Studies of Non-A, Non-B Hepatitis and Characterization of the Hepatitis C Virus in Chimpanzees

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1 Introduction

This year marks the end of the second decade of research on post-transfusion non-A, non-B hepatitis (or PT-NANBH), now primarily known to be caused by hepatitis C virus (HCV; Choo et al. 1989). Approximately 10 years ago, the successful molecular cloning of HCV was publicly announced and ushered in a new era of epidemiological, medical, and laboratory research virtually unparalleled by any other area in virology. During the last 10 years, according to a January 1998 search of the NLM-Medline-PubMed database, more than 14,000 publications listing hepatitis C have been cited. This astounding growth was a result of the diligent and persistent efforts of at least two major laboratories and the accumulated knowledge generated by many other scientists in other laboratories who struggled to isolate,

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characterize, and clone the major agent (virus) responsible for PT-NANBH. One Australian scientist who previously worked in the field of PT-NANBH remarked (in 1990) that "over 80 laboratories at one time or another worked on non-A, non-B hepatitis...and that by the end of 1987 virtually only three or four laboratories were seriously pursuing research in this area." This perspective highlights the extremely frustrating nature of earlier research in the field of PT-NANBH and reveals the trend of a rapidly diminishing number of active laboratories over time during the mid-1980s.

PT-NANBH was first recognized by PRINCE et al. (1974) and ALTER et al. (1975) as a form of viral hepatitis that was distinct from disease caused by hepatitis A virus (HAV) or hepatitis B virus (HBV). Unlike HAV and HBV, however, the agent responsible for PT-NANBH proved to be elusive and defied the efforts of electron microscopists to visualize disease-associated virus-like particles and also remained undetectable by even the most sensitive serologic tests available at the time. Research in the late 1970s to early to mid-1980s revealed the true nature of the problem. The titer(s) of the infectious agent was so low in infectious plasma, serum, blood products, and liver tissue that many investigators began to accept the fact that they were not likely to isolate and characterize the presumptive viral agent.

This chapter summarizes much of what was learned about the major agent responsible for human PT-NANBH through the use of chimpanzees, the only biomedical model for this disease. More expansive reviews of the broad area of PT-NANBH by DIENSTAG (1983) and FAGAN and WILLIAMS (1984) provide a contemporaneous overview of this field.

2 Development of Primate Model

The chimpanzee is the only primate proven to be susceptible to infection with human NANBH viruses, primarily HCV (hepatitis E virus, HEV, is not considered within the scope of this review). Although chimpanzees had been used previously for experimental transmission of human HAV and HBV, it was not until 1978 that it was discovered that this species of primate was also susceptible to infection with one or more presumptive viral agents of NANBH. Prior to the latter studies, the use of untreated factor VIII concentrates (anti-hemophilic factor, FVIII) for the treatment of hemophilia A was known to carry with it the risk of transmitting either (or both) HBV or NANBH. In the latter instance, HRUBY and SCHAUF (1978) reported the transmission of short-incubation period NANBH in pediatric patients following infusion of FVIII concentrates, while CRASKE and SPOONER (1978) reported evidence for the existence of two distinct types of FVIII-associated non-B hepatitis in transfused hemophiliacs. ALTER et al. (1978) and TABOR et al. (1978) first reported the susceptibility of chimpanzees to human NANBH. HOLLINGER et al. (1978) also showed that human NANBH could be readily transmitted to chimpanzees. Laboratory studies initiated at the U.S. Centers for Disease Control

