Detection of Defective Genomes in Hepatitis A Virus Particles Present in Clinical Specimens

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SUMMARY

Hepatitis A virus (HAV) particles harbouring a physically defective RNA genome have been reported to occur in all HAV-infected cell culture systems analysed so far. The most prominent defects consist of three distinct overlapping deletions in the region of the HAV genome encoding the structural proteins. By probing for the endpoints of these deletions in RNA samples using S1 nuclease and exonuclease VII mapping, we obtained suggestive evidence for the existence also of defective genomes in HAV particles present in faecal specimens, in viraemic blood collected in the course of hepatitis A virus infection in man, as well as in the liver of an experimentally infected marmoset monkey. The deletions identified extend from nucleotide (nt) 1200 to nt 3820 and from nt 1200 to nt 3240 of the HAV genome. They are compatible with two of the deletions detected in particles grown in vitro in cell cultures and shown to interfere with the replication of standard hepatitis A virions.

In recent studies aimed at the factors operating in the establishment and maintenance of persistent hepatitis A virus (HAV) infection in cell culture, we obtained evidence for the widespread occurrence of HAV particles harbouring a physically defective RNA genome (Nüesch et al., 1988). The most prominent defects consisted of three distinct types of deletions spanning a considerable part of or the whole region encoding the viral structural proteins. Moreover, genomes with 3' proximal truncations of variable size were detected. Further experiments yielded suggestive evidence that such defective particles interfere with the replication of standard hepatitis A virions in susceptible cell cultures (J. P. F. Nüesch et al., unpublished results). Thus, they show all properties of defective interfering (DI) particles.

DI particles have been detected in cell culture harvests of almost all viruses (for review, see Barrett & Dimmock, 1986). Their ability to modulate replication of standard virus in vitro and in vivo, as well as their role in the establishment of persistent infection have been studied extensively in various experimental systems. However, there is not yet an unequivocal report on the isolation of DI virus from clinical specimens in the course of natural infection of man or animals. Our studies now provide evidence that the defective genomes previously identified in vitro in defective and evidently interfering HAV particles are also present in HAV particles in vivo.

Our analysis was based on consecutive faecal samples collected during the incubation period from patient MBB, who acquired the infection when crossing the Mediterranean Sea from North Africa to Italy (Frösnér et al., 1979; Siegl et al., 1984), also on faecal samples from two diseased individuals (KMW and CLF) in two separate outbreaks of hepatitis A in Switzerland (Schilt et al., 1982; Siegl et al., 1984), on a sample of viraemic blood in a case of transfusion-associated hepatitis A (Hollinger et al. 1983), as well as on extracts of the liver of a marmoset monkey experimentally infected with HAV derived from a sample of human stool (CR326)

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Fig. 1. Northern blot analysis of HAV RNA extracted from particles in human faecal specimens (MBB 11/4, MBB 11/5, MBB 11/7, MBB 11/9, KMW 1 collected 6, 5, 3 and 1 day before, and 2 days after the onset of clinical disease, respectively) and in the liver of an experimentally infected marmoset monkey. Suspensions of the materials (10%) were centrifuged at 16500 g at 4 °C for 15 min. The resulting supernatants were extracted twice with chloroform and digested with DNase (50 μg/ml, at 37 °C for 2 h) and RNase (20 μg/ml, at room temperature for 30 min). Particles were then pelleted by ultracentrifugation (100000 g, 16 h) and viral RNA was recovered after pretreatment with proteinase K (400 μg/ml, 0.1 M-Tris-HCl pH 7.2, 1% SDS, at 37 °C for 30 min) by extraction with phenol : chloroform (1 : 1) and precipitation with ethanol. Northern blot transfers onto GeneScreen-Plus and hybridization were performed as described previously (Nüesch et al., 1988). Numbers indicate nucleotides.

collected in Costa Rica (Provost et al., 1975). All these viruses were available as cell culture isolates and some of the isolates have been shown to contain defective HAV particles (Nüesch et al., 1988). Virus particles contained in the clinical samples were purified and viral RNA was extracted as described in the legend to Fig. 1. As illustrated in this figure, Northern blotting of viral RNA revealed that the standard viral genome and the subgenomic viral RNA were present in all specimens. However, the quantity of both RNA species varied with the origin of the sample and, where consecutive specimens were available, with the time of infection.

The appearance of shorter than genome length RNAs could reflect either the presence of subgenomic viral RNAs in defective HAV particles or the breakdown of molecules during extraction; it could also be due to contamination with non-viral RNA molecules that cross-hybridize with HAV cDNA probes. The latter possibility appears likely from results of previous experiments (de Chastonay & Siegl, 1987) as well as from observations that cDNA of various picornaviral genomes detect eukaryotic rRNA even under stringent conditions of hybridization (McClure & Perrault, 1986). To test for this, 18S and 28S rRNA from uninfected MRC-5 cells were electrophoretically separated. Multiple identical blots of this RNA were then hybridized separately to the 32P-labelled RNA transcripts of six regions of the cloned HAV genome (Fig. 2). They were used for hybridization in both the positive and the negative polarity. All positive-stranded RNA probes failed to detect rRNA sequences. However, negative-stranded probes specific for the 5' untranslated region, the P2 region, and for the P3 region of the HAV genome, detected 28S rRNA to various degrees with sequences from the P3 region leading to the most pronounced reactions. No cross-hybridization could be recorded with probes spanning most of the HAV sequences specifying viral structural proteins.

In a second control experiment we added extracts of rRNA to faecal samples devoid of HAV particles and subjected this mixture to the standard purification. Hybridization of Northern blots derived from such materials never gave a detectable signal on autoradiographs. Therefore, it can be assumed that the purification procedure effectively removes any non-encapsidated, non-viral RNA from the faecal extracts and it may be concluded that the signals observed in the experiments depicted in Fig. 1 are indeed specific for HAV RNA.

