X-100) and tested for HIV reverse transcriptase (RT) activity as described before (Eberle and Knopf, 1996) using the RT assay chemiluminescence kit according to Manufacturer's recommendations (Roche). RT activity was expressed in relative light units (RLU). mAb 2F5 (final concentration 25 μg/ml) was used as a positive control. As negative control, a commercially available normal guinea pig serum (Calbiochem) was used at a similar dilution (1:40).

2.12. Genetic analysis of the rescued viral genome

Viral RNA was extracted with Trizol (Invitrogen) from Huh-7 cells infected either with marmoset's feces (first passage) or with extracts of infected cells (second passage). The *C. therm.* polymerase one-step system (Roche) and anti-sense primer 5′-CCGCTCGAGTTACTGAGTCCTTAACTCCATCATTCTG-3′ was used to generate cDNA (70 °C, 1 h). The VP1-2A region was further PCR amplified with the sense primer 5′-ACAC-AAGGAGAACAGGGAAC-3′ applying the amplification program recommended by the Manufacturer. The RT-PCR amplification products were sequenced with the reverse primer 5′-CCGGTACCCTCCTCAGTATAAAAAGAGAAATATTGGC-3′ (AGOWA, Berlin). The PCR product derived from the chimeric genome was distinct by its additional SacI restriction site.

3. Results

3.1. Replication of the chimeric virus HAV-gp41 in cell culture

Recombinant expression of chimeric virus-like HAV particles with the neutralizing HIV 2F5 epitope as part of the viral protein 2A was recently shown (Beneduce et al., 2002). In order to prepare fully infectious chimeric particles, a reverse genetic approach was applied (Kusov et al., 2005). Huh-T7 cells were

Table 1
Antigenicity and genomic stability of the chimeric virus HAV-gp41

	Viral passage no.								
	0			1			2		
ELISA[a]/RT-PCR[b]	HAV	gp41	Epitope seq.	HAV	gp41	Epitope seq.	HAV	gp41	Epitope seq.
Transfected cDNA									
pT7-HAV-gp41	1.02	0.40	2F5	1.56	0.52	2F5	2.02	0.61	2F5
pT7-18f	2.2	–	wt	2.9	–	wt	nd	nd	nd

[a] After cDNA transfection into Huh-T7 cells (passage 0) followed by two passages in Huh-7 cells, the HAV and gp41 antigenicity was determined with the 7E7 and 2F5 ELISA, respectively. The OD signals were normalized to protein concentration.
[b] Viral RNA was extracted, reverse transcribed and PCR amplified. RT-PCR amplification product was sequenced at 2A/2B junction as described in Section 2.

transfected with various amounts of cDNA pHAV-gp41 and its specific infectivity was compared with the parental construct pT7-18f. Viral replication was determined by quantification of the HAV antigen that accumulated over a 20-day incubation period. Compared to pHAV-gp41, the parental genome produced approximately two times more HAV antigen (not shown) indirectly suggesting that the chimeric 2A had a negative effect on the viral life cycle. These data are in line with earlier reports showing that deletions within the C-terminus of HAV 2A limited the replication efficiency (Harmon et al., 1995; Cohen et al., 2002).

Next we showed that the 2F5e insertion in 2A was antigenically active and stably expressed over various viral passages. As follows from the data presented in Table 1, both antigens were detectable in cell lysates 15 days post transfection, implying that the chimeric virus was not only viable, but also able to express the 2F5e on native particles. The rescued chimeric virus was further passaged twice without loss of either antigenicity, demonstrating that the insertion was stably integrated into the HAV genome. The presence of the 2F5e in the 2A region was confirmed by Western blot (not shown, but see Fig. 2) and by sequencing after RT-PCR amplification of the chimeric virus genome rescued after two passages in Huh-7 cells (Table 1).

To obtain sufficient material for both, a live and subunit vaccine, a stock of the chimeric virus was prepared starting from the third viral passage. As described in Section 2, fully infectious mature virions (160S) and empty virus-like particles (70S) that are both produced in infected cells were separated by velocity sedimentation in sucrose (not shown). The mature particles (160S) were subsequently used for inoculation of marmosets (live vaccine), whereas the empty virus-like particles that sedimented at 70S and contained the chimeric VP1-2A-gp41 precursor were mixed with adjuvant for the immunization of guinea pigs (subunit vaccine).

3.2. Replication of the chimeric virus HAV-gp41 in marmosets

Mature 160S particles purified from HAV-gp41-infected cells were given simultaneously per os and intravenously to two marmosets. Feces and sera collected until 90 days post inoculation (dpi) were analyzed for the presence of chimeric virus, its genome and specific antibodies (see below) and for liver enzymes. Changes in liver enzyme activity or viral genomes were undetectable in sera of marmosets inoculated either with chimeric virus HAV-gp41 or with parental HAV (data not shown). As direct detection of the HAV and gp41 antigen in feces was unsuccessful with the described ELISA (Beneduce et al., 2002; data not shown), fecally shed HAV was isolated in cell culture (Fig. 1). Until 16–18 dpi, infectious virus from feces of both marmosets was identified by HAV antigen accumulation in Huh-7 cells. At later time points, no infectious virus was isolated. To demonstrate the expression of the fusion protein VP1-2A-gp41, cells inoculated with fecal extracts were lysed before the HAV particles were fully matured (8 dpi) and the cell lysates were analysed by Western blot (Fig. 2). In line with

