A recombinant vaccinia virus expressing hepatitis A virus structural polypeptides: characterization and demonstration of protective immunogenicity

Peter Karayiannis,1* Sarah O'Rourke,1 Michael J. McGarvey,1 Shashi Luther,1 Jenny Waters,1 Robert Goldin2 and Howard C. Thomas1

Departments of 1Medicine and 2Histopathology, St Mary's Hospital Medical School, Imperial College of Science, Technology and Medicine, London W2 1NY, U.K.

A recombinant vaccinia virus containing most of the P1 region of hepatitis A virus (HAV) was constructed. Cell lysates of cultures infected with the virus contained HAV proteins detectable by radioimmunoassay. Western blot analysis revealed the presence of a single protein of Mr 60K to 62K, bearing epitopes from structural polypeptides VP4, -3 and -2, and the N terminus of VP1. The size of the protein suggests that at least some of the vaccinia virus thymidine kinase is also expressed. Inoculation of tamarin monkeys with the recombinant virus resulted in the development of a specific anti-HAV immune response which was protective against challenge with a virulent strain of HAV. Recombinant viruses expressing the above region of HAV or the proteins expressed by such viruses may be useful in the development of a vaccine suitable for use in man.

Introduction

Hepatitis A virus (HAV) remains an important cause of morbidity and occasionally mortality, both in developing countries where it is endemic, and in industrialized nations where it is normally sporadic. As sanitary conditions in developing countries improve, the prevalence of HAV seropositivity decreases, creating a large pool of susceptible individuals. These individuals, together with high risk groups such as day-care centre staff, parents and siblings of toddlers attending such centres, promiscuous homosexual men, and military personnel and tourists in endemic areas, would benefit from an effective HAV vaccine. Formalin-inactivated (Provost & Hilleman, 1978; Provost et al., 1986; Binn et al., 1986; Flehmig et al., 1989) and live attenuated (Provost et al., 1982, 1983, 1986; Feinstone et al., 1983; Karron et al., 1988; Mao et al., 1989) virus vaccines have been produced and shown to be protective in both primates and human volunteers. A candidate subunit vaccine based on the VP1 structural polypeptide has also been produced in Escherichia coli by recombinant DNA techniques, but, although immunogenic in rabbits (Johnston et al., 1988), the antibody produced reacted only with denatured VP1 and not with whole virus.

Recombinant vaccinia viruses expressing foreign genes have been constructed recently, opening the way to a new approach to vaccine development (Smith et al., 1984; Mackett et al., 1985a; Elango et al., 1986). In this study, we report the characterization of a recombinant vaccinia virus (vHAV) containing an HAV cDNA fragment encoding structural polypeptides VP4, VP2 and VP3, and the N terminus of VP1. Tamarin monkeys inoculated with this recombinant developed protective immunity against HAV infection.

