Recent advances in the identification of hepatitis viruses*

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Summary

Resulting directly from the discovery of virus-related antigens, rapid progress has marked the last decade of viral hepatitis research. The hepatitis B virion has been tentatively identified as a DNA virus with an endogenous DNA polymerase, and new serological markers for type B hepatitis have been discovered. Hepatitis A antigen has been identified on a virus-like particle thought to be the hepatitis A virion. Progressively more sophisticated assays for hepatitis antigens and antibodies have been applied to the study of viral hepatitis epidemiology and biochemical-biophysical characterization of the agents. Most recently, knowledge learned from such studies has been exploited to develop a prototype non-infectious but immunogenic hepatitis B vaccine using hepatitis B surface antigen (HB_sAg) purified in large quantities from chronic HB_sAg carriers. Especially exciting is the prospect, suggested by serological studies of viral hepatitis, that hepatitis viruses besides hepatitis A and B viruses will be identified.

Introduction

Although hepatitis has been recognized clinically for centuries and epidemics of hepatitis have been considered viral in origin for decades, and despite recent advances in tissue culture techniques, hepatitis viruses have not been cultivated *in vitro* or positively identified. Within the last 6 years, however, virus-like structures associated with type B (Dane, Cameron and Briggs, 1970) and type A (Feinstone, Kapikian and Purcell, 1973) hepatitis have been found, and evidence supporting their aetiological roles is accumulating. Little more than a decade has elapsed since the serendipitous discovery of Australia antigen (Blumberg, Alter and Visnich, 1965) and its ultimate association with type B viral

hepatitis (Prince, 1968; Blumberg *et al.*, 1967). The study of Australia antigen, other antigens associated with hepatitis B virus (HBV) infection, and the immune response to these antigens has provided an ever expanding understanding of the disease and its aetiological agents. Now a similar series of new insights about type A hepatitis has begun.

Viral hepatitis, type B

Hepatitis B surface antigen

Based on electron microscopic studies, hepatitis B surface antigen (HB_sAg) represents a specificity on three morphologically distinct forms: spherical particles approximately 22 nm in diameter, tubules of similar diameter and of variable length (Bayer, Blumberg and Werner, 1968), and a 42-nm double-shelled structure, the Dane particle (Dane *et al.*, 1970), which is thought to represent the hepatitis B virion (Fig. 1). The outer coat of the Dane particle measures 7 nm in width and is antigenically identical to the 22-nm spheres and tubules present in abundance and thought to represent excess viral coat material (Table 1). Residing on each HB_sAg particle or component is a group-reactive determinant, *a*, and, generally, mutually exclusive allelic subdeterminants *d*, *γ* (Le Bouvier *et al.*, 1972), and *w*, *r* (Bancroft, Mundon and Russell, 1972), as well as numerous other recently discovered but less well characterized sub-determinants (Le Bouvier and Williams, 1975). As a result, four virus-determined major subtypes of HB_sAg are recognized: *adw*, *ayw*, *adr*, and *ayr*. In infected liver cells, HB_sAg reactivity is localized to the cytoplasm (Edgington and Ritt, 1971), and 22-nm spheres can be detected there by electron microscopy (Huang *et al.*, 1974).

Hepatitis B core antigen

Within the Dane particle and exposed by detergent or lipid solvent treatment is a 27-nm core (Almeida, Rubenstein and Stott, 1971), called hepatitis B core antigen (HB_cAg), which does not cross-react antigenically with HB_sAg and which elicits a distinct

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FIG. 1. Hepatitis B surface antigen components purified from the serum of a chronic carrier (2% phosphotungstic acid negative stain). The bar represents 100 nm.

TABLE 1. Antigens associated with hepatitis viruses

Virus	Antigen	Morphological form(s) and their presumed relationship to virus		Antibody
Hepatitis B	Hepatitis B surface antigen (HB _s Ag)	22 nm spheres Tubules of variable length Coat of 42 nm Dane particle	Excess viral coat material Virion (surrounds core)	Anti-HB _s
	Hepatitis B core antigen (HB _c Ag)	27 nm core within Dane particle	Viral core (contains DNA and DNA polymerase)	Anti-HB _c
	e Antigen	None identified (soluble)	Unknown	Anti-e
Hepatitis A	Hepatitis A antigen (HA Ag)	27 nm particle	Virion	Anti-HA
Others	None identified	None identified		None identified

immune response (Hoofnagle, Gerety and Barker, 1973; Gerety, Hoofnagle and Barker, 1974; Barker *et al.*, 1974). Staining infected liver tissue with fluorescein-labelled antibody to HB_cAg (anti-HB_c) (Barker *et al.*, 1973) as well as electron microscopic observation (Barker *et al.*, 1973; Huang, 1972) localize HB_cAg to the hepatocyte nucleus (Table 1).