Finally, we tried to obtain direct proof for the presence of defective genomes in HAV particles purified from human faecal samples, from extracts of marmoset liver, as well as from viraemic
Fig. 2. Hybridization of HAV cRNA representative of distinct regions of the HAV genome to cellular ribosomal RNA. Multiple aliquots of rRNAs were either slot-blotted or electrophoresed and transferred onto nitrocellulose. These membranes were hybridized individually to 32P-labelled HAV cRNA probes, transcribed by SP6 polymerase or T7 polymerase from subcloned HAV cDNA as indicated by arrows in (a). (b) Hybridization of negative-stranded RNA transcripts to 5 μg aliquots of rRNA extracted from BGM cells. Autoradiography of these blots was standardized and the results were compared by videodensitometry. The respective intensities are included under (b). In (c), 2 μg samples of rRNA extracted from MRC-5 cells were electrophoresed and, after transfer to nitrocellulose, were hybridized to the negative-stranded HAV cRNA probes used for (b). Hybridization with positive-stranded transcripts always gave negative results. None of the probes hybridized to 18S rRNA. Asterisk (*) denotes corrected intensity; pictured is an overexposed autoradiogram of a slot blot hybridization.
Fig. 3. Mapping of the 3′-terminal breakpoint of deletion C within the capsid protein gene region. Viral RNA prepared from clinical specimens was hybridized to a cDNA probe labelled at the 5′ end of the negative strand at the Avail site at position 3480. Hybrids were digested with S1 nuclease and protected cDNA fragments were resolved on a sequencing gel. During digestion with S1 nuclease, protection by standard viral RNA yields a fragment of 440 nt, whereas partial protection by the defective genome of type C results in a smaller fragment of 240 nt. The corresponding deletion breakpoint is located at nt 3240. All other fragments shown in these S1 mapping experiments, and probably due to incomplete homology between viral RNA and labelled probe, were not confirmed by exonuclease VII mapping (data not shown). M, DNA size markers (pBR322/HpaII); X, position of label on the S1 hybridization probe.

B also yielded a characteristic yet rather faint band on the autoradiograph. Documentation of such bands on a photographic print proved to be extremely difficult, therefore they are not shown. However these and other faint bands could be reproduced regularly whenever the quantity of clinical material permitted repetitions of experiments. Similar results were obtained for the 3′ terminus of deletion B at nt 3820 with RNA from samples 11/4, 11/5 and 11/9 of patient MBB. For RNA derived from MBB 11/5 the breakpoint at nt 3820 was confirmed by exonuclease VII digestion (data not shown). The search for the 5′ end of deletion C (nt 1370 to nt 3240) with various cDNA probes yielded negative results with all viral samples. In contrast, the 3′ terminus of this deletion could be identified readily in HAV particles contained in human stool, marmoset liver, and even human viraemic blood (Fig. 3). The presence of only one identifiable 5′-terminal breakpoint together with two demonstrable 3′-terminal breakpoints suggests that the most prominent internal deletions in defective HAV particles in clinical specimens extend between nt 1200 and nt 3820 (deletion B) and between nt 1200 and nt 3240. Fig. 4 illustrates that defective genomes with the ‘C′-3′ endpoint are found both in virus particles...
Fig. 4. Mapping of the 3'-terminal breakpoint of deletion C in virus from marmoset liver infected with HAV strain CR326 (Mar/L: O), from stool of patient CLF (O), and from early passage of these HAV strains in cell culture (C). Viral RNA was prepared as described and hybridized to a cDNA probe labelled at the 5' end of the negative strand at the AvaII site at position 3480. Hybrids were digested with S1 nuclease and protected cDNA fragments were resolved on a sequencing gel. For further details see Fig. 3.

isolated from clinical specimens and in particles produced after a few passages in vitro in cell cultures infected with such materials. However control experiments, in which two HAV-negative human stools were subjected to standard procedures for extraction and analysis, always yielded negative mapping results.

We never obtained evidence for the presence of deletions other than B and C. In full agreement with previous findings (Nüesch et al., 1988), this underlines the predominance of some regions of the HAV genome for the formation of internal deletions. Whether or not 3'-proximal truncations (Nüesch et al., 1988) could also contribute to the size spectrum of subgenomic HAV RNA in vivo was not investigated owing to the lack of sufficient clinical material.

In conclusion, our data support the existence in vivo of defective HAV particles which, when present in cell cultures, are able to interfere with the replication of standard HAV. From experimental studies there is convincing evidence that comparable DI particles of various viruses can influence the course of acute infections. Apparently their presence, even in a barely detectable quantity, can ameliorate the effects of the replication of virulent virus, and favourably modulate the immune response of the host to virus infection, but also can facilitate the establishment of persistent infection (Barrett & Dimmock, 1986). What then is the role of such particles in an HAV infection? Hepatitis A is a relatively benign, usually self-limiting disease with no tendency (or at least no well documented tendency) for the development of a
persistent infection or chronic disease. Compared to the course of infections with better known members of the picornavirus family, the most conspicuous characteristics are the relatively extended incubation period of up to 36 days and the usually well developed immune response at the time that the infection becomes clinically manifest. As far as clinical materials and the sensitivity of experimental techniques permitted a detailed analysis, HAV DI particles are present both early and late during infection and in all specimens (liver, blood, faeces) known to yield infectious HAV at some point during infection. It remains to be shown to what extent (if any) this omnipresence of DI particles is related to the distinguishing features of HAV infection mentioned above.

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REFERENCES


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