Methods

Vectors. An insertion vector (pGS62) containing a vaccinia virus early promoter (7.5K) (Mackett et al., 1984) was linearized with BamHI followed by alkaline phosphatase treatment (Sambrook et al., 1989). A BamHI fragment from plasmid pHAV/J (Ticehurst et al., 1987) (obtained from Drs J. Ticehurst and R. H. Purcell, National Institutes of Health, Bethesda, Md., U.S.A.) corresponding to positions 634 to 2375 of the HAV genome (Cohen et al., 1987) was ligated into linearized pGS62 immediately after the 7.5K promoter, forming a chimeric plasmid (pGS62/HAV) containing the 1742 bp HAV cDNA fragment under the control of this promoter. There were 101 nucleotides from the 5' untranslated region of the HAV genome between the cloning site and the ATG of the open reading frame encoding the polyprotein (Fig. 1). The remainder of the genome encodes structural polypeptides VP4, VP2 and VP3, and the first 57 amino acids from the N terminus of VP1. Transformed E. coli colonies carrying the chimeric plasmid were identified by hybridization with a 32P-labelled HAV cDNA probe (Karayiannis et al., 1988) and the correct orientation of the insert with respect to the vaccinia virus 7.5K promoter was determined by dideoxynucleotide sequencing. The primer used (5' ATTGCACGGTAAAGGAAG) bound to a region upstream from the cloning site (Mackett et al., 1984).
Recombinant vaccinia virus. Recombinant vHAV was generated by transfection of pGS62/HAV into Vero cells already infected with wild-type vaccinia virus (strain WR, a gift from Dr G. L. Smith, Oxford, U.K.) as previously described (Mackett et al., 1985b). Homologous recombination between the chimera and vaccinia virus resulted in the interruption of the thymidine kinase (TK) gene, and the resultant TK-recombinant viruses were selected as previously described (Mackett et al., 1985b). The presence of the foreign gene in recombinant vHAV was confirmed by dot-blot hybridization (Karayiannis et al., 1988) and Southern blot analysis (Mackett et al., 1985b). In the latter, vHAV DNA was digested with BamHI to release the 1.7 kb HAV cDNA insert. HAV mRNA transcripts were detected by Northern blot analysis, identified a fragment of the 1.7 kb HAV insert, and undigested and digested HAV DNA was probed with 32P. Southern blot analysis, identified a fragment of the 1.7 kb HAV insert, and undigested and digested HAV DNA was probed with 32P.

Expression of HAV protein. Expression of HAV proteins was established by solid phase RIA (Karayiannis et al., 1986) of cell lysates and immunostaining of vHAV-infected monolayers with human anti-HAV IgG. Plaques expressing HAV proteins were detected using rabbit anti-human and swine anti-rabbit antisera in turn. The latter serum was labelled with biotin and alkaline phosphatase (Dakopatts, ABComplex.AP).

Further characterization of the expressed proteins was achieved by immunoblotting following separation of the proteins by 10% SDS-PAGE and blotting onto nitrocellulose filters, which were then blocked with 5% milk (Marvel) in TBS (10 mM Tris–HCl pH 7.4, 150 mM NaCl) containing 1% NP40 at room temperature for 1 h. The filters were then incubated at 37°C for 1 h in TBS with guinea-pig antisera (gift from Dr D. Sangar, Wellcome Biotechnology, Beckenham, U.K.) against HAV peptides coupled to keyhole limpet haemocyanin representing amino acid positions 10 to 30, 40 to 59, 31 to 47 and 7 to 23 in VP1, 2, 3 and 4 respectively. Following several washes with TBS/NP40, the filters were incubated at 37°C for 1 h with horseradish peroxidase-conjugated rabbit anti-guinea-pig immunoglobulins in TBS. The TBS in all antibody binding steps contained 5% milk. Bands were visualized using an enhanced chemiluminescence method (Amersham) following exposure of the filters to photographic film.

Animal experiments. To test the ability of vHAV to protect against subsequent HAV exposure, two tamarin monkeys (animals 104 and 123) were inoculated intradermally in the upper part of their back with 10⁷ p.f.u. of vHAV in 0.1 ml of saline, following a bleed for baseline serology and biochemistry. Similarly, one other animal (tamarin 125) was inoculated with 10⁷ p.f.u. of wild-type vaccinia virus (strain WR); the dose of virus was reduced to limit the severity of the dermal lesions, which tend to be more severe with wild-type virus than with TK-recombinants. The animals were allowed to recover and mount an immune response, and were bled 8 weeks later. Details on housing, diet, anaesthesia and experimental procedures have been reported elsewhere (Karayiannis et al., 1986). No other animals were present and no other experimental HAV infections were undertaken in the isolation room during the experiment.

HAV challenge. Ten weeks after vHAV inoculation, both the immunized and the control animals were challenged with 0.4 ml of HAV strain HM175 by intravenous injection. HAV was grown in PLC/PRF/5 cells (passage 15) and its capacity to cause disease was tested in one animal (tamarin 87). The titre of the virus was 4 × 10⁶ radioimmunofocus-forming units/ml of inoculum, as estimated in tissue culture by a radioimmunofocus assay (Dr D. J. Wood, National Institute of Biological Standards, South Mimms, U.K.).