in 1977, in response to an outbreak of NANBH among recipients of three different lots of commercially manufactured FVIII, led to the experimental transmission of disease (NANBH) to four colony-born chimpanzees in early 1978 using the above implicated lots of FVIII materials. BRADLEY et al. (1979) showed that intravenous infusion of 15–30ml of one or more lots of FVIII induced relatively short incubation period NANBH as evidenced by the rapid rise of alanine aminotransferase (ALT) activity following inoculation. Examination of acute-phase liver biopsy specimens revealed histologic evidence of disease that was consistent with a diagnosis of viral hepatitis. WYKE et al. (1979) showed that contaminated factor IX concentrates could also induce acute NANBH that was consistent with a diagnosis of viral hepatitis. Shortly thereafter, YOSHIZAWA et al. (1980) reported the transmission of NANBH to chimpanzees using suspect fibrinogen linked to the transmission of disease in humans. The above studies conclusively proved the extraordinary value of chimpanzees as a biomedical model for human NANBH. Numerous (largely unreported) attempts by our (Centers for Disease Control, CDC) laboratory and other laboratories to induce human-origin NANBH in other species, including tamarins, marmosets, and cynomolgus macaques, failed to demonstrate that any species of primate, other than the chimpanzee, was susceptible to infection with one or more proven human NANBH agents.

3 Pathogenesis and Course of Disease in Chimpanzees

Long-term follow-up studies of NANBH infected chimpanzees by BRADLEY et al. (1981, 1982) provided unequivocal evidence for the occurrence of persistent viremia and disease that was remarkably similar to that observed in human PT-NANBH, as described by KORETZ et al. (1976), GALBRAITH et al. (1979), RAKELA and REDEKER (1979), BERMAN et al. (1979), and ALTER et al. (1982). BRADLEY et al. (1981, 1982) also showed that approximately 70% of chimpanzees observed at CDC for 5–7 years after acute NANBH were found to display enzymatic, histologic, and electron microscopic evidence of chronic disease or silent infection (see below) (Fig. 1). One frequently observed pattern of disease development (pathogenesis) in chimpanzees was the initial rise and fall of ALT values accompanied by apparent histologic resolution of disease. However, longer-term studies of numerous chimpanzees revealed that while "resolution" of overt disease could be seen for up to 2 to 3 years (after inoculation) in some animals, others demonstrated slowly increasing and/or intermittent elevations of ALT activity indicative of persistent infection. Consistent with the above findings by BRADLEY et al. (1981, 1982), TABOR et al. (1980) and ALTER and DIENSTAG (1984) reported that persistent viremia could exist in the absence of elevated ALT activity in human blood donors. In fact, ALTER and DIENSTAG (1984) estimated that approximately 70% of donors capable of transmitting NANBH may not demonstrate abnormal ALT activity. These projections were corroborated by BRADLEY et al. (1982), who proved that several

