Review

Hepatitis D: Thirty years after ☆

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The key to the discovery of the Hepatitis D Virus (HDV) was the description in Turin, Italy in the mid-1970s of the delta antigen and antibody in carriers of the hepatitis B surface antigen. The new antigen was first thought to be a marker of the Hepatitis B Virus (HBV) and in view of its intricate true nature, it would have possibly died away as another odd antigenic subtype of HBV, like many that were described in the 1970s. Fortunately, instead, a collaboration started in 1978 between the Turin group, and the National Institute of Health and Georgetown University in the US. With American facilities and expertise this collaboration led just a year later, in 1979, to the unfolding of an unexpected and amazing chapter in virology. Experiments in chimpanzees demonstrated that the delta antigen was not a component of the HBV but of a separate defective virus requiring HBV for its infection; it was named the hepatitis D virus to conform to the nomenclature of hepatitis viruses and classified within the genus Deltavirus. The animal experiments were also seminal in proposing to future clinical interpretation, the paradigm of a pathogenic infection (hepatitis D), that could develop only in HBV-infected patients, was mainly transmitted by superinfection of HDV on chronic HBV carriers and had the ability to strongly inhibit the helper HBV. The discovery of the HDV has driven three directions of further research:

(1) The understanding of the replicative and infectious mechanisms of the HDV.
(2) The assessment of its epidemiological and medical impact.
(3) The search for a therapy for chronic hepatitis D (CHD).

This review summarizes the progress achieved in each field of research in the thirty years that have passed since the discovery of HDV.

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1. Progress in HDV virology

The genetic structure of the new virus appears rudimentary at first glance. The HD virion is a hybrid particle made up of the HD-Antigen (HD-Ag) and an RNA species enclosed within a HBsAg coat derived from HBV [1–3]. The virion contains a circular minute RNA genome of negative polarity, folding in native conditions into a nearly complementary rod-like structure. In the liver, the corresponding antigenomic circular strand of positive polarity contains only one open reading frame; this encodes the HD-Ag, the sole known protein of HDV, through the transcription of a 0.8 Kb messenger RNA. The very measure of the genome size indicated that HDV was not a conventional virus, as it was distinctly smaller than all known animal viruses [3,4]. Its size, and the folded RNA loop, were instead similar to the size and structure of the RNA viroids of higher plants; this first suggested that HDV may have originated from the plant rather than the animal world.

A second unique feature of HDV was the recognition in 1989 that both the genomic and antigenomic strands contained a ribozyme, a RNA segment of less than 100

bases that retained the genetic information but was also able to self-cleave and self-ligate the circular HDV genome [5]. The HDV ribozymes have been crystallized [6] and shown to catalyse cleavage of the HDV–RNA backbone with a rate of enhancement of 10^6- to 10^7-fold over the uncatalyzed rate. They use several catalytic strategies including the use of metal ions [7], intrinsic binding energy and acid-base catalysis under physiological conditions; the latter ability appears to be unique among ribozymes [8]. Ribozymes are also present in viroids and this was taken as further evidence to support an origin of HDV [9] from plants. However, viroids do not code for protein and the structure of the "hammerhead" ribozymes described in plant viruses is different from the HDV ribozymes, indicating that the catalytic domain of HDV–RNA represents a different ribozyme motif. More intriguing, the finding that the $CPEB_3$ ribozyme, a conserved mammalian sequence residing in an intron of the $CPEB_3$ gene, is structurally and biochemically related to the HDV ribozyme, suggests that HDV could derive from the human trascriptome and may have evolved in modern protein-dominated organisms rather than from an ancestral RNA world [10]. Alternatively, human HDV may have evolved from a viroid-like satellite RNA of smaller original size, that acquired the RNA encoding the HD-Ag and the ribozyme through recombination with the human transcriptome [11].

The dilemma confronting early research on HDV was how this virus could replicate; its minute genome coded only for the HD-Ag and could not possibly code for any of the complex enzymatic functions required for independent replication. It was intuitive that HDV exploited HBV and cellular functions, but what were these functions and what role did the HDV play in the replication process? Both questions have been answered in the last 30 years by the discovery of a surprisingly articulate sequence of cellular and viral interactions leading to HDV synthesis [12,13].