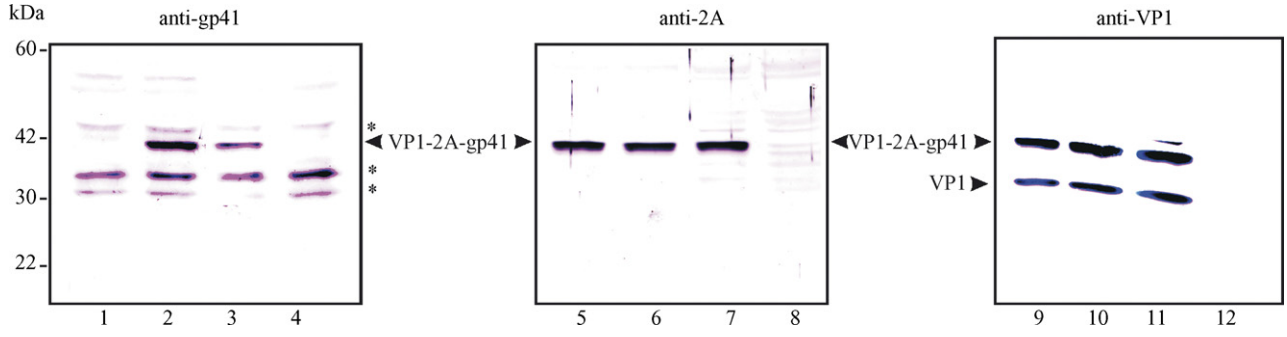
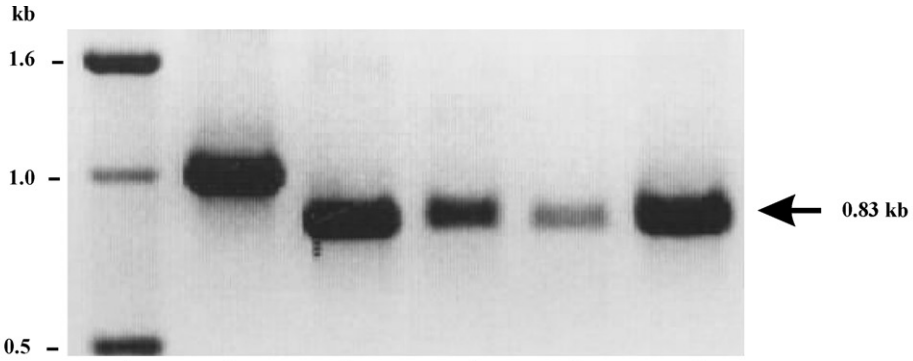


Fig. 2. Identification of fusion protein VP1-2A-gp41 in lysates of Huh-7 cells inoculated with fecal extracts of marmoset #1 (lanes 2, 6, and 10) and marmoset #2 (lanes 3, 7, and 11) collected at 18 and 11 dpi, respectively (see Fig. 1). After separation by SDS-PAGE, the proteins were transferred onto a nitrocellulose membrane and probed with the indicated antibodies as described previously (Beneduce et al., 2002). The extracts of HAV-infected (lanes 1, 5, and 9) and mock-infected Huh-7 cells (lanes 4, 8, and 12) harvested at the same time post inoculation (8 dpi) were analysed. Identified proteins are indicated by arrowheads. Host proteins unspecifically reactive with anti-gp41 are indicated by asterisks. The mobility of marker proteins is indicated on the left.



(A)

Chimeric HAV-gp41:

```
..gaa gtt GAG CTC GAT AAA TGG GCA AGT  -   -   -  gaa ttg...
.. E   V   E   L   D   K   W   A   S   -   -   -   E   L ...
```

(B)

Parental HAV:

```
...gaa gtt ggg aaa caa aga ctc aag tat gct cag gaa gaa ttg...
... E   V   G   K   Q   R   I   K   Y   A   Q   E   E   L ...
```

(C)

Fig. 3. Genetic stability of HAV-gp41 shed in feces of infected marmosets. (A) Electrophoretic mobility of the RT-PCR amplification product derived from the virus shed at day 18 and 11 post inoculation of marmoset #1 and #2, respectively. Lane 1—DNA ladder, lanes 2 and 3—RT-PCR amplification product prepared from wild-type HAV cDNA and cDNA encoding HAV-gp41, respectively. Lanes 4 and 5—RT-PCR amplification product derived from the virus fecally excreted by marmoset #1 and marmoset #2 and rescued after two passages in cell culture, respectively. The SacI restriction fragment (0.83 kb) of the RT-PCR product prepared from the virus suspension used for marmoset inoculation is shown in lane 6. (B) Nucleotide and amino acid sequence of the RT-PCR-amplification product of the chimeric virus HAV-gp41 shown in A (lane 4). (C) Nucleotide and amino acid sequence of the RT-PCR amplicon derived from parental HAV after sequencing of the RT-PCR amplification product shown in A (lane 2). The HAV 2A nucleotide sequence is shown with small letters, the gp41 sequence is shown in boldface and capital letters. The recognition site of the restriction enzyme SacI is underlined. Note that three codons are missing in the recombinant HAV-gp41 chimeric virus as compared to the wild-type HAV, strain 18f.