Biochemical and serological investigations. Alanine aminotransferase (ALT) levels were measured using a Reflotron System with the appropriate test strips (Boehringer Mannheim). Anti-HAV antibody was detected with commercially available kits (Havab and Havab-M; Abbott Laboratories). HAV antigen and HAV RNA in faecal samples were detected as described previously (Karayiannis et al., 1986, 1988).

Results

Characterization of vHAV

Digestion of vHAV DNA with BamHI, followed by Southern blot analysis, identified a fragment of the...
A recombinant vaccinia-hepatitis A virus

Expression of HAV proteins

HAV antigen was detected in cell lysates of vHAV-infected cultures by RIA (infected cell:control cell, lysate, 5:1). The presence of HAV antigens was confirmed by immunostaining of similar monolayers. Uninfected cells in the same monolayers and wild-type vaccinia virus-infected cultures were HAV antigen-negative.

Immunoblotting of vHAV-expressed proteins separated by SDS–PAGE revealed the presence of a single band of Mr, 60K to 62K which was reactive with antisera to the VP4, -3 and -2 peptides (Fig. 3), and with antiserum to a VP1 peptide from the N terminus (residues 10 to 30), but not to one from the C terminus (residues 210 to 230) of the polypeptide which was not encoded by the HAV cDNA insert. Strong signals were obtained with anti-

VP1 (not shown) and anti-VP4 antisera, whereas the other two peptide antisera gave a weaker signal (not shown). A further band (47K) binding anti-VP2 antiserum represented a non-specific reaction because it was also present in immunoblots of proteins from control cultures.

vHAV inoculation of tamarins

Pre-vaccination serum samples were negative for anti-HAV antibodies. The animals developed small erythematous lesions at the site of inoculation but no secondary lesions were observed. Sera from all three animals were retested for anti-HAV antibodies 8 and 10 weeks after vaccination. Both vHAV recipient animals were found to be positive, with anti-HAV antibody titres of 1/40 and 1/100 (tamarins 104 and 123 respectively) recorded in the 10 week serum sample. Tamarin 125, which received wild-type vaccinia virus, was negative for anti-HAV antibody.

HAV challenge

Ten weeks after vaccination, all three animals, which had normal liver biochemistry and histology, were challenged by intravenous injection with live HAV strain HM175 grown in tissue culture. The dose and natural history of infection with this inoculum were predetermined in tamarin 87 (Fig. 4). Tamarin 125 (vaccinated with wild-type vaccinia virus) developed the same biochemical changes as tamarin 87 (Fig. 5). There were ALT abnormalities between weeks 1 and 3, and a liver biopsy taken at week 3 showed heavy infiltration of portal tracts by mononuclear cells, some lobular inflammation and liver cell swelling in periportal areas (Karayiannis et al., 1986). HAV antigen was detected in
the faeces between days 4 and 12, and HAV RNA between days 3 and 15 (Karayiannis et al., 1986). In contrast, the two animals vaccinated with vHAV did not show any elevation in ALT levels (Fig. 5) and there were no histological changes in the liver biopsies performed 3 weeks post-challenge; HAV antigen and RNA were absent from the stools.

Anti-HAV IgM was detected in the control animal between weeks 2 and 6; no anti-HAV IgM response was detected in the vaccinated animals, but there was a secondary response following challenge with live virus. Titres rose to 10^2 and 10^3 (animals 104 and 123, respectively) within a week of challenge and remained at these levels throughout the follow-up period. In the control animal (tamarin 125), anti-HAV antibody titres did not plateau until week 6 (Fig. 6).

**Discussion**

The recombinant vHAV generated produces a single 60K to 62K HAV protein which is immunogenic in the tamarin model. This immune response is protective against HAV challenge.