Crucial to the understanding of the respective role of HBV and host cells was the report by Dr. Fu and Dr. Taylor in 1993 [14] that hepatocytes nuclear extracts were able to replicate faithfully the whole genomic and antigenomic strand of HDV without any extraneous factor. The conclusion that HDV replication can proceed in host cells in the absence of HBV proteins was confirmed by *in vitro* and *in vivo* transfection experiments; the first have shown that cDNA constructs of HDV induce genome replication when transfected into cultured cells [15], the second that DNA copies of the HDV–RNA and of the RNA itself injected into the tail vein of mice elicit typical HDV replication with a transient expression of HDV in several tissues of the rodent [16], besides the liver. Therefore, mammalian cells can autonomously support efficient HBV replication. Avian cells do not [17]; however, the fusion of mammalian to avian cells rescues HDV synthesis, suggesting that avian cells lack

factors permissive to HDV replication that are restored by mammalian cells.

Clearly, HDV needs HBV only to borrow the HBsAg capsid whereby it attaches to hepatocytes and propagates infection [18]. Within cells, HDV–RNA is associated with multiple copies of the HD proteins to assemble into a ribonucleoprotein complex, which is exported by the HBV envelope through the budding into the lumen of a pre-Golgi compartment before being secreted [18]. Though the small HBsAg alone can package the HDV ribonucleoprotein and assemble into virions [19], the large HBsAg protein bearing in S1 a receptor to cells, is required to confer infectivity [20,21].

The HBsAg coat is not exclusive, as HDV can coat also within the woodchuck hepatitis virus (WHV) surface antigen and *in vivo* HDV has been passed from chimpanzees to the rodent [4]. However, in apparent contradiction to the *in vivo* transmission of primate HDV to woodchucks, woodchuck-HDV was reported to infect cultured primary woodchuck hepatocytes as well as cultures of primary human hepatocytes, but human HDV could not infect woodchuck hepatocytes [22]; the different host-range specificities seems determined by the different recognition of WHV and HBV Pre S1 proteins on human hepatocytes.

Conventional RNA viruses undergo replication by a virus encoded RNA-dependent RNA-polymerase which replicates the viral genome; they cannot use cellular RNA-polymerases, as these accept only DNA templates. Hepatitis D virus does not possess any polymerase, therefore, the next enigma was how it replicated in the host. The explanation is that host RNA polymerase II (Pol II), is deceived by HDV and becomes redirected to read and copy its RNA. This was confirmed by transcriptional run-on experiments which have demonstrated inhibition of viral synthesis after the addition of low-dose amanitin [23], a toxin that selectively blocks the accumulation of RNA Pol II transcripts. Presumably the rod-like conformation of the viral RNA is recognized as double-stranded DNA for RNA Pol II binding. Immunoprecipitation experiments using blocking monoclonal antibodies to RNA Pol II [24] have shown that human RNA Pol II binds directly to the terminal stem-loop regions of HDV–RNA and the interactions of the HD-Ag with the RNA Pol II clamp, a mobile structure that holds DNA and RNA in place, may facilitate the unusual RNA synthesis [25]. Once redirected to copy HDV–RNA, RNA Pol II carries out unchecked its transcriptional activity, elongating a multimeric linear transcript of either the genome or the antigenome over the viral RNA circular template of the other; this elementary double-rolling circle mechanism of replication is unknown to animal viruses but operative in viroids [13].

The last step requires the rearrangement of the linear multimeric strand into genomic and antigenomic HDV–

RNAs. This is where the HDV finally steps in; its ribozymes first cleave the redundant transcription product into a genomic/antigenomic size monomer and then ligate the linear monomer into the infectious circular form.

The intricate RNA-cellular interactions are only part of the replicative story of HDV. Crucial and incredibly complex for such a small molecule are also the functions of the HD-Ag. The HD-Ag contains several functional domains [26]; a coiled-coil domain facilitates protein–protein interactions [27], a bipartite nuclear-localization signal drives the HD proteins to the nucleus [28], an oligomerization domain and two arginine rich motifs support the binding to the RNA. It is edited by a cellular double-stranded adenine-deaminase into a small HD-Ag (SHD-Ag, 195 amino acid residues) and a large HD-Ag (LHD-Ag, 214 amino acid residues), the first promoting replication, the second virion assembly [29]. Many other functions of the HD-Ag isoforms have been recognized and added to the list in the last years [12,13,30].

The dilemma is again how the two small HDV proteins can escort the HDV genome through different cellular compartments and become involved in the different steps of replication, transcription and formation of progeny virions. The answer is that the biological activities of the HD-Ag depend on a host of ordered protein–protein interactions regulated by post-translational modifications of the HD-Ag [31]; as a one gene–genome the HDV offers a unique opportunity to determine what these are and how they affect the functional roles of the HDV proteins.