previously reported data (Beneduce et al., 2002), the fusion protein VP1-2A-gp41 was detected by Western blot. Only in lysates of cells inoculated with fecal extracts of the HAV-gp41-infected marmosets, the 2F5 epitope was unambiguously identified by anti-gp41 (mAb 2F5, lanes 2 and 3), along with 2A (lanes 6 and 7) and VP1 (lanes 10 and 11) confirming the expression of the fusion protein VP1-2A-gp41. Neither in mock- (lane 4) nor in HAV-infected cells (lane 1), specific interaction with anti-gp41 antibody was detectable, albeit some unspecifically interacting cellular proteins were also developed using anti-gp41 (indicated by asterisks in lanes 1–4). To find out whether the fecally excreted virus contained the chimeric genome with the 2F5e sequence in 2A, RNA of the virus isolated after the second passage in cell culture was reverse-transcribed and PCR-amplified using HAV-specific primers targeting the VP1-2B region (Fig. 3A). As a genetic marker, a SacI recognition site had been inserted into the sequence encoding 2F5e and was used to distinguish the chimeric genome (Fig. 3B) from that of the parental strain (Fig. 3C). The SacI fragment of the RT-PCR product derived from RNA found in the fecal samples collected at day 11 (marmoset #1) and day 18 (marmoset #2) post inoculation (Fig. 3A, lanes 4 and 5, respectively) had the same size as that of the chimeric virus HAV-gp41 used for inoculation (lane 6) or that obtained after PCR amplification of the chimeric cDNA (lane 3). In contrast, the SacI restriction fragment of the RT-PCR product amplified from the parental genome was 1.0 kb in size (Fig. 3A, lane 2). Final confirmation of the chimeric nature of the fecally shed virus was obtained by nucleotide sequence determination of the RT-PCR amplification products (Fig. 3B). Since the inoculated virus is usually shed in feces until 4 days after HAV inoculation (Asher et al., 1995), these data indicate the replication of the chimeric virus in vivo. Taken together, the kinetics of virus excretion and analysis of the viral genomes isolated in cell culture from feces of infected marmosets strongly suggest that the inoculated chimeric HAV-gp41 replicated in marmosets (see also Zamjatina et al., 1990; Asher et al., 1995; Emerson et al., 2002)

3.3. Immunogenic properties of the chimeric HAV-gp41

3.3.1. Immune response in infected marmosets

3.3.1.1. Anti-HAV response. Blood samples of marmosets inoculated with HAV-gp41 were collected until 90 days and analysed serologically. The anti-HAV response is shown in Fig. 4, with anti-HAV IgM at the top of panels A and B. Both, anti HAV IgM (lines at the top) and total anti-HAV IgG + IgM (bars) were present starting 20 dpi. Anti-HAV IgM was detectable until

60 dpi in marmoset #1 (panel A) and until 45 dpi in marmoset #2 (panel B).

Although high titers of IgM antibody are usually elicited after infection, low titers of this antibody can also be found in individuals immunized with a killed vaccine (Jilg et al., 1992; Shouval et al., 1993). In order to distinguish between an active infection and immunization as a response to the inoculum given orally and parenterally, the immune response to viral non-structural proteins was analyzed. To this end, the marmosets 'sera were tested for the presence of antibodies to HAV proteinase 3C. As follows from the data presented in Fig. 4, a specific interaction with immobilized HAV 3C was detectable in sera of marmoset #1 taken between 34 and 90 dpi (lanes on the top of panel A). A similar interaction was detected in sera of marmoset #2 starting 13 dpi (not shown). The induction of antibodies directed to viral non-structural proteins (such as 3C) is a direct indication for infection of marmosets with chimeric virus HAV-gp41, due to its active in vivo replication (see also Jia et al., 1992; Stewart et al., 1997; Kabrane-Lazizi et al., 2001).

3.3.1.2. Anti-gp41 response. To detect anti-gp41 antibody, we first applied an ELISA utilizing the synthetic peptide

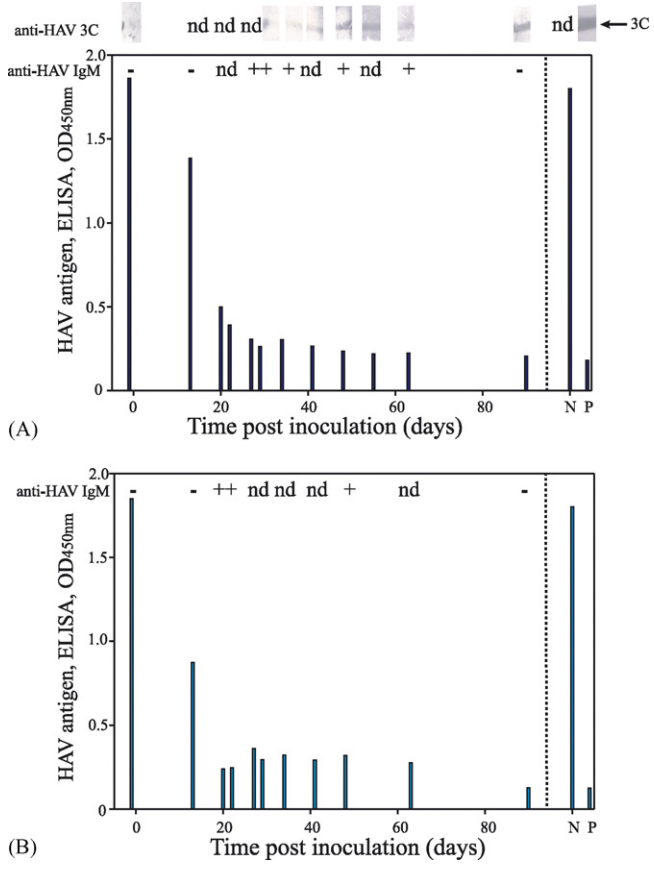


Fig. 4. Kinetics of anti-HAV IgM + IgG (bars, competitive assay), IgM (lines on the top: (−) negative, (+) positive, (nd) not done), and anti-3C (lanes above the diagram in panel A) response in marmosets #1 (panel A) and #2 (panel B) inoculated with the chimeric HAV-gp41 (160S particles). N—anti-HAV negative monkey serum, P—serum of a HAV-infected monkey, used as a positive control for interaction with immobilized HAV 3C (lane P above the diagram) and anti-HAV IgM + IgG (bar P).

