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Hepatitis Viruses: Characterization and Diagnostic Techniques

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Two human hepatitis viruses have been identified and characterized, but one or more additional agents exist. Hepatitis B virus (HBV) is a complex 42-nm predominantly double-stranded DNA virus with distinct surface and core antigens and an endogenous DNA polymerase. Hepatitis A virus (HAV) is a 27-nm RNA virus with enterovirus-like properties. Progressively more sensitive and specific immunologic assays have been applied to the study of viral hepatitis and are available for routine diagnostic purposes. As a result we recognize distinct serologic response patterns to infection, new antigenic markers, biochemical-biophysical characteristics of the viruses, and their epidemiologic features. Recombinant DNA technology has permitted the cloning of HBV genetic material and gene products in *E. coli*, but the virus has not been cultivated *in vitro*. In contrast, successful *in vitro* cultivation of HAV has finally been accomplished. Application of sensitive serologic tests for HAV and HBV has revealed that "non-A, non-B" agents account for a substantial proportion of transfusion-associated hepatitis as well as hepatitis occurring in the absence of percutaneous exposure. These agents have been transmitted to chimpanzees, and several putative virus antigen-antibody systems have been described; however, a specific association between these virus antigens and non-A, non-B hepatitis has not been established.

INTRODUCTION

Traditional classification of viral hepatitis was based on epidemiologic observations and studies in volunteers which suggested the existence of two types of infection. One had a short incubation period (two to six weeks), was spread by the fecal-oral route, occurred in large common-source outbreaks, and, considered highly infectious, was designated "infectious hepatitis." The other, labelled "serum hepatitis," was associated with percutaneous inoculation of blood or instruments contaminated with blood, had a long incubation period (six weeks to six months), and was not spread as readily from person to person. The discovery of virus antigens and animal models and the development of serological tests for the agents of viral hepatitis, however, have changed radically our traditional concepts and continue to broaden our understanding of the viruses which cause hepatitis in man.

VIRAL HEPATITIS, TYPE B

Australia antigen, discovered in the early 1960s, is known today to represent an antigenic specificity, hepatitis B surface antigen (HBsAg), on the surface of three

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morphologically distinct forms: spherical particles approximately 22 nm in diameter, tubules of similar diameter but of varying length, and the 7-nm outer coat of a 42-nm double-shelled structure, the hepatitis B virion (Dane particle). The 22-nm spheres and tubules are thought to represent excess viral coat protein and circulate in sufficient abundance to be detected by relatively insensitive immunologic techniques. In infected liver cells, HBsAg activity is localized to the cytoplasm where it can be demonstrated in chronically infected persons by immunofluorescence or immunoperoxidase staining techniques; by the presence in paraffin sections of "ground-glass" hepatocytes, orcein or aldehyde-fuchsin staining; or by electron microscopy. Hepatitis B surface antigen consists of approximately seven polypeptides as well as lipid and carbohydrate components. Residing on each HBsAg particle is a group-reactive determinant, *a*, and, generally, mutually exclusive allelic subdeterminants *d*, *y* and *w*, *r* as well as a number of other recently described but less well-characterized subdeterminants. As a result, four virus genome-determined major subtypes of HBsAg were recognized: *adw*, *ayw*, *adr*, and *ayr*, but the recent recognition of w variants w_1, w_2, w_3, and w_4 as well as other specificities has expanded this group of major subtypes. Different subtypes are characteristic of different geographic locales and tend to distinguish certain special epidemiologic settings, but, with the exception of an association between *ayw* strains and infantile papular acrodermatitis, the virus-coded subtype does not correlate with severity, outcome, or extrahepatic syndromes of hepatitis B virus (HBV) infection.