Phosphorylation [32,33], acetylation [34], methylation [35], prenylation [36] and several other post translational modifications of the HD antigens [37–39] have been shown to orchestrate the life cycle of the virus. Different phosphorylation patterns of the small and large HD-Ag account for their distinct biological functions [32] and phosphorilated SHD-Ag increases replication from antigenomic to genomic RNA [33]. Acetylation and deacetylation of HD-Ag may provide a molecular switch for the synthesis of the different RNA species [34], and interactions with the multifunctional transcription factor YY1 and its associated acetyltransferase selectively modulates replication of the genomic and antigenomic forms [39]. Methylation at arginine residues regulates the subcellular localization and also RNA replication [35]. Farnesylation of the LHD-Ag is essential to target this protein to cellular membranes containing the HBsAg in order to trigger viral assembly [40]. More intriguing, the participation of HDV proteins in the regulation of cellular gene expression could trigger HDV-related liver damage. It was reported that isoprenylation of LDH-Ag regulates Transforming-Growth Factor -β-induced signal-transduction and through this mechanism may induce liver fibrosis [41].

2. Epidemiological and clinical impact

Indirect antibody assays for the diagnosis of HDV became generally available at the beginning of the 1980s, prompting worldwide studies on the epidemiology and medical impact of the infection. Molecular assays for the direct measure of HDV–RNA in serum were developed but have not been made commercially available and are limited to specialized laboratories only; the current PCR assays are specific and have a sensitivity threshold of 10–100 copies of the HDV genome [42,43], per ml of serum.

Surveys in the 1980s showed that HDV is endemic worldwide, though with prevalences and patterns of infection varying in different areas; globally the number of HDV carriers was estimated at 15,000,000 at that time [44]. Medical scrutiny confirmed that CHD usually runs a severe and progressive course [45]. The prototype patient with CHD carried the HBsAg in blood, had elevated ALT, a liver biopsy exhibiting aggressive hepatitis but no markers of HBV replication; the discrepancy between a florid HBsAg-disease and the lack of HBV synthesis provided the best harbinger to the suspicion of an underlying HDV infection [46]. However, in epidemiological pockets as far away as the Greek island of Archangelos and Pacific Samoa, HDV caused no or insignificant disease, indicating that the course of HDV infections may span a clinical spectrum ranging from asymptomatic carriage of the virus to very severe disease [47].

A factor that may influence the course of disease is the genotype of the HDV. Currently this virus is divided into eight major genotypes differing as much as 40% in nucleotide sequence [48]. Genotype I is the most frequent worldwide and has variable pathogenicity. In a study from Taiwan CHD patients infected with genotype I HDV had a lower remission rate and more adverse outcomes [49] than those with genotype II HDV. Genotypes II and IV were found in East Asia causing relatively mild disease [50]; however, a genotype II b variant isolated from HDV patients from the Miyako Island, Okinawa (II b-M) was associated with greater progression to cirrhosis then the II b genotype prevalent in Taiwan [51]. Genotype III was associated with the genotype F of HBV and fulminant hepatitis in South America [46]. While the genotype of HBV does not seem to affect the interaction of HBsAg with HDV, the genotype of HDV may influence the efficiency of assembly with the HBsAg into virions. Aminoacid sequence variations in HBsAg expressed by naturally occurring variants of HBV diminished the assembly efficiency of genotype II and IV of HDV but not of genotype I [52]; the more efficient interactions between genotype I of HDV and HBV, might play a role for the wide distribution of HDV I in the world.

Since the 1990s the circulation of HDV has declined significantly in Europe, consistent with the hypothesis that the virus was discovered at the time of a major epidemic that has been brought under control by now [53]. Sequential surveys in Italy, each one in several hundreds of HBsAg carriers with liver disease documented an anti-HD rate of 24.6% in 1983 [54], of 23% in 1987 [55], of 14% in 1992 [56] and of 8.3% in 1997 [57]. Consistent declines in the prevalence of HDV were also reported in Spain [58], Taiwan [59] and Turkey [60]. In Turkey the overall prevalence of anti-HD in HBsAg carriers with liver disease and in those with cirrhosis diminished, respectively, from 31% to 11% and from 43.3% to 24% in the period 1980–2005.

Along with the decline in the circulation of HDV, the clinical scenario of hepatitis D has also changed. While the majority of hepatitis D patients observed in Italy in the 1980s had a florid chronic active hepatitis, and cirrhosis was seen in fewer than 20% of cases, by the end of the 1990s, the proportion of cirrhosis residual to burnt-out inflammation had increased to 70% [61,62].