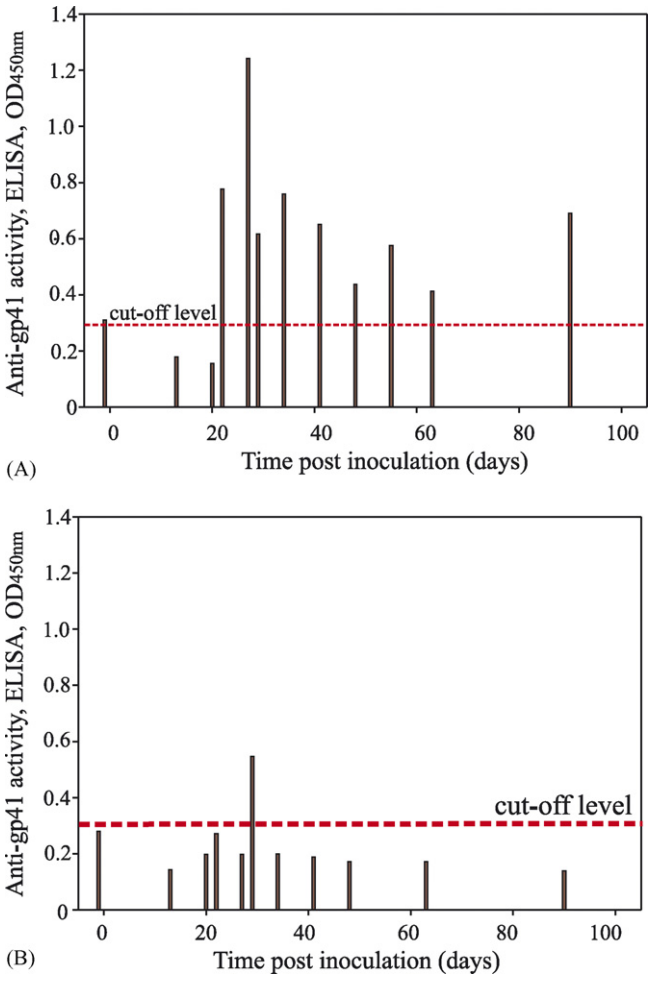


Fig. 5. Anti-gp41 response of marmosets #1 and #2 as judged by an ELISA with the synthetic peptide GGGLELDKWASLW (see Section 2). Sera of four non-infected marmosets were used as a negative control for cut-off level calculation (see caption to Fig. 1).

GGGLELDKWASLW (see Section 2). The data shown in Fig. 5 indicate that in marmoset #1 a distinct anti-gp41 response was mounted starting 21 dpi (panel A). In marmoset #2, the ELISA did not exceed the cut-off value by a factor of 2.1 (panel B). In order to enhance specificity, we used a commercially available assay that is applied as a confirmatory test for the HIV infection. Indeed, the data obtained with the INNO-LIA HIV confirmation system indicated that sera of marmoset #1 collected 20 dpi and later contained anti-gp41 (Fig. 6A, lanes 3–5) while very weak signals were observed for sera of marmoset #2 (data not shown). As expected, the serum of an HIV-infected individual, used as a positive control, interacted with immobilized proteins sgp120, gp41, and p31 (panel A, lane 1). No immune response to gp41 was detected in pre-immune sera (lane 2) and in the sera of naïve and HAV-infected marmosets (lane 6 and not shown). As mature 160S particles contain only minute amounts of 2A with 2F5e exposed at their surface (not shown), the above findings imply that the inoculated material had initiated the complete viral life cycle with the transient production of immature chimeric particles exposing the 2F5e. These data support the notion that the chimeric virus HAV-gp41 is infectious in marmosets.

3.3.2. Immune response in immunized guinea pigs

As an alternative to the live vaccination strategy, guinea pigs were used to test the immunogenicity of 70S particles also obtained from cells infected with chimeric HAV-gp41. Although this fraction of HAV-gp41 might still contain some infectious particles, we did not expect viral replication in guinea pigs, as this virus was not specifically adapted to replicate in these animals (Hornei et al., 2001). For immunization, guinea pigs were injected subcutaneously and intramuscularly three times with 2 weeks intervals. Two weeks later, the anti-HAV and anti-gp41 response was tested. Immunization with the commercially available HAV vaccine (HAVRIX) served as control for the vaccination scheme, antibody induction, and specificity of the antibody detection system. A competitive ELISA similar to that applied for the marmoset sera was used to detect anti-HAV in guinea pig sera. All guinea pigs immunized with HAV-containing material (HAVRIX, guinea pigs #3–5, or HAV-gp41, #6–9), but not the control animals (#1 and #2), responded with an equally strong antibody response indicating that the immunization scheme and detection system were appropriate (Table 2). Anti-2F5e was determined with the in-house made peptide-based ELISA that was successfully used for the marmoset sera. Two guinea pig sera (animals #7 and #8) showed detectable levels of anti-gp41 (Table 2). These sera were also reactive in a modified INNO-LIA HIV confirmation test where we replaced the anti-human with an anti-guinea pig conjugate as the second antibody (Fig. 6B, lanes 7 and 8). In contrast, none of the guinea pigs immunized with the HAV vaccine HAVRIX recognized the 2F5e proving the specificity of the assay (lanes 4–6). No interaction with gp41 was detected in a serum of a HIV-negative individual (lane 1) and in that of a control guinea pig (lane 3).