Within the hepatitis B virion and exposed by detergent or lipid solvent treatment is a 27-nm nucleocapsid core, called hepatitis B core antigen (HBcAg), immunologically unrelated to HBsAg and to which the host mounts a distinct immune response. In chronically infected persons, HBcAg is localized by immunologic techniques primarily, but not exclusively, to the hepatocyte nucleus, where 27-nm particles can be visualized by electron microscopy. Hepatitis B core antigen consists of two, perhaps three, polypeptides and there is no subtype heterogeneity to this antigen. Within the core is a circular DNA genome with a molecular weight of approximately 2×10^6 daltons of which 70 percent is double-stranded and the remainder single-stranded [1,2]. This genome with double- and single-stranded segments is unique to HBV and to the virus of a recently discovered agent in woodchucks, woodchuck hepatitis virus, which is morphologically similar to HBV and is associated with acute and chronic hepatitis and hepatocellular carcinoma in this species [3]. Also within the core is a HBV-specific DNA-dependent DNA polymerase which uses HBV DNA as a primer template and which has the potential *in vitro* to make double-stranded the single-stranded region of the genome [1]. The recent application of recombinant DNA technology has accelerated characterization and allowed more accurate restriction endonuclease mapping of the HBV genome [4,5]. Furthermore, the elaboration of HBcAg by *E. coli* which had incorporated parts of the HBV genome has been described [4]. Thus, despite refractoriness of HBV to cultivation in tissue culture, elaboration of viral gene products can be studied *in vitro*. In the same vein, establishment of a HBsAg-secreting cell line, PLC/PRF/5, derived from a patient with hepatic cell carcinoma and HBV infection [6], provides another *in vitro* model of virus gene product synthesis.

Two subpopulations of hepatitis B virions (Dane particles) have been described [7]. One appears "full" when examined by electron microscopy, has a buoyant density in cesium chloride (CsCl) of 1.22 g/cm^3, and contains DNA and DNA polymerase. The other appears "empty," has a lighter density of 1.20 g/cm^3, is devoid of or has an incomplete genome and lacks DNA polymerase. The existence of these incomplete

virions is reminiscent of defective interfering particles described in association with such agents as vesicular stomatitis virus and lymphocytic choriomeningitis virus. These defective particles could compete with complete virions for hepatocyte receptor sites or require cooperation from a complete virion for replication. In well-defined systems of defective interference, these incomplete particles appear to facilitate establishment of a non-cytopathic persistent infection tolerated by the host. It is attractive to speculate that similar interference by lighter virions contributes to the modulation of HBV infection which eventuates in a chronic carrier state.

During the course of HBV infection, a well-defined sequence of serological events occurs. The earliest detectable finding, occurring several weeks after exposure, is HBsAg in serum. Shortly after HBs antigenemia begins, antibody to the core of HBV (anti-HBc) becomes detectable in high titer, long before appreciable levels of anti-HBs can be detected and roughly coincident with the transient appearance of DNA polymerase activity, days to weeks before or coincident with elevation of serum aminotransferase activity. (Hepatitis B core antigen circulates within the intact virion; therefore, it is immunologically sequestered and not detectable in the circulation.) In self-limited acute cases, HBsAg levels diminish gradually, anti-HBc titers fall, and appreciable levels of antibody to HBsAg (anti-HBs) become detectable several weeks to months after the disappearance of HBsAg. Both anti-HBs and anti-HBc remain detectable for years after infection but at relatively low titers. In approximately 10 percent of cases, HBsAg never reaches detectable levels ($<10^9$ particles/ml); in such instances a diagnosis of HBV infection is made by demonstration of an anti-HBc/anti-HBs response. In fact, anti-HBc may be the only indicator of HBV infection during the interval or "window" between disappearance of HBsAg and emergence of anti-HBs [8]. Moreover, the fact that HBsAg-negative, anti-HBs-negative blood containing anti-HBc has been implicated in the transmission of type B hepatitis to transfusion recipients [9] and the demonstration that anti-HBc-positive blood may contain HBsAg at levels beneath the sensitivity threshold of radioimmunoassay suggest that high titer anti-HBc is a sensitive indicator of ongoing hepatitis B viral replication even in the absence of detectable circulating HBsAg.