Characterization of the recombinant virus by Southern blotting indicates the presence of an insert of the correct size (1.7 kb) which hybridizes to the relevant HAV cDNA fragment. Moreover, a dominant HAV-specific transcript of 2.8 kb was detected. This is longer than the HAV insert containing the sequences encoding the structural polypeptides (VP4, -2 and -3, and part of VP1) because the transcript terminates in flanking vaccinia virus sequences downstream from the HAV insert. Although smaller transcripts were also detected, their significance is unknown; they may represent degradation products of the main RNA transcript.

The HAV structural polypeptides are expressed as a single polyprotein, a 60K to 62K protein bearing epitopes from all four structural polypeptides. The size of this protein suggests that apart from the HAV polypeptides the polyprotein may contain some of the C terminus of the interrupted vaccinia virus TK. There is no evidence that the polyprotein is processed to yield the individual structural polypeptides. This is not surprising in the absence of the HAV-encoded protease; cellular proteases do not appear to have modified the protein post-translationally.

The protein expressed is expected to be a fusion product of the HAV polyprotein with the C terminus of vaccinia virus TK. Since there is no stop codon at the end of the HAV-specific sequence, translation is expected to continue to the first translational stop codon, at the end of the TK gene; the predicted size of the fusion protein would be between 60K and 65K. This agrees with the estimated size of the protein obtained by immunoblotting.

The animal experiments show that vaccination with recombinant vHAV results in an immune response to HAV structural proteins and that animals are protected when subsequently challenged with a live HAV strain capable of inducing hepatitis, as shown in two control animals not immunized with vHAV (tamarins 87 and 125). VP1 has been shown in studies with neutralizing monoclonal antibodies (MAbs) to contain at least some neutralizing antigenic sites (Hughes et al., 1984). Furthermore, a synthetic peptide incorporating amino
acids 11 to 25 of VP1 has been shown to induce anti-
HAV neutralizing antibodies (Emini et al., 1985). More
recently, studies with a range of anti-HAV MAbs in
neutralization escape mutant experiments suggested that
the capsid protein, VP3, also contributes to an immuno-
dominant domain on the HAV capsid. It is suggested
that this conformational domain is formed by VP3 and
VP1 (Ping et al., 1988). If this is correct, then the recombinant vHAV constructed not only expresses HAV
capsid proteins but also presents them in the correct
context for the formation of at least part of this
conformational epitope. The cDNA fragment intro-
duced into vaccinia virus has the complete coding
sequence of VP4, VP2 and VP3, and the N-terminal 57
amino acids of VP1. This N-terminal portion of VP1
would, therefore, also incorporate the epitope described
by Emini et al. (1985) and, as shown by the immunoblot
experiments, the VP1 peptide antibody to this epitope
bonds to the expressed HAV polyprotein.

MAbs to HAV compete successfully with labelled
polyclonal serum for binding to HAV and, as shown,
these MAbs, which are virus-neutralizing, are directed
mainly to the single conformational epitope (Hughes
et al., 1984; Stapleton & Lemon, 1987). Polyclonal anti-
HAV antiserum was used for the detection of the expres-
sed protein in cell lysates by RIA and immuno-
staining of infected cultures, although the same anti-
bodies failed to bind to denatured vHAV protein follow-
ing separation on SDS–polyacrylamide gels (results not
shown). Moreover, anti-HAV antibody in post-vaccina-
tion tamarin sera was detected in the Havab test, which
is a competitive inhibition assay. These results lend
further support to the hypothesis that the vHAV protein
may represent part of the conformational HAV epitope
to which the HAV antibodies employed in the assays
described may bind.

vHAV-inoculated tamarin monkeys develop a specific
anti-HAV response following a single injection of the
recombinant virus. This contrasts with the need for three
doses of formalin-inactivated or live attenuated virus
vaccines (1-10) to produce an adequate response. A
secondary response was seen on challenge with HAV
and, although no HAV antigen or HAV RNA was
detectable in the faeces, a subclinical infection cannot be
ruled out.

Recombinant vaccinia virus expressing the region of the
HAV genome used here or the proteins expressed by
such viruses may form the basis for a vaccine in man.