Dane particle DNA and DNA polymerase

Among the most exciting recent advances in the study of type B hepatitis are the localization of an HBV-specific DNA-dependent DNA polymerase (Kaplan *et al.*, 1973; Robinson and Greenman,

1974) and a double-stranded circular DNA molecule within the core of the Dane particle (Robinson and Greenman, 1974). From Dane particles with DNA polymerase activity, double stranded circular DNA molecules have been extracted; their length corresponds to a molecular weight of 1.6×10^6 daltons which, theoretically, can code for about three average size proteins, and evidence suggests that these DNA molecules serve as the primer-template for synthesis of new DNA by the DNA polymerase (Robinson, Clayton and Greenman, 1974). Recently, Gerin, Ford and Purcell (1975) and Kaplan *et al.* (1976) described the existence of two sub-populations of

Dane particles, one with a higher density (1.22 g/cm³ in caesium chloride) which contains DNA and DNA-polymerase, and a lighter one (1.20 g/cm³) devoid of DNA and lacking DNA polymerase. Thus, in addition to 22-nm spheres and filaments, empty Dane particles may represent defective interfering particles which, on the one hand, compete with infectious complete virions for hepatocyte receptor sites but, on the other hand, may facilitate the establishment of a non-cytopathic persistent infection tolerated by the host (carrier state). The presence of defective interfering particles, the complexity of DNA replication imposed by the closed circular model, and the uniquely small size of the genome—smaller than the double-stranded DNA of any known virus—may explain why hepatitis B virus is so difficult to cultivate under artificial conditions. Although infectivity studies to prove the point are lacking, the candidacy of the Dane particle for the hepatitis B virion is supported by its virus-like structure including an immunologically distinct inner core, its association with a specific DNA polymerase, and the presence within its core of DNA. It is reasonable to postulate, in addition, that HB_sAg-positive sera with high Dane particle counts and elevated DNA polymerase activity are likely to be more infectious than HB_sAg-positive sera lacking these findings.

Serological events during type B viral hepatitis

During acute type B viral hepatitis, HB_sAg appears in the blood for days to months before elevation of serum aminotransferase activity and

morphological lesions in the liver. Shortly after HB_s antigenaemia begins, DNA polymerase can be detected transiently (Krugman *et al.*, 1974; Kaplan, Gerin and Alter, 1974); at approximately the same time, anti-HB_c appears and often coincides with biochemical and clinical evidence of acute hepatitis (Hoofnagle *et al.*, 1973; Hoofnagle, Gerety and Barker, 1975). Usually not until HB_sAg disappears does anti-HB_s appear (Lander *et al.*, 1972), following which it can be detected by sensitive assays for years after illness. Thus, during acute type B hepatitis, a brief interval may exist between the disappearance of HB_sAg and the appearance of anti-HB_s during which anti-HB_c is the only indicator of HBV infection (Hoofnagle *et al.*, 1975) (Fig. 2).

The degree and duration of each of these serological findings may vary among acutely affected individuals, and the course of events differs considerably among chronic HB_sAg carriers. Since HB_sAg persists in the blood, no anti-HB_s is detected. Furthermore, persistence of elevated DNA polymerase activity and rising titres of anti-HB_c (Hoofnagle *et al.*, 1975) reflect continuous virus replication. Theoretically, a chronic HBV carrier with levels of HB_sAg too low to detect even with sensitive test procedures may be discovered by testing for anti-HB_c; however, in practice, this has not been verified

e Antigen

The most recently popularized addition to our ever growing awareness of the complexity of HBV is the 'e' antigen system (Magnius and Espmark, 1972a, b). Unlike HB_sAg, e is a soluble protein and