The most important variation on the theme of standard serologic events is the chronic HBsAg carrier state. In carriers, HBsAg remains detectable in the circulation indefinitely, anti-HBc increases in titer, but anti-HBs is generally not detectable with currently available assays. These observations and deductions derived from sero-epidemiologic investigations suggest that anti-HBc is not protective but reflects recent or current viral infection, especially when present in high titer. In contrast, anti-HBs appears to be the protective antibody, and its presence in the circulation is the goal of strategies for passive and active immunization. Currently, trials are under way to evaluate a HBV vaccine prepared from plasma of chronic carriers by purifying 20-nm HBsAg particles to render them free of complete virions by ultracentrifugation [10,11].

A less well-characterized antigen associated with HBV infection, but one which has been the recent object of intense study, is hepatitis B e antigen (HBeAg) [12]. This antigen, or group of antigens (e_1, e_2, and e_3 have been identified), is a soluble protein found in HBsAg-positive serum and correlates with the presence of intact virions, DNA-polymerase activity, and HBsAg in high titer, i.e., with ongoing viral synthesis. As such, HBeAg identifies HBsAg-positive persons whose infectivity is likely to be high [13,14]. Individuals with HBV infection who are anti-HBe-positive are much less likely to be infectious. No longer believed to correlate well with severity or chronicity of liver disease, HBeAg appears early, transiently during acute type B hepatitis [15].

Although its biological significance is not fully appreciated, HBeAg, which is immunologically distinct from HBsAg and HBcAg, appears to be associated with and to share polypeptides of HBcAg [16]. Preliminary reports localize HBeAg to the nucleus of infected hepatocytes.

Recently, a number of new antigenic markers have been associated with HBV infection, including "delta" antigen [17] and an antigen unique to the surface of the intact virion [18]; however, these have not been studied extensively. A most intriguing discovery is that HBsAg particles from some individuals bear receptors for polymerized human or chimpanzee serum albumin [19]. Concentration and presence of these receptors correlate with the presence of HBeAg, and, therefore, the presence of these receptors is an indirect indicator of infectivity. Additional study of these receptors may elucidate the hepatotropism of HBV and the limitation of its host range to humans and chimpanzees.

Advances in the study of HBV have evolved from immunologic identification of virus antigens and antibodies. Once identified in serum with such relatively insensitive methods as agar gel immunodiffusion (AGD), counterelectrophoresis (CEP), and complement fixation (CF), HBsAg is now detected by one of a variety of sensitive "third-generation" immunologic methods. Those that have achieved the greatest reliability and widest use for large-scale, rapid screening are solid-phase radioimmunoassay (RIA) [20], immune adherence hemagglutination (IAHA) [21], and enzyme-linked immunosorbent assay (ELISA) [22]. Agar gel diffusion and CEP are too insensitive to detect anti-HBs in any but the most strongly positive sera. Instead, passive hemagglutination (PHA) [23] and several modifications of RIA, including radioimmunoprecipitation [24], are the most sensitive and widely used. In addition, IAHA and ELISA are being applied to anti-HBs testing. Methods applied widely to detection of anti-HBc, in ascending order of sensitivity, are CF, CEP, IAHA, and RIA. For several years, insensitivity of methods to detect HBeAg and anti-HBe remained an obstacle to the investigation of this antigen-antibody system. Use of AGD to detect HBeAg or anti-HBe required lengthy incubations and yielded very faint immunodiffusion lines; results obtained by CEP were obtained more quickly but without increased sensitivity and even perhaps at the expense of specificity. Today, techniques of "third-generation" sensitivity, PHA, RIA, and ELISA, are being applied to the study of this antigen-antibody system. With these newer techniques, laboratory diagnosis of viral hepatitis type B has been simplified. A diagnosis of type B hepatitis is based on demonstration of serum HBsAg or de novo appearance of anti-HBc and anti-HBs. The presence of HBeAg in HBsAg-positive serum suggests that the serum is likely to be infectious for contacts.

Finally, although a discussion of the epidemiology of HBV [25] is beyond the present scope, several salient points deserve mention. Parenteral inoculation with contaminated blood products was once thought to be the only mode of HBV transmission; however, more recent studies implicate or suggest many non-percutaneous routes as well. Hepatitis B surface antigen has been found in virtually every body secretion, some of which are transferred to contacts without introduction via the percutaneous route. Among such modes of transmission, vertical (from mother to infant), venereal, and oral spread are probably the most important. Given the fact that nonpercutaneous routes and other secretions have been implicated in transmission of HBV, the term "serum" hepatitis is no longer appropriate to describe hepatitis type B. In fact, serologic testing has shown that approximately one-half of all sporadic cases lacking percutaneous exposure and once designated as "infectious" hepatitis are actually caused by HBV [26].