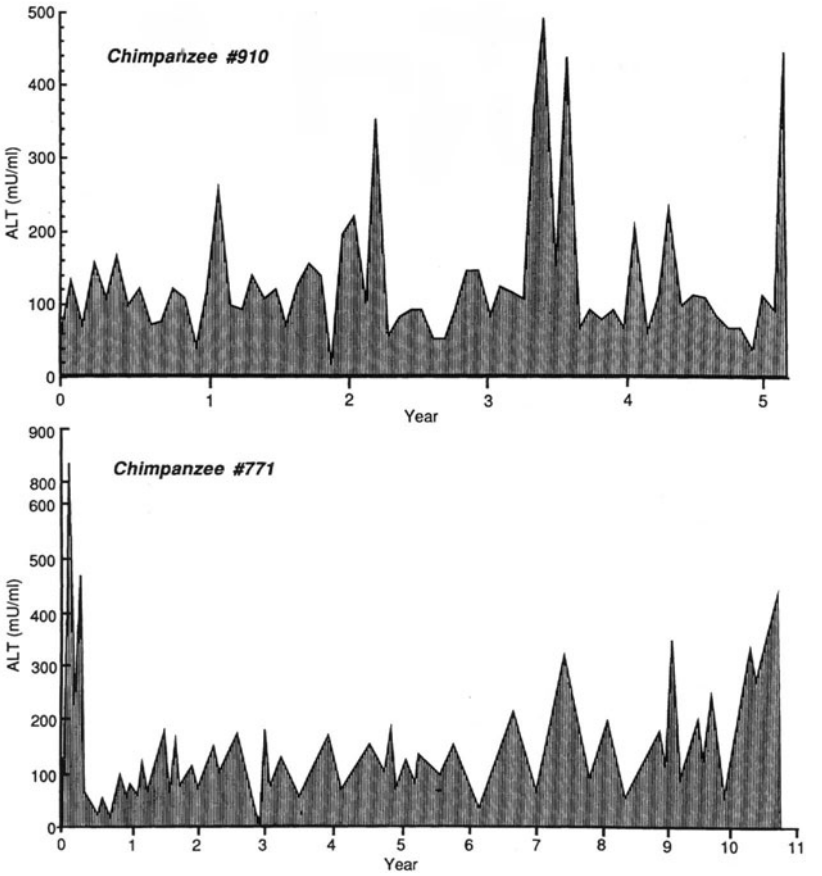

Fig. 1. Alanine aminotransferase (ALT) patterns in two chimpanzees persistently infected with the major agent of post-transfusion non-A, non-B hepatitis (PT-NANBH; HCV)

plasma units obtained from four animals with neither electron microscopic nor enzymatic evidence of persistent infection were infectious when intravenously inoculated into colony-born chimpanzees. Bradley et al. (unpublished findings) also demonstrated the transmission of biochemically and histologically "silent" disease to two chimpanzees using 1×10^{-7}g of NANBH-infected liver tissue. Electron microscopic (EM) evidence of infection was found in one of the above chimpanzees after careful examination of serial (weekly) liver biopsy specimens obtained over a period of months. The remaining animal showed no EM evidence of infection with the major agent of NANBH, but was shown to be refractory to infection by proven-infectious inocula (see Sect. 9, Viral Interference, below). The above findings suggested that the major etiologic agent of PT-NANBH could establish a subclinical course of infection in chimpanzees, and presumably in humans, in the absence of elevated ALT activity and histologic or EM evidence of infection.

Although these earlier studies could also be interpreted to mean that prior infection with the agent of NANBH induced neutralizing (protective) antibodies, the combined findings more strongly suggested that the agent of PT-NANBH normally causes persistent infection in which additional homologous challenge virus does not perturb or otherwise cause any alteration in the established disease state.

Spontaneous recrudescence of relatively severe disease was documented by BRADLEY et al. (1982) in persistently infected chimpanzees. In fact, one NANBH-infected chimpanzee developed both enzymatic and EM evidence of acute disease after a 2-year period of quiescence, a surprising and sobering finding that indicated that resolution of overt disease does not necessarily signal recovery from infection. The observation by BROTMAN et al. (1985) and BURK et al. (1984), that rechallenged chimpanzees exhibited renewed elevations of ALT activity due to reinfection in the absence of neutralizing antibodies, was at one time interpreted to mean that the co-occurrence of elevated enzymes was due to exacerbation of chronic, underlying disease. More recent studies, however, have shown that reinfection can occur after either homologous or heterologous virus challenge, as evidenced by the appearance of type-specific nucleotide sequences of HCV in serum or plasma. In spite of the latter findings, studies conducted by BRADLEY et al. at the CDC showed that NANBH-infected chimpanzees not subjected to rechallenge exhibited a variety of histologic, enzymatic, and EM patterns of disease and that silent infections, recurrent disease, and persistent infection with or without elevated ALT activity and histologic evidence of viral hepatitis could occur. All of the above findings, taken together, suggested that if there were any parallels between the course of disease observed in chimpanzees and humans, it was reasonable to assume that total resolution of NANBH may occur in only a small proportion of infected individuals.