FIG. 2. Idealized representation of serological events in a typical case of acute hepatitis B virus infection. Hepatitis B surface antigen (HB_sAg, ...), antibody to HB_sAg (anti-HB_s, ---), antibody to hepatitis B core antigen (anti-HB_c, —), serum glutamic pyruvate transaminase or alanine aminotransferase (SGPT). (Reproduced with permission of Dr P. Holland, Dr H. Alter, and W. B. Saunders Co., *Medical Clinics of North America*, 59, 849. 1975.)

is antigenically distinct from HB_sAg of all subtypes. These antigens (at least two, e₁ and e₂, have been identified) are found only in serum containing HB_sAg, do not cross-react at all with HB_sAg (Magnius and Espmark, 1972a, b) or HB_cAg (Takahashi *et al.*, 1976), and appear within the first several weeks of clinically recognizable illness (Magnius *et al.*, 1975) (Table 1). Preliminary reports associate the presence of e antigen in serum with the development and perpetuation of chronic hepatitis or cirrhosis (Nielsen, Dietrichson and Juhl, 1974), with the presence of Dane particles, DNA polymerase (El Sheikh *et al.*, 1975; Nordenfelt and Kjellen, 1975) and relatively high infectivity of HB_sAg serum. Anti-e, on the other hand, appears to correlate with resolution of liver disease, better prognosis, and relatively low infectivity. Although these correlations are not absolute, studies of the properties of e and its epidemiology promise to unravel still more of the mysteries of HBV.

Since, at present, there is no widely available method to abort chronic carriage of HBV, the question of infectivity of chronic carriers has practical significance. Do we limit the activities of health care personnel who are chronic carriers? Can we determine which among them are highly infectious? Ideally, infectivity of chronic carrier serum could be tested in chimpanzees; however, the shortage of susceptible animals and the expense involved make this approach impractical. Perhaps, studies of e antigen and DNA polymerase will be useful in detecting highly infectious carriers and distinguishing them from carriers who pose less of a threat to others.

Newer tests for hepatitis B virus antigens and antibodies

Little would have been learned about type B hepatitis without the development of ever more sophisticated serological tests for detecting its antigens and antibodies. Although a review of these procedures is beyond the present scope, several of the most reliable, sensitive, and widely used newer tests deserve mention. For the detection of HB_sAg, radioimmunoassay techniques, with appropriate specificity testing, are the most authoritative (Ling and Overby, 1972). A less costly method, often of comparable sensitivity, immune adherence haemagglutination has been used widely in Japan to screen donor units before transfusion (Mayumi, Okochi and Nishioka, 1971).

Agar gel diffusion and counter-immunoelectrophoresis (CEP), the earliest tests used to identify HB_sAg are too insensitive to detect anti-HB_s in any but the most strongly positive sera. Passive haemagglutination (Vyas and Shulman, 1970) and several modifications of radioimmunoassay, including radio-

immunoprecipitation (Lander, Alter and Purcell, 1971), are very sensitive and are used currently for definitive determinations. Immune adherence haemagglutination, also useful for sensitive, practical anti-HB_s testing, is being increasingly accepted (Mayumi *et al.*, 1971).

Shortage of HB_cAg precludes the commercial availability of assays to detect anti-HB_c. The methods used in investigative laboratories, in ascending order of sensitivity, are complement fixation (Hoofnagle *et al.*, 1973), CEP (Huang and Groh, 1973), immune adherence haemagglutination (Tsuda *et al.*, 1975), and radioimmunoassay (Purcell *et al.*, 1974; Moritsugu *et al.*, 1975).

Epidemiology

Equipped with a vast array of test procedures for hepatitis B antigens and antibodies, investigators have learned much about the transmission of type B hepatitis, as reviewed recently by Mosley (1975) and Szmunn (1975). Although parenteral inoculation with contaminated blood products is probably the most common mode of transmission, many non-percutaneous routes have been implicated or suggested. HB_sAg has been found in virtually every body secretion including saliva and semen, and several possible routes of spread have been suggested including venereal, oral, droplet, contact, arthropod-borne, and vertical transmission, in addition to overt parenteral and concealed parenteral modes of spread. Among non-percutaneous modes of transmission, oral, venereal, and vertical spread are probably the most important.