We would like to thank Drs J. Ticehurst and R. H. Purcell
(NIH) for the provision of pHAV/J, Dr G. L. Smith for the provision of the
pGS62 vector and the wild-type vaccinia virus, and Dr J. Saldanha for
useful discussion. In addition, we would like to thank Dr D. Sangar
(Wellcome Biotechnology) for the provision of the peptide antibodies.
This work was supported by a grant from the World Health
Organization.

References

Binn, L. N., Bancroft, W. H., Lemon, S. M., Marchwicki, R. H.,
Leduc, J. W., Trahan, C. J., Staley, E. C. & Keenan, C. M.
(1986). Preparation of a prototype inactivated hepatitis A virus
vaccine from infected cell cultures. Journal of Infectious Diseases
153, 749–756.

Cohen, J. L., Ticehurst, J. R., Purcell, R. H., Buckler-White, A. &
Baroudy, B. M. (1987). Complete nucleotide sequence of wild-type
hepatitis A virus: comparison with different strains of hepatitis A

Elango, N., Prince, G. A., Murphy, B. R., Venkatesan, S.,
Chanock, R. M. & Moss, B. (1986). Resistance to human respiratory
syncytial virus (RSV) induced by immunization of cotton
rats with a recombinant vaccinia virus expressing RSV G
glycoprotein. Proceedings of the National Academy of Sciences, U.S.A.

Induction of hepatitis A virus neutralizing antibody by a virus-

Live attenuated vaccine for hepatitis A. Developments in Biological
Standardization 54, 429–432.

of a killed hepatitis A vaccine in seronegative volunteers. Lancet i,
1039–1041.

Hughes, J. V., Stanton, L. W., Tomassini, J. E., Long, W. J. &
Scolnick, E. M. (1985). Neutralizing monoclonal antibodies to
hepatitis A virus: partial localization of a neutralizing antigenic site.

Johnston, J. M., Harmon, S. A., Binn, L. N., Richards, O. C.,
genetic properties of a hepatitis A virus capsid protein expressed in

Karayiannis, P., Jonett, T., Enzicott, M., Pignatelli, M., Brenes,
replication in tamarins and host immune response in relation to
pathogenesis of liver cell damage. Journal of Medical Virology 18,
261–276.

Karayiannis, P., McGarvey, M. J., Fry, M. A. & Thomas, H. C.
(1988). Detection of hepatitis A virus RNA in tissues and faeces of
experimentally infected tamarins by cDNA hybridization. In Viral
Hepatitis and Liver Disease (pp. 117–120). Edited by A. J. Zuckerman.
New York: Alan R. Liss.

Karron, R. A., Daemer, R., Ticehurst, J., D'Hondt, E., Popper,
Studies of prototype live hepatitis A virus vaccines in primate

Kieny, M. P., Lathe, R., Drilien, R., Spenser, D., Skory, S.,
Expression of rabies virus glycoprotein from a recombinant vaccinia

the production and selection of infectious vaccinia virus recombinants

recombinants: expression of cDNA genes and protective immuniza-

Mackett, M., Smith, G. L. & Moss, B. (1985b). The construction and
caracterisation of vaccinia virus recombinants expressing foreign
genes. In DNA Cloning: A Practical Approach, pp. 191–211. Edited

Mao, J. S., Dong, D. X., Zhang, H. Y., Chen, N. L., Zhang, X. Y.,
Huang, H. Y., Xie, R. Y., Zhou, T. J., Wang, Z. J., Weng, Y. Z.,
He, Z. H., Cao, Y. Y., Li, F. K. & Chu, C. M. (1989). Primary study
of attenuated live hepatitis A vaccine (H2 strain) in humans. Journal
of Infectious Diseases 159, 621–624.

recombinant vaccinia virus protects chimpanzees against hepatitis

Ping, L. H., Jansen, R. W., Stapleton, J. T., Cohen, J. I. & Lemon,


(Received 19 February 1991; Accepted 29 May 1991)