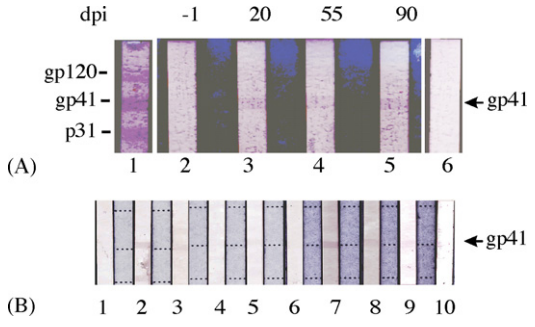


Fig. 6. Anti-gp41 response analysed with the INNO-LIA HIV confirmation test. (A) The reaction of antisera of marmoset #1 collected at the indicated time-points with immobilized gp41 (lanes 2–5) is indicated by an arrow on the right side. The interaction of the serum of the normal marmoset with immobilized gp41 is presented in lane 6. A similar result was obtained when the serum of a HAV-infected monkey was analysed (not shown). The proteins recognized by the serum of an HIV-positive individual (lane 1) are indicated on the left. dpi—days post inoculation. (B) Anti-gp41 response of guinea pigs immunized with virus-like 70S particles of HAV-gp41. Human anti-HIV negative and positive sera are used in lanes 1 and 2, respectively. Lanes 3–10 show the detection of immobilized gp41 by the sera of guinea pigs #3–5 (see Table 2) immunized with HAVRIX (lanes 4–6), chimeric HAV-gp41 virus-like 70S particles (lanes 7–10, animals #7, #8, #9, and #6, respectively), or control guinea pig #1 (lane 3). Note that due to the cross-reaction with the anti-guinea pig conjugate used in the modified INNO-LIA HIV confirmation test, the immobilized gp41 is recognized by the human HIV-positive serum (lane 2). Protein gp41 recognized by the animals' sera is indicated by arrows on the right side.

Table 2
Anti-HAV and anti-gp41 response in sera of guinea pigs immunized with chimeric 70S particles of HAV-gp41

Nr.	Immunized with	Anti-HAV[a]	Anti-gp41[b]
#1	PBS	–	–
#2	PBS	–	–
#3	HAVRIX	+	–
#4	HAVRIX	+	–
#5	HAVRIX	+	–
#6	HAV-gp41	+	±
#7	HAV-gp41	+	+
#8	HAV-gp41	+	+
#9	HAV-gp41	+	±

[a] Competition assay using a polyclonal anti-HAV to coat the immunoplate.
[b] Using the peptide GGGLELDKWASLW for coating an immunoplate; +, positive reaction (inhibition >85%); –, no competition; ±, weak positive reaction (competition ~50%).

Next to the immunogenicity, the induction of a neutralizing antibody response is an important property of an efficacious vaccine. Therefore, we assessed the HIV neutralizing response of the animal sera by measuring the reduction of reverse transcriptase activity in HIV-infected cells (Eberle and Seibl, 1992). Specific, albeit weak, HIV-neutralizing activity was detectable in the serum of guinea pig #7 (mean value of HIV RT activity was 944 RLU), but not in the negative guinea pig serum (3413 RLU). Specific neutralizing effect was less obvious for the marmoset sera (not shown). The neutralizing activity of mAb 2F5 used as a positive control was more pronouced (229 RLU). The HIV neutralization ability seems to be unspecifically affected by high protein concentrations (data not shown). Therefore, specific low-titer activity might have been masked as reported previously (Yusibov et al., 1997). Collectively, the data show that the chimeric HAV-gp41 is replication-competent and able to induce an anti-epitope immune response either due to viral replication in HAV-susceptible non-human primates or due to immunization of small animals (guinea pigs).

4. Discussion

Since its introduction in 1992, the hepatitis A vaccine containing the inactivated virus has proven highly efficacious in preventing viral infection (and replication) worldwide. Compared to other killed or subunit vaccines, HAV seems to be particularly stable and immunogenic, producing a neutralizing antibody response of both the IgM and IgG class in vaccines and providing protection for more than 10 years (Andre et al., 2002). In addition, a cellular immune response is also elicited (Schmidtke et al., 2005). Furthermore, recent vaccination studies suggest that the dose of the inactivated virus can be reduced underlining the exceptional immunogenicity of the HAV particle (Pancharoen et al., 2005). An attenuated live vaccine has been employed and shown promising results in effectively preventing HAV infection in a hepatitis A outbreak (Zhao et al., 2000; Wang et al., 2004). Combined these particular features of the

anti-HAV preventive measures had convinced us to test HAV as a live and subunit carrier of the HIV 2F5 epitope. In cell culture HAV replication results in the production of both fully matured (160S) and immature or empty (70S) particles, providing us with the opportunity to use the mature chimeric particles for infection of marmosets as a prototype of a live vaccine and the immature particles for immunization of guinea pigs (subunit vaccine).

As a prerequisite for a chimeric live vaccine and in extension of our earlier work, we demonstrated in a first set of experiments that the chimeric HAV genome with the 2F5e insertion in the C-terminal part of 2A was fully infectious and genetically stable in cell culture (Table 1) and in animals (Fig. 3B). This finding was not unexpected, as others and we had shown that HAV particle assembly requires the N-terminal half of 2A and that the C-terminal part is mostly dispensable for viral replication in vitro and in vivo (Harmon et al., 1995; Probst et al., 1999; Cohen et al., 2002). In addition, we have recently shown that 2A is exposed to the particle's surface and might thus efficiently present the foreign epitope for a humoral immune response (Probst et al., 1999; Beneduce et al., 2002). The results presented here and before clearly demonstrate that the 2A signal function in HAV particle assembly is not much affected by C-terminal deletions or the insertion of the 2F5e or another epitope into this part of 2A (Beneduce et al., 2002, and data not published). During HAV maturation, the primary assembly signal 2A is for the most part removed from the surface of immature particles by a yet unidentified host proteinase (Cohen et al., 2002; Rachow et al., 2003). Removal of 2A seems not to be complete, as small quantities of VP1-2A were detected in infectious particles (Borovec and Anderson, 1993). Moreover, anti-2A antibodies were found in sera of HAV-infected patients providing indirect evidence that 2A or its structural precursor VP1-2A is presented to the immune system (Khudyakov et al., 1999).