4 Evidence that PT-NANBH Was Caused by a Virus

As noted and described above, there were several convincing lines of experimental and clinical evidence that suggested the etiologic agent of PT-NANBH was virus-like in nature. This evidence included the transmissibility of disease to both humans and chimpanzees by intravenous or percutaneous inoculation of contaminated donor blood, blood fractions, acute-phase liver homogenates, red blood cells, or "purified" chronic-phase plasma preparations, as previously reported by ALTER et al. (1978), TABOR et al. (1978), HAUGEN (1979), BRADLEY et al. (1979, 1980, 1981), BRADLEY and MAYNARD (1983), WYKE et al. (1979), YOSHIZAWA et al. (1980), and HOLLINGER et al. (1978). Infectivity of the major etiologic agent (also previously referred to as the "tubule forming agent," or TFA, due to the formation of peculiar hepatocyte cytoplasmic tubules in chimpanzees; see below) was also shown be destroyed by treatment with chloroform (BRADLEY and MAYNARD 1983; FEINSTONE et al. 1983), treatment with 1:1000 formalin (TABOR and GERETY 1980; YOSHIZAWA et al. 1982), inactivation with β-propiolactone and ultraviolet irradi-

ation (Prince et al. 1985), or heating at 100°C for 60min (Yoshizawa et al. 1982) or 60°C for 10h (Hollinger et al. 1984). Other studies (some described below) demonstrated that the TFA would pass through an 80nm sharp cut-off polycarbonate filter (Bradley et al. 1985a) or partially through a 50nm filter (He et al. 1987). It is also worth noting that persistent PT-NANBH infection in chimpanzees was shown by Bradley et al. (1983c), Tsiquaye et al. (1983), and Brotman et al. (1983) to interfere with superinfection by two other hepatotropic viruses, namely HAV and HBV. The latter phenomenon strongly suggested that viral interference was the culprit and further supported the growing (or accepted) notion that the TFA was a virus. Furthermore, liver biopsy specimens obtained from both human patients and experimentally infected chimpanzees revealed a variety of characteristic light microscopic changes that were entirely consistent with a diagnosis of acute, chronic, or persistent viral hepatitis, as described by Dienes et al. (1982), Bianchi and Gudat (1983), and Hoofnagle and Alter (1984). Finally, the hepatocyte ultrastructural alterations observed in PT-NANBH infected chimpanzees were reported by Bradley et al. (1985b) to be identical or similar to those found in cells infected by RNA, but not DNA viruses (see below).

5 Evidence for the Existence of Multiple NANBH Agents

Aside from the enterically transmitted form of NANBH, now known to be caused by hepatitis E virus (HEV; Bradley et al. 1988; Bradley 1990; Reyes et al. 1990), earlier studies suggested that PT-NANBH might be caused by a variety of unrelated hepatotropic agents (viruses). This view was supported by: (1) the observation of multiple attacks of viral hepatitis in patients transfused with blood products, as reported by Mosley et al. (1977), Hruby and Schauf (1978), Craske and Spooner (1978), and Norkrans et al. (1980); (2) the occurrence of both long and short incubation period disease in transfused patients, as reported by Craske et al. (1975), Aach et al. (1978), and Guyer et al. (1979); (3) the observation of unique or distinct patterns of ALT activity seen in infected patients, as reported by Tateda et al. (1979); (4) the results of cross-challenge studies in chimpanzees that had been experimentally infected with a variety of infectious materials, including blood, blood fractions, or blood products, as reported by Bradley et al. (1980), Hollinger et al. (1980), Tsiquaye and Zuckerman (1979), Yoshizawa et al. (1981), and Tabor et al. (1980); (5) the appearance of characteristic hepatocyte ultrastructural alterations in some, but not all, NANBH-infected chimpanzees, as reported by Bradley et al. (1983), Yoshizawa et al. (1981), and Shimizu et al. (1979); and (6) the observation by Bradley et al. (1983) of two, sequential episodes of NANBH in chimpanzees after the inoculation of chloroform-resistant and chloroform-sensitive agents, respectively (see below).