A large proportion of sporadic hepatitis unrelated to known exposure and once considered 'infectious hepatitis' can be identified as type B hepatitis on the basis of tests for HB_sAg, anti-HB_s, and anti-HB_c. Exposure to hepatitis B virus varies with geographical location, ethnic background and occupation, increases directly with age and inversely with rising socioeconomic class. The chronic carrier rate after infection is approximately 5–10% (Redeker, 1975). As mentioned earlier, tests for DNA polymerase activity, Dane particle numbers, e antigen and antibody may provide prognostic clues, and tests for HB_sAg subtypes are useful as general epidemiological tools. In addition, serological studies have suggested, but not proved, a role for HBV in polyarteritis nodosa (Gocke *et al.*, 1970; Sergeant *et al.*, 1976), glomerulonephritis (Brzosko *et al.*, 1974), and hepatic cell carcinoma (Williams, 1975).

Control of viral hepatitis type B

Experimental studies in chimpanzees and epidemiological studies in man suggest that the presence of anti-HB_s correlates with immunity to HBV infection. Although trials of passive immunization

with hepatitis B immune globulin have recently been reported (Iwarson *et al.*, 1975; Couroucé-Pauty, Delons and Soulier, 1975; Desmyter *et al.*, 1975; Seeff *et al.*, 1975; Surgenor *et al.*, 1975; Grady and Lee, 1975; Prince *et al.*, 1975; Redeker *et al.*, 1975), more promising is the progress toward development of an HBV vaccine for active immunization. Unique among modern viral vaccines, an HBV vaccine has been prepared without cultivating HBV *in vitro*: instead, the 22-nm spherical forms of HB_sAg were purified from the plasma of chronic HB_sAg carriers (Purcell and Gerin, 1975; Hilleman *et al.*, 1975). Separated from Dane particles and tubular forms of HB_sAg, then inactivated with formalin, the 22-nm particle vaccine has been shown to be immunogenic but non-infectious. In a preliminary study, vaccinated chimpanzees were protected against HBV infection when challenged 24 weeks after vaccination, whereas non-vaccinated chimpanzees developed hepatitis (Purcell *et al.*, 1975). Another approach to vaccine development, the use of polypeptides derived from HB_sAg, is also being investigated (Dreesman *et al.*, 1975; Shih and Gerin, 1975). The relatively poor immunogenicity of these polypeptides and the difficulty of large scale purification may limit the applicability of this method. Currently, extensive efficacy and long-term safety tests in chimpanzees are under way for the 22-nm particle vaccine, as are detailed evaluations of the immune response—both humoral and cellular—to the new vaccine. A successful trial of the 22-nm particle vaccine in man was recently reported by Maupas *et al.* (1976), who inoculated patients and staff in a haemodialysis unit.

More recently Greenberg *et al.* (1976), Desmyter *et al.* (1976), and Purcell *et al.* (1976a) have presented data indicating that exogenously administered interferon or interferon inducers may limit HBV replication in HB_sAg chronic carriers.

Viral hepatitis, type A

Although nature has bestowed upon man a hepatitis virus less virulent than type B, she has deprived him of an abundant type A viral antigen as a serological marker. Consequently, and because hepatitis A virus (HAV) has not been cultivated *in vitro* either, progress in its study lagged considerably behind. Studies in humans in the 1940s, 1950s and 1960s and experimental infection of marmosets (tamarins) in the 1960s and early 1970s characterized HAV as a small virus resistant to ether and heat, transmissible in faeces and blood collected during the incubation period and acute illness, and immunologically distinct from HBV (Voegt, 1942; MacCallum and Bradley, 1944; Findlay and Wilcox, 1945; Paul *et al.*, 1945; Neefe, Gellis and Stokes, 1946; Havens, 1945a, b, 1946; Krugman, Ward and Giles, 1962; Krugman, Giles and Hammond, 1967;

Mascoli *et al.*, 1973; Provost *et al.*, 1973). Although infectivity of blood and faeces was demonstrated in these pioneering studies, HAV was not cultivated from or identified in any of these specimens.