After administration of a live virus, antibodies to viral particle might be directly induced by the inoculated material or by virus newly synthesized in the liver during viral replication. As the marmosets used here were orally and parenterally inoculated, the immune response to viral particles does not allow differentiating between these alternatives. As active viral replication is a prerequisite for the late appearance of infectious viral particles in the animals' stool, the detection of virus in feces up to 18 dpi is clear evidence of viral replication in vivo. Viral particles that may have passed through the animals' intestinal tract are usually shed in feces within 1–4 dpi (Asher et al., 1995). Furthermore, demonstration of antibodies directed to the viral non-structural protein 3C (Fig. 4) presents unambiguous evidence for chimeric virus replication in the infected animals (Jia et al., 1992; Kabrane-Lazizi et al., 2001).

Over the past years, both subviral particles and attenuated viral strains that are in prophylactic use or considered as potential vaccines were tested as carriers of foreign antigens. Both the recombinant surface (HBsAg) and core antigen (HBcAg) of hepatitis B virus (HBV) efficiently presented foreign epitopes to the immune system (Pumpens et al., 1995; Yang et al., 2005). However, presentation of the 2F5e on recombinant HBsAg was unable to elicit a neutralizing immune response (Eckhart et al., 1996), whereas presentation on the hemagglutinin of a chimeric influenza virus induced a neutralizing antibody response that inhibited HIV syncytium formation (Muster et al., 1994). By the same assay system, the neutralizing capacity of antisera directed to a chimeric plant virus displaying 2F5e was documented (Marusic et al., 2001). These observations underline the notion that the ability of the 2F5e to elicit neutralizing antibodies depends on the molecular context in which it is presented to the host immune system and the neutralizing assay system used. These studies are representative for our observations reported here. A neutralizing antibody response was clearly detectable in guinea pigs, but less well in marmosets. For the human monoclonal antibody 2F5 it is assumed that it neutralizes HIV infectivity by preventing a conformational change required for full infectivity (Barbato et al., 2003). In support of these findings, the data described here clearly indicate that the 7 amino acids of the 2F5e are presented on the HAV particles' surface in a stable conformation able to elicit a neutralizing response. The particular location on the carrier and thus limited accessibility of the 2F5e might be a major determinant for variable neutralization ability (Muster et al., 1994; Eckhart et al., 1996; Marusic et al., 2001). Moreover, the experimental animal used for antibody production and the neutralization assay system certainly will have an effect on the ability to demonstrate a protective immune response.

An attenuated HAV strain has been successfully shown to prevent overt and asymptomatic hepatitis A and thus to serve as a live vaccine (Zhao et al., 2000). Although not directly tested, it can be assumed that this vaccine might elicit a mucosal immunity. Shown in mice, rectal immunization with the HAV vaccine induced a strong systemic (spleen cells, humoral antibodies) and local (Peyer's patch cells) immune response (Mitchell and Galun, 2003). Moreover, ingestion of HAV by the gastrointestinal tract stimulates IgA production due to contact of the virus with the mucosa-associated lymphoid tissue (Dotzauer et al., 2000). Mucosal tissues are also the major sites of HIV entry and initial replication (Lehner et al., 1999). Therefore, an effective strategy to prevent HIV infection must consider the use of a vaccine that elicits both systemic and mucosal immunity. Secretory immunoglobulins A, in fact, may function as a first line of defense, preventing the transmission of the virus through the mucosa.

Using the replication-competent chimeric HAV described here, it will be intriguing to determine whether this virus induces a mucosal immune response protecting both against HAV and HIV infections.

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References

Anderson, D.A., Ross, B.C., 1990. Morphogenesis of hepatitis A virus: isolation and characterization of subviral particles. J. Virol. 64, 5284–5289.

Andre, F., Van Damme, P., Safary, A., Banatvala, J., 2002. Inactivated hepatitis A vaccine: immunogenicity, efficacy, safety and review of official recommendation for use. Expert Rev. Vaccines 1, 9–23.

Asher, L.V., Binn, L.N., Mensing, T.L., Marchwicki, R.H., Vassell, R.A., Young, G.D., 1995. Pathogenesis of hepatitis A in orally inoculated owl monkeys (Aotus trivirgatus). J. Med. Virol. 47, 260–268.

Barbato, G., Bianchi, E., Ingallinella, P., Hurni, W.H., Miller, M.D., Ciliberto, G., Cortese, R., Bazzo, R., Shiver, J.W., Pessi, A., 2003. Structural analysis of the epitope of the anti-HIV antibody 2F5 sheds light into mechanism of neutralization and HIV fusion. J. Mol. Biol. 330, 1101–1115.

Beneduce, F., Kusov, Y., Klinger, M., Gauss-Müller, V., Morace, G., 2002. Chimeric hepatitis A virus particles presenting a foreign epitope (HIV gp41) at their surface. Antiviral Res. 55, 369–377.