In view of the reported high incidence of persistent PT-NANBH in many infected individuals and experimentally infected chimpanzees, it now appears that

many of the earlier cross-challenge studies were most likely confounded by the probable effects of viral interference. Other, more recent discoveries of other blood-borne viruses, namely, hepatitis G virus (HGV), reported by LINNEN et al. (1996), and GBV-C (closely related to HGV), reported by SIMONS et al. (1995), were foreshadowed in a review by ALTER and BRADLEY (1995) that described a form of NANBH unrelated to what is now known as HCV. The latter review described evidence for and against the existence of another major agent of NANBH. While the "true" hepatotropic nature and importance of the above two viruses still remain in doubt, it is clear that other agents of PT-NANBH may still be lurking in our blood supply system. In contradistinction to the above (and much more recent) findings, the turbulent course of PT-NANBH observed in many patients and chimpanzees could also explain the occurrence of many so-called second bouts of NANBH that in reality were simply exacerbations of chronic, underlying disease.

6 Attempts to Detect NANBH (Virus)-Specific Antigens, Antibodies, and Enzymes

Numerous laboratories during the late 1970s and early 1980s worked in vain to develop methods to detect virus-specific antigens and antibodies. Other laboratories sought, and even reported finding, virus-specific enzyme activities such as particle-associated reverse transcriptase activity (see below). Although numerous reports of antigen-antibody systems associated with NANBH appeared in the early literature, confirmation of their specificity was always problematic, if not impossible. Studies performed at the CDC (Bradley et al., unpublished work) encompassed the use of presumed convalescent human and chimpanzee sera, acute-phase sera, and chronic-phase sera (as well as the individual IgG and IgM fractions derived from these materials). More than 30,000 individual tests embodying RIA, immunodiffusion, counterimmunoelectrophoresis, or immunofluorescent probe configurations were employed. A variety of plasma and serum concentrates as well as liver homogenates (or fractions thereof) were tested by the above methods and all results were uniformly negative. Scientists in other major laboratories around the world suffered a similar sense of paroxysmal indignity when they unsuccessfully applied the then state-of-the-art methodologies to solve one of the most perplexing problems of the day.

As noted earlier, studies at the CDC and elsewhere strongly suggested that acute PT-NANBH frequently, if not usually, progressed to persistent liver disease and/or viremia. BRADLEY and MAYNARD (1983) and BRADLEY (1984) surmised from the above findings that antibody (regardless of source and type) of sufficient avidity and potency (titer) for the development of sensitive serologic tests for virus-specific antigens (and/or antibodies) was simply not available or could not be readily identified. It was generally known at the time that RIA and ELISA procedures required the use of high-avidity and/or high-titered antibody for optimum

sensitivity. It was also known at the time that antibodies that could readily function in far less sensitive procedures, such as immunoelectronmicroscopy, agar gel diffusion, fluorescent probe assays, and (somewhat later) western-blotting, were unsuitable for use in RIA and ELISA tests. BRADLEY (1984) speculated that even if antibodies of sufficient avidity and/or titers were available for use in the above procedures, viral antigens (or virus) would still not be detectable due to the low titers of circulating virus in known infectious plasmas, sera, liver homogenates, and blood fractions. BRADLEY concluded that the root of the enigma was not necessarily the source of antibody, but rather, that the titer of the PT-NANBH agent (virus) was below the level of detectability using the current methodologies. The latter hypothesis was supported by available titration data in chimpanzees that showed the majority of inocula had titers less than 1×10^3 chimpanzee infectious doses (CID) per ml. For example, the FVIII materials used by BRADLEY et al. (1979) in their first primate transmission studies were found to have a titer of less than 1×10^3CID/ml (BRADLEY et al. 1983b). The NANBH "F" strain used by FEINSTONE et al. (1981) was a chronic-phase plasma and was shown to have a titer of less than 1×10^2CID/ml. YOSHIZAWA et al. (1982) described a fibrinogen preparation with a titer greater than 1×10^2 but less than 1×10^4CID/ml; similarly, Tabor and Gerety (personal communication) found that one of their inocula had a titer of approximately 1×10^2CID/ml. Of great interest was the finding by Overby et al. (Abbott Laboratories, N. Chicago; unpublished studies of the late 1970s and early 1980s) that none of ten human acute-phase plasma units had a titer greater than 1×10^3CID/ml. One acute-phase human plasma ("H"-strain agent) described by FEINSTONE et al. (1981), was found to have a titer of approximately 1×10^6CID/ml; however, plasma taken from the same individual 1week later was found to be noninfectious when intravenously inoculated into a presumably naive chimpanzee (H.J. Alter, personal communication). The latter finding was puzzling, but indicated that titers of the NANBH agent (virus) could change significantly over a short period of time during the acute phase of disease. Taken together these findings supported the view by some investigators that the difficulties in developing a standard serologic assay for NANBH (virus)-specific antigen was not going to be accomplished until and unless a much more sensitive assay was developed.