VIRAL HEPATITIS, TYPE A

Studies in volunteers and epidemiologic investigations demonstrated that hepatitis A virus (HAV) is excreted in feces late in the incubation period and early during the acute phase of illness; however, attempts to identify the virus in stool were not successful until Feinstone et al. [27] applied the technique of immune electron microscopy (IEM) to the study of type A hepatitis. Convalescent serum or immune serum globulin, known to contain antibody to the virus, was incubated with acute phase stool specimens from individuals experimentally infected with the MS-1 strain of HAV. In the immune precipitate that formed, virus-like particles were identified by electron microscopy; the specificity of the association between these particles and HAV was proven by serological means and upheld by reports of immunologically indistinguishable particles in stools of naturally infected persons. Hepatitis A virus particles were also detected in liver and serum of experimentally infected marmoset monkeys [28].

Hepatitis A virus is 27 nm in diameter, has a range of buoyant densities in CsCl from 1.27–1.45 g/cm^3 but a principal density of 1.34 g/cm^3, and a sedimentation coefficient of 160S. The non-enveloped virion has cubic symmetry and may appear "full" (unpenetrated by stain) or "empty" (penetrated by stain). Within the hepatocyte, HAV is localized to the cytoplasm, where it has been visualized in vesicular structures. Its genome is a single-stranded RNA with a molecular weight of 1.9×10^6 daltons, and its four polypeptides are similar in molecular weight to those of enteroviruses. Like other enteroviruses, HAV is ether- and acid-resistant, but the heat stability of HAV is uncharacteristic of this group of viruses. In general, however, HAV most closely resembles the enterovirus subgroup of picornaviruses. To date, all HAV strains described have been immunologically indistinguishable and only one antigen has been identified with the virion.

Recent advances in hepatitis A research have been fostered by the development of immunologic assays to detect virus antigen and antibody. Hepatitis A virus has been purified from stool and liver by ultracentrifugation, electrophoresis, gel filtration, and affinity chromatography for use as antigen in serological tests. In addition to IEM, which can be used to detect and quantitate both HAV and anti-HAV, more simple, practical tests have emerged, including CF [29], IAHA [30], RIA [31–33], and ELISA [34,35]. Moreover, HAV can be demonstrated in tissue by immunofluorescence [36] and immunoperoxidase [37] staining.

With these techniques investigators have defined the following sequence of detectable virologic and serologic events during HAV infection in humans and nonhuman primates: within one to two weeks after exposure, HAV can be detected by immunofluorescence in the liver where it remains detectable for several weeks, generally outlasting the brief period of fecal HAV shedding and elevated serum aminotransferase activity. Viremia is also an early event which, demonstrated by infectivity of serum, is most pronounced during the late incubation period and persists no more than a few days after the onset of illness. Generally, the concentration of HAV in serum is too low for detection by *in vitro* immunologic techniques. Shedding of the virus in feces is one of the earliest virologic events detectable with *in vitro* techniques in routinely available clinical material. Fecal excretion of HAV coincides with the onset of non-specific symptoms (malaise, fatigue, anorexia, etc.) and peaks in intensity several days to more than a week before biochemical and histologic evidence of acute hepatitis. By the time a patient presents to a physician with jaundice the bulk of fecal HAV excretion will have already occurred [38].

Serum antibody to HAV appears during the onset of acute illness, increases in titer

toward a peak two to three months later, falls gradually, but remains relatively high and readily detectable years after infection. Although anti-HAV can be detected during acute illness by most of the techniques described above, there is a delay of from one to four weeks after acute illness before anti-HAV is detectable by IAHA. Theoretically, then, a combination in acute phase serum of a negative IAHA test for anti-HAV with anti-HAV detectable by one of the other serologic techniques provides presumptive evidence for acute HAV infection [39]. Antibody which appears during early acute illness is predominantly IgM, which appears transiently. Antibody of the IgG class develops more gradually, reaches high levels during convalescence, and persists for years, if not indefinitely. Anti-HAV is protective; its presence in serum indicates previous HAV infection and immunity to reinfection.