Borovec, S.V., Anderson, D.A., 1993. Synthesis and assembly of hepatitis A virus-specific proteins in BS-C-1 cells. J. Virol. 67, 3095–3102.

Bradley, D.W., Maynard, J.E., Hindman, S.H., Hornbeck, C.L., Fields, H.A., McCaustland, K.A., Cook Jr., E.H., 1977. Serodiagnosis of viral hepatitis A: detection of acute-phase immunoglobulin M anti-hepatitis A virus by radioimmunoassay. J. Clin. Microbiol. 5, 521–530.

Cohen, L., Benichou, D., Martin, A., 2002. Analysis of deletion mutants indicates that the 2A polypeptide of hepatitis A virus participates in virion morphogenesis. J. Virol. 76, 7495–7505.

Dotzauer, A., Gebhardt, U., Bieback, K., Göttke, U., Kracke, A., Mages, J., Lemon, S.M., Vallbracht, A., 2000. Hepatitis A virus-specific immunoglobulin A mediates infection of hepatocytes with hepatitis A virus via asialoglycoprotein receptor. J. Virol. 74, 10950–10957.

Dotzauer, A., Brenner, M., Gebhardt, U., Vallbracht, A., 2005. 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. J. Gen. Virol. 86, 2747–2751.

Eberle, J., Seibl, R., 1992. A new method for measuring reverse transcriptase activity by ELISA. J. Virol. Methods 40, 347–356.

Eberle, J., Knopf, C.W., 1996. Nonisotopic assays of viral polymerases and related proteins. Methods Enzymol. 275, 257–276.

Eckhart, L., Raffelsberger, W., Ferko, B., Klima, A., Purtscher, M., Katinger, H., Ruker, F., 1996. Immunogenic presentation of a conserved gp41 epitope of human immunodeficiency virus type 1 on recombinant surface antigen of hepatitis B virus. J. Gen. Virol. 77, 2001–2008.

Emerson, S.U., Huang, Y.K., Nguyen, H., Brockington, A., Govindarajan, S.St., Clair, M., Shapiro, M., Purcell, R.H., 2002. Identification of VP1/2A and 2C as virulence genes of hepatitis A virus and demonstration of genetic instability of 2C. J. Virol. 76, 8551–8559.

Finnegan, C.M., Berg, W., Lewis, G.K., DeVico, A.L., 2002. Antigenic properties of the human immunodeficiency virus transmembrane glycoprotein during cell–cell fusion. J. Virol. 76, 12123–12134.

Gauss-Müller, V., Deinhardt, F., 1988. Immunoreactivity of human and rabbit antisera to hepatitis A virus. J. Med. Virol. 24, 219–228.

Harmon, S., Emerson, S.U., Huang, Y.K., Summers, D.F., Ehrenfeld, E., 1995. Hepatitis A viruses with deletions in the 2A gene are infectious in cultured cells and marmosets. J. Virol. 69, 5576–5581.

Hornei, B., Kammerer, R., Moubayed, P., Frings, W., Gauss-Müller, V., Dotzauer, A., 2001. Experimental hepatitis A virus infection in guinea pigs. J. Med. Virol. 64, 402–409.

Jia, X.Y., Summers, D.F., Ehrenfeld, E., 1992. Host antibody response to viral structural and nonstructural proteins after hepatitis A infection. J. Infect. Dis. 165, 273–280.

Jilg, W., Bittner, R., Bock, H.L., Clemens, R., Schatzl, H., Schmidt, M., Andre, F.E., Deinhardt, F., 1992. Vaccination against hepatitis A: comparison of different short-term immunization schedules. Vaccine 1, 126–128.

Kabrane-Lazizi, Y., Emerson, S.U., Herzog, C., Purcell, R.H., 2001. Detection of antibodies to HAV 3C proteinase in experimentally infected chimpanzees and in naturally infected children. Vaccine 19, 2878–2883.

Khudyakov, Y.E., Lopareva, E.N., Jue, D.L., Fang, S., Spelbring, J., Krawczynski, K., Margolis, H.S., Fields, H.A., 1999. Antigenic epitopes of the hepatitis A virus polyprotein. Virology 260, 260–272.

Kusov, Y., Gauss-Müller, V., 1999. Improving proteolytic cleavage at the 3A/3B site of the hepatitis A virus polyprotein impairs processing and particle formation, and the impairment can be complemented in trans by 3AB and 3ABC. J. Virol. 73, 9867–9878.

Kusov, Y.Y., Gosert, R., Gauss-Müller, V., 2005. Replication and in vivo repair of the hepatitis A virus genome lackung the poly(A) tail. J. Gen. Virol. 86, 1363–1368.

Lehner, T., Bergmeier, L., Wang, Y., Tao, L., Mitchell, E., 1999. A rational basis for mucosal vaccination against HIV infection. Immunol. Rev. 170, 183–196.

Malcolm, B.A., Chin, S.M., Jewell, D.A., Stratton-Thomas, J.R., Thudium, K.B., Ralston, R., Rosenberg, S., 1992. Expression and characterization of recombinant hepatitis A virus proteinase. Biochemistry 31, 3358–3363.

Markel, H., 2005. The search for effective HIV vaccines. New Engl. J. Med. 335, 753–757.

Marusic, C., Rizza, P., Lattanzi, L., Mancini, C., Spada, M., Belardelli, F., Benvenuto, E., Capone, I., 2001. Chimeric plant virus particles as immunogens for inducing murine and human immune responses against human immunodeficiency virus type 1. J. Virol. 75, 8434–8439.