The common denominator in the control of HDV is the spectacular control of HBV achieved in the last 15 years, which is depriving the defective HDV of the HBV network necessary to propagate its infection [63]. By contrast, it is unlikely that HDV is being brought under control in the many HDV hyperendemic areas where HBV remains still unchecked, and a significant presence of hepatitis D has been reported in the last three years from Pakistan [64], India [65], Mongolia [66], Iran [67,68], Vietnam [69], Tajikistan [70], Tunisia [71], Mauretania (Francoise Lunel, personal communication).

The dramatic decline of HDV in Europe at the end of the 1990s has raised the hope that in this area hepatitis D would soon be cancelled from the list of communicable diseases [57]. This perspective, however, now appears too optimistic and certainly premature. In London [72], the prevalence of HDV has remained stable (around 8.5%) between 2000 and 2006; most HDV patients were from Southern or Eastern Europe, Africa and the Middle East, and their mean age was 36 years. In Hannover [73], the prevalence declined from 18.6% in 1992 to 6.8% in 1997 but no further decrease was recorded thereafter; from 1999 onwards, between 8% and 14% of the HBsAg carriers were positive for anti-HD, the majority were immigrants from Turkey and the ex-Soviet Union. In France [74] there is still a residual HDV population represented mostly by African immigrants; within this epidemiological niche the infection seems to be increasing. In the most recent survey of 1179 HBsAg carriers conducted in Italy in 2006, the prevalence of anti-HD was 8.1% [75], similar to the prevalence found in 1997, suggesting that the decline of HDV has not continued.

Thus the consistent reservoir of HDV remaining in Europe is sustained by two different pools of HDV-infected patients. One is composed of the residual ageing domestic pool that survived the brunt of the hepatitis D epidemic in the 1970–1980s. The other is made up by a population of young patients with recent HDV infections migrating to Europe from areas where HDV remains endemic [76]. HDV may be returning not only by import but also by spreading in loco. Immigrants from many parts of the world are also heavily infected with HBV and segregation of these HBsAg carriers into metropolitan, overcrowded ghettos may locally restore a dense HBsAg network, providing a fertile terrain to the secondary spreading of HDV.

3. Therapy; beyond Interferon, the problems and the perspectives

Alfa-Interferon was first used in CHD 25 years ago and still remains the only available treatment. Results, however, are far from optimal [77]. Standard treatment given for one year achieves remission of CHD in about 20–25% of the patients, but the rates of HDV–RNA clearance are distinctly lower. Somewhat better results are obtained with a more aggressive schedule and higher dosage of IFN; liver chemistry may ameliorate in about 50% of the patients while on therapy, yet again the clearance of HDV [78] is sustained only in a minority. Prolonging therapy up to two years did not increase the rate of sustained response over 1 year of treatment [79].

Peg-IFN may be more beneficial; in a study of CHD patients treated with Peg IFN alfa 2b 1.5 µg/kg weekly for 12 months, 6 of 14 (43%) achieved a sustained clearance of serum HDV–RNA [80]. However, in two other groups of patients treated with the same Peg-IFN, a sustained viral response was obtained only in 17% [81] and 21% of the patients [82]. The small number of patients included in all treatment series makes it difficult to discriminate a true therapeutic advantage of IFN from a more benign spontaneous course of the disease. Though there are anecdotal reports of resolution of HBV/HDV infections and even of fibrosis in HDV patients treated with IFN [78], no controlled study has been carried out to determine whether IFN induces a significant long-term effect; of note disease resolution may occur in the natural history of HDV infections and in a follow-up over many years the rate of spontaneous clearance of the HBsAg was higher in HDV-positive than negative carriers of the HBsAg [83]. Treatment failures might result from the interference of HDV with IFN-alfa intracellular signalling; in HDV-transfected hepatoma cells the blocking of the activation of Tyk2 by HDV resulted in the selective impairment of activation and translocation to the nucleus of STAT1 and STAT2 [84].