For practical purposes, clinical laboratory diagnosis usually depends on demonstration of a serologic response in paired acute and convalescent serum samples or of anti-HAV of the IgM class [40,41] during acute illness.

Eluding investigators for several decades, successful cultivation of HAV *in vitro* has finally been accomplished. Provost and Hilleman [42] recently reported serial propagation of the CR326 strain of HAV, which had been passaged extensively in marmosets, in primary explant cultures of adult *Saguinus labiatus* marmoset livers and, even more efficiently, in a fetal rhesus monkey kidney cell line (FRhK6). Unlike other enteroviruses which are cytopathic in cell culture, HAV was not cytopathic in these cell lines; its presence was monitored by immunofluorescence. Although direct inoculation of clinical specimens from man into tissue culture is not yet feasible as a diagnostic tool, this work does represent an important breakthrough for hepatitis A research and for hepatitis research in general.

Seroepidemiologic studies have shown that exposure to HAV is widely distributed and increases as a function of increasing age and decreasing socioeconomic class. In modern urban societies, a decrease in the incidence of HAV infection has occurred, and age-related differences in prevalences of HAV exposure reflect this change. Type A hepatitis is transmitted almost exclusively by fecal-oral spread, which is enhanced by poor personal and environmental hygiene. There is no virologic, epidemiologic, or clinical evidence for the existence of viremic or intestinal HAV carriers, and the virus is rarely, if ever, spread in nature by parenteral mechanisms. Perpetuation of HAV in nature appears to depend on non-epidemic, inapparent infection [43,44].

NON-A, NON-B VIRAL HEPATITIS

Once sensitive serologic tests for both HBV and HAV infection became available, their application revealed that, today, 80–90 percent of cases of transfusion-associated hepatitis are not caused by either of these recognized human hepatitis viruses [45,46]. These cases, designated "non-A, non-B" hepatitis, are also unrelated to other known viruses which occasionally affect the liver and occur in a variety of non-transfusion epidemiologic settings paralleling those implicated in the transmission of HBV. Chronic hepatitis may be seen in 30–40 percent of patients with transfusion-associated hepatitis non-A, non-B, and epidemiologic evidence favors the existence of an asymptomatic chronic carrier state for this agent. Actually, there is also evidence to suggest the existence of more than one such non-A, non-B agent [47,48].

The most convincing evidence for the existence of non-A, non-B agents derives from transmission studies in volunteers and successful serial transmission in chimpanzees. Although a number of putative non-A, non-B ("hepatitis C") virus antigen-antibody systems have been defined by such techniques as agar gel diffusion

[49], electron microscopy [50], and immunofluorescence [51], no viral agent or immunologic marker has been identified which fulfills accepted serologic criteria for a *specific* association with non-A, non-B hepatitis.

IN THE FUTURE

In the years to come, new virologic markers for HBV will be better defined and studied on a larger scale. Hepatitis B vaccines, some already produced, will be tested and made available to high-risk populations, and attempts will be made to abort chronic HBV carriage with antiviral chemotherapy. Improved understanding of the molecular virology and viral genetics of HBV will derive from studies of woodchuck hepatitis virus and from recombinant DNA and monoclonal antibody technologies. Additional work will also be required to pursue the intriguing association between HBV and hepatocellular carcinoma.

Serologic tests for HAV infection are now commercially available and will be applied to diagnosis and epidemiologic investigation. The ability to cultivate HAV *in vitro* will provide limitless new horizons for its study and should answer questions remaining about the composition of its genome and its capsid polypeptides.

For non-A, non-B hepatitis, the most pressing task is identification of the agent(s) and development of simple, sensitive, specific serologic tests. Availability of such tests is expected to curtail the incidence of transfusion-associated hepatitis and allow characterization of the elusive agents.

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