Matoba, N., Magerus, A., Geyer, B.C., Zhang, Y., Muralidharan, M., Alfsen, A., Arntzen, C.J., Bomsel, M., Mor, T.S., 2004. A mucosally targeted subunit vaccine candidate eliciting HIV-1 transcytosis-blocking Abs. Proc. Natl. Acad. Sci. U.S.A. 101, 13584–13589.

Mitchell, L.A., Galun, E., 2003. Rectal immunization of mice with hepatitis A vaccine induces stronger systemic and local immune responses than parenteral immunization. Vaccine 21, 1527–1538.

Muster, T., Guinea, R., Trkola, A., Purtscher, M., Klima, A., Steindl, F., Palese, P., Katinger, H., 1994. Cross-neutralizing activity against divergent human immunodeficiency virus type 1 isolates induced by the gp41 sequence ELDKWAS. J. Virol. 68, 4031–4034.

Pancharoen, C., Mekmullica, J., Thisyakorn, U., Kasempimolporn, S., Wilde, H., Herzog, C., 2005. Reduced-dose intradermal vaccination against hepatitis A with an aluminum-free vaccine is immunogenic and can lower costs. Clin. Infect. Dis. 41, 1537–1540.

Probst, C., Jecht, M., Gauss-Müller, V., 1999. Intrinsic signals for the assembly of hepatitis A virus particles. Role of structural proteins VP4 and 2A. J. Biol. Chem. 274, 4527–4531.

Pumpens, P., Borisova, G.P., Crowther, R.A., Grens, E., 1995. Hepatitis B virus core particles as epitope carriers. Intervirology 38, 63–74.

Purcell, R.H., Wong, D.C., Shapiro, M., 2002. Relative infectivity of hepatitis A virus by the oral and intravenous routes in 2 species of nonhuman primates. J. Infect. Dis. 185, 1668–1671.

Purtscher, M., Trkola, A., Gruber, G., Buchacher, A., Predl, R., Steindl, F., Tauer, C., Berger, R., Barrett, N., Jungbauer, A., Katinger, H., 1994. A broadly neutralizing human monoclonal antibody against gp41 of human immunodeficiency virus type 1. AIDS Res. Hum. Retroviruses 10, 1651–1658.

Rachow, A., Gauss-Müller, V., Probst, C., 2003. Homogeneous hepatitis A virus particles Proteolytic release of the assembly signal 2A from procapsids by factor Xa. J. Biol. Chem. 278, 29744–29751.

Schmidtke, P., Habermehl, P., Knuf, M., Meyer, C.U., Sanger, R., Zepp, F., 2005. Cell mediated and antibody immune response to inactivated hepatitis A vaccine. Vaccine 23, 5127–5132.

Shouval, D., Ashur, Y., Adler, R., Lewis, J.A., Armstrong, M.E., Davide, J.P., McGuire, B., Kuter, B., Brown, L., Miller, W., Elkana, Y., Nalin, D.R., 1993. Single and booster dose responses to an inactivated hepatitis A virus vaccine: comparison with immune serum globulin prophylaxis. Vaccine 1, 9–14.

Schultz, D.E., Honda, M., Whetter, L.E., McKnight, K.L., Lemon, S.M., 1996. Mutations within the 5′ nontranslated RNA of cell culture-adapted hepatitis A virus which enhance cap-independent translation in cultured African green monkey cells. J. Virol. 70, 1041–1049.

Sikuler, E., Keynan, A., Hanuka, N., Friedman, M.G., Sarov, I., 1983. Detection and persistence of specific IgA antibodies in serum of patients with hepatitis A by capture radioimmunoassay. J. Med. Virol. 11, 287–294.

Sompayrac, L., 2002. Hepatitis A virus—a virus that detours. In: How Pathogenic Viruses Work. John and Bartlett Publishers, Boston, Toronto, London, Singapore, pp. 46–49.

Stewart, D.R., Morris, T.S., Purcell, R.H., Emerson, S.U., 1997. Detection of antibodies to the nonstructural 3C proteinase of hepatitis A virus. J. Infect. Dis. 176, 593–601.

Wang, X.Y., Xu, Z., Yao, X., Tian, M., Zhou, L., He, L., Wen, Y., 2004. Immune responses of anti-HAV in children vaccinated with live attenuated and inactivated hepatitis A vaccines. Vaccine 22, 1941–1945.

Yang, H.J., Chen, M., Cheng, T., He, S.Z., Li, S.W., Guan, B.Q., Zhu, Z.H., Gu, Y., Zhang, J., Xia, N.S., 2005. Expression and immunoactivity of chimeric particulate antigens of receptor binding site-core antigen of hepatitis B virus. World J. Gastroenterol. 11, 492–497.

Yusibov, V., Modelska, A., Steplewski, K., Agadjanyan, M., Weiner, D., Hooper, D.C., Koprowski, H., 1997. Antigens produced in plants by infection with chimeric plant viruses immunize against rabies virus and HIV-1. Proc. Natl. Acad. Sci. U.S.A. 94, 5784–5788.

Zamjatina, N.A., Andjaparidze, A.G., Balayan, M.S., Sobol, A.V., Titova, I.P., 1990. Susceptibility of *Macaca rhesus* monkey to hepatitis A virus strains isolated from man and monkeys. Vopr. Virusol. 1, 30–32.

Zhao, Y.L., Meng, Z.D., Xu, Z.Y., Guo, J.J., Chai, S.A., Duo, C.G., Wang, X.Y., Yao, J.F., Liu, H.B., Qi, S.X., Zhu, H.B., 2000. H2 strain attenuated live hepatitis A vaccines: protective efficacy in a hepatitis A outbreak. World J. Gastroenterol. 6, 829–832.