In most HDV patients, HBV is spontaneously repressed. Nevertheless Famciclovir, Lamivudine and Adefovir, which are inactive against HDV but active against the helper HBV, were used in monotherapy or in combination with IFN, in an attempt to further diminish the replication level of HBV and totally deprive the defective HDV of the helper effect. Neither one nor their combination with IFN, where more efficacious than IFN [77,85]. The problem confronting HDV therapy is that there is no specific enzymatic function to target, such as the polymerases and proteases of HBV and HCV. It should be considered that HDV depends on the HBsAg and not on HBV replication, therefore its synthesis is not influenced by the level of HBV-DNA in serum; the secretion of HDV *in vitro* [86] and the levels of HDV–RNA *in vivo* [87] are directly correlated to the amount of serum HBsAg, not to the titer of HBV-DNA. Potential targets to therapy are offered by the process of HD virion assembly. On the HBV side, this requires the integrity of critical regions of the HBsAg. Deletion of residues 24–28 on the small HBsAg led to an envelope mutant deficient for production of HD virions [88] and the interactions of the LHD-Ag with HBsAg were ablated by the substitution of phenylalanine for tryptophan at positions 196, 199 or 201 of the small HBsAg [89]. Likewise, mutations or conformational changes in cysteines on the antigenic loop of the HBsAg (which bears the conserved <u>a</u> determinant and is involved in HDV infectivity) created a block to the entry of HDV into cells [90]. This evidence would suggest that there are several sites on the HBsAg central to HDV maturation, the disruption of which may offer a target to antiviral therapy. Interestingly, lamivudine-induced mutations in the polymerase region, which confer HBV resistance to this therapy, are associated with changes in the overlapping envelope gene products; a change in SW 196 L/S of the small HBsAg, carried by mutation M 204 I in the reverse transcriptase of HBV, was found to inhibit the secretion of HDV particles in cell cultures, suggesting that lamivudine therapy may indirectly abrogate an underlying HDV infection [91].

On the HDV side, critical to the interaction of LHD-Ag with the HBV envelope protein is farnesylation (prenylation), of the last four aminoacids Cys-Arg-Pro-Gln-COOH on the LHD-Ag (so-called CXXX box; C for cysteine, X for any amino-acid) [92]. By preventing the association of the HDV ribonucleoprotein with the HBsAg and, therefore, virion assembly, disruption of prenylation might form the basis of a new therapeutic strategy against HDV; experiments *in vitro* have shown that HDAg prenylation can be inhibited by the prenylation inhibitor BZA-5B and *in vivo* the prenylation inhibitors FTI – 277 and FTI – 2153 were highly effective at clearing HD viremia in a mouse model of HDV infection [93].

4. Conclusions and perspectives

Most rewarding were the studies on the nature of HDV, which have unravelled an unsuspected maze of viral and cellular interactions induced by a novel human virus, unique in size, in possessing a ribozyme, in replicating by a rolling circle mechanism, in redirecting to its advantage the cellular replicative machinery.

Paradoxically, the most outstanding feature of this highly pathogenic agent is currently attracting attention as a potential therapeutic tool in the development of gene inactivation systems based on the direct targeting of RNAs. As the only catalytic cleaving RNAs in humans, the HDV ribozymes function properly in the low-ionic environment of human cells, are more stable, have a longer half-life than the other non-human ribozymes, and should not be recognized by the immune system as an external, invading RNA [94].

The adaptation to the human cell environment makes HDV ribozymes suitable RNA silencing tools and strategies are being developed for their therapeutic use in the inhibition of viral activity, cancer growth and inherited genetic diseases.

The circulation of HDV and its clinical impact have greatly diminished in Europe, decreasing interest in its infection. To the point that the sensitive HDV–RNA assays developed in the last years have not become widely available and that testing of HBsAg carriers for HDV is often neglected even in high-rank laboratories. This was the case in London [72], where a number of metropolitan viral laboratories were contacted and none of them routinely tested HBV-infected patients for anti-HD.

HDV remains a major health problem in the many underdeveloped areas of the world where HBV is uncontrolled; the price extolled by globalisation and the increasing migratory fluxes is the reconstitution of a HDV reservoir and the recapitulation of florid HDV disease among immigrants to Europe. Awareness of this new threat should enforce more vigilance in order to provide accurate contemporary scenarios of HDV and plan adequate prevention.

Therapy of hepatitis D remains the most unsolved business. It is discouraging that the therapy available today is not different from the limited IFN treatment attempted more than 20 years ago. The problem is formidable as HDV has no enzymatic proteins to be targeted by conventional antiviral therapy. However, the lesson that antiviral drug therapy improves with the increase of the knowledge of the replication mechanisms of the virus, augers well also for the prospects of HDV therapy. The accelerated progress in the understanding of the molecular mechanisms of HDV, in particular of the HBsAg interactions between the large HD antigen and the HBsAg in the assembly of the HD virion, may provide a potential therapeutic target, unconventional as the HDV itself.

Acknowledgements

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