Hepatitis A antigen

Data and clinical specimens generated in early volunteer and marmoset studies, however, were instrumental to the ultimate visualization of a virus-like particle with hepatitis A antigen activity. In 1973, Feinstone *et al.* (1973) used immune electron microscopy to examine stool specimens taken from prison volunteers infected with the Willowbrook MS-1 strain of HAV. In these studies, Feinstone incubated convalescent serum from one of the volunteers with filtrates of acute phase stool specimens and detected aggregates and single 27-nm virus-like particles coated with antibody (Fig. 3). The association of these particles, designated hepatitis A antigen (HA Ag), with HAV infection was supported by the failure to detect immune aggregates when pre-inoculation or convalescent stools were used as an antigen source or when acute phase stools were incubated with pre-inoculation serum from patients with type A hepatitis or convalescent serum from patients with type B hepatitis or viral gastroenteritis. Furthermore, serological responses to HA Ag were demonstrated in paired serum samples of patients with experimental and natural type A hepatitis.

FIG. 3. Antibody-coated aggregate of hepatitis A antigen particles purified from the stools of a patient with acute type A hepatitis (2% phosphotungstic acid negative stain). The bar represents 100 nm.

Reports soon followed describing studies by Provost *et al.* (1975b) in which similar virus-like particles were observed in the sera and livers of *Sanguinus mystax* (marmosets) experimentally infected with the CR326 strain of HAV, which had been extensively characterized at the Merck Institute (Mascoli *et al.*, 1973; Provost *et al.*, 1973). Since particles were observed by thin section electron microscopy within cytoplasmic vesicles, since buoyant density determinations and preliminary nucleic acid determinations were suggestive, Provost *et al.* (1975b) proposed that HAV is an RNA-containing enterovirus. The multiple densities for HA Ag detected by others, however (Feinstone *et al.*, 1974; Bradley *et al.*, 1975; Moritsugu *et al.*, 1976), and the heat stability of HAV are more characteristic of parvoviruses, small viruses containing single stranded DNA; additional characterization of HAV is needed before final classification of virus type can be made.

Identification of HA Ag particles in experimental infection of man and marmoset was confirmed by the detection of antigenically and morphologically indistinguishable particles in the stools of naturally infected humans (Gravelle *et al.*, 1975; Locarnini *et al.*, 1974; Dienstag *et al.*, 1975c) and in the liver, bile and stool of experimentally infected chimpanzees (Schulman *et al.*, 1976), a species of non-human primates recently shown to be susceptible to HAV infection (Dienstag *et al.*, 1975b). Preliminary evidence indicates that HA Ag isolates from different geographical areas and from different sources are immunologically related, if not identical, suggesting that there is one serotype of HAV. The HA Ag particle probably is the virus of type A hepatitis (Table 1).

Newer tests for hepatitis A virus antigen and antibody

Although immune electron microscopy is a specific, sensitive, and quantitative assay for HA Ag and antibody to HA Ag (anti-HA), its practicality for large scale testing is limited. With HA Ag purified from marmoset livers, Provost *et al.* (1975a) developed a complement fixation test and Miller *et al.* (1975) an even more sensitive immune adherence haemagglutination (IAHA) test. Since there is a shortage of marmosets, other sources of HA Ag have been tapped and, recently, Moritsugu *et al.* (1976) reported the successful use of stools from humans with naturally-acquired HAV infection as an HA Ag source for IAHA. Of the few techniques available today, IAHA has proved the most economical, simple, and widely used; however, it is still not commercially available. Another useful test, also limited to research laboratories, is a solid phase radioimmunoassay, which can be modified to detect both HA Ag and anti-HA (Hollinger *et al.*, 1975; Purcell *et al.*, 1976b).

Detectable events during type A viral hepatitis

After a brief incubation period of approximately 2–4 weeks, the first detectable laboratory index of HAV infection is excretion of HA Ag particles in stool. Faecal HA Ag shedding coincides with the onset of non-specific symptoms (malaise, abdominal pain, anorexia, etc.) and usually reaches a peak before significant elevation of serum aminotransferase activity and histological evidence of liver damage can be detected. Since HA Ag shedding occurs so early and usually disappears before jaundice becomes biochemically and clinically apparent, patients rarely have detectable HA Ag in their stools when they present with obvious hepatitis to a physician (Dienstag *et al.*, 1975a). As Krugman observed in studies among children at the Willowbrook State School, faeces are infectious early during incubation and not much later than the initial appearance of jaundice (Krugman *et al.*, 1962). Antibody to HA Ag can be detected by immune electron microscopy or complement fixation during acute illness; however, the appearance of anti-HA cannot be detected for from 1 to 4 weeks after acute illness by IAHA. Subsequently anti-HA titres rise gradually and reach a peak, in man, about 2–3 months after acute illness. Although titres fall afterwards, relatively high titres may persist even 10 years after infection (Moritsugu *et al.*, 1976; Gravelle *et al.*, 1975; Locarnini *et al.*, 1974; Dienstag *et al.*, 1975c, 1975b; Provost *et al.*, 1975a; Miller *et al.*, 1975; Krugman, Friedman and Lattimer, 1975; Dienstag, Alling and Purcell, 1976a) (Fig. 4).

Epidemiology

Recently available serological techniques have been applied to investigate modes of HAV transmission and prevalence of HAV infection. Serological evidence for infection with HAV has been demonstrated in common-source water-borne (Feinstone *et al.*, 1973; Purcell *et al.*, 1975) and food-borne outbreaks (Feinstone *et al.*, 1973; Gravelle *et al.*, 1975; Dienstag *et al.*, 1975c; Purcell *et al.*, 1975), shellfish-associated hepatitis (Dienstag *et al.*, 1976c), intra-family and intra-institutional outbreaks (Feinstone *et al.*, 1973; Purcell *et al.*, 1975), and non-human primate-associated hepatitis (Dienstag *et al.*, 1976b). A significant proportion of sporadic hepatitis has also been attributed serologically to HAV. On the other hand, although HAV can be transmitted experimentally by parenteral inoculation, type A hepatitis has not been confirmed serologically as a significant contribution to transfusion-associated hepatitis (Feinstone *et al.*, 1975; Knodell *et al.*, 1975), haemodialysis-associated hepatitis (Zuckerman, Couroucé and Szmuness, unpublished data), drug abuse-related hepatitis (Mosley *et al.*, 1977), or other forms of percutaneous transmission via blood and

FIG. 4. Serial determinations of antibody to hepatitis A antigen by immune adherence (IA) haemagglutination in a patient with viral hepatitis, type A. (Reproduced with permission of Dr Saul Krugman and the *New England Journal of Medicine*, 292, 1141. 1975.)

blood products. The rarity of percutaneous spread of HAV infection is consistent with the lack of a chronic HAV carrier state.

Serological surveys have also confirmed epidemiological observations which suggest that frequency of exposure to HAV increases proportionally with age (Purcell *et al.*, 1975; Szmuness *et al.*, 1976; Villarejos *et al.*, 1976) and inversely with rising socio-economic status (Miller *et al.*, 1975; Szmuness *et al.*, 1976; Villarejos *et al.*, 1976).

Control of viral hepatitis, type A

Passive immunization with immune serum globulin is the only available method to prevent or modify HAV infection. Since non-infectious virus antigens have not been identified, and since HAV has not been purified in large enough quantities from clinical material, the development of a vaccine must await *in vitro* cultivation of HAV.

Viral hepatitis, type non-A, non-B

The availability of sensitive serological tests for HAV and HBV made possible definitive aetiological classifications of viral hepatitis. Post-transfusion hepatitis was once considered to be exclusively type B hepatitis; however, a significant proportion of these cases have no serological evidence for HBV infection. Initially, it was thought, these patients were infected with HBV but serological tests were not sensitive enough to detect HBsAg or anti-HBs. Today, however, sensitivity of testing procedures is adequate to make or exclude a diagnosis of type B hepatitis with almost complete confidence, if serial evaluations are performed after transfusion and

throughout convalescence. Application of these sensitive tests revealed that many transfusion-associated cases were not caused by HBV, and recent serological testing for anti-HA has failed to incriminate HAV also (Feinstone *et al.*, 1975; Knodell *et al.*, 1975). Cases of hepatitis which are serologically neither type A nor type B also occur among drug abusers (Mosley *et al.*, 1977), haemodialysis patients (Zuckerman and Couroucé, unpublished data), intra-family contacts (Villarejos *et al.*, 1975), and intra-institutional contacts (Ogra, unpublished data). Both cytomegalovirus and Epstein-Barr virus have also been excluded as the aetiological agent in most of these cases. Thus, the occurrence of what has been designated 'non-A, non-B' hepatitis suggests that one or more other hepatitis viruses exist. To date, however, no virus or virus antigen has been identified in these cases of human 'non-A, non-B' hepatitis.

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