Evanescent excitement regarding the possible identification of a retrovirus-like agent responsible for human PT-NANBH was generated by the nearly simultaneous reports of particle-associated reverse-transcriptase (RT) activity in human sera and in cultured NANBH-infected chimpanzee liver cells, as reported by PRINCE et al. (1984), SETO et al. (1984), and IWARSON et al. (1985). SETO et al. (1984) detected RT activity in four human NANBH serum specimens and in two plasma products, all of which had been shown to transmit disease to chimpanzees. RT activity was also detected in 12 of 12 human acute- and chronic-phase NANBH sera, but not in 47 of 49 sera from healthy donors. NANBH infectivity was also found at a buoyant density of 1.14g/cm^3 in a sucrose gradient that was coincident with a peak of RT activity. In spite of these earlier, potentially exciting findings, workers at the CDC and in other laboratories were unable to detect elevated RT activity in well-documented cases of human PT-NANBH using pedigreed panels of

sera. Furthermore, pelleted acute-phase human plasma fractions that contained the equivalent of at least 1×10^6CID of the NANBH TFA were shown to be completely devoid of RT activity (A.J. Weiner, and M.A. Houghton 1984, personal communication). These findings, combined with the knowledge that the NANBH TFA was < 80nm in diameter (see below), strongly suggested that the agent was not a retrovirus. Furthermore, use of hybridization probes specific for conserved DNA polymerase gene sequences of both hepadnaviruses (i.e., HBV) and retroviruses (replicative intermediates) including human T-cell lymphocytotropic virus type III (HTLV-III), MuLV, RSV, and cauliflower mosaic virus failed to detect homology between any of these viruses with total and polyadenylated RNAs extracted from several, proven-infectious chimpanzee livers (M.A. Houghton, A.J. Weiner, D.W. Bradley 1984, unpublished findings).

7 Infectivity and Virus-Like Particles: Peripheral Blood Lymphocytes, Cultured Chimpanzee Liver Cells, Liver Homogenates, and Blood Fractions

In addition to conventional studies with human and chimpanzee sera and plasmas, NANBH infectivity was sought in peripheral blood cell preparations from both humans and chimpanzees. Inoculation of a colony-born chimpanzee at the CDC with 2.2×10^7 peripheral blood lymphocytes (PBLs) obtained from a proven NANBH carrier did not induce elevated ALT activity up to 95 days after inoculation, nor was there any electron microscopic evidence of infection by the NANBH TFA in serial (weekly) liver biopsy specimens obtained during the study period (Bradley et al., unpublished data). Challenge of this animal with a NANBH chronic-phase liver homogenate resulted in short-incubation period disease accompanied by the appearance of characteristic hepatocyte ultrastructural alterations. In contrast to these early findings, Hellings et al. (1985) reported the transmission of NANBH to a chimpanzee using mononuclear leukocytes from a patient with chronic NANBH. This discrepancy in experimental findings was never resolved.

Prince et al. (1984) reported finding 85–90nm diameter virus-like particles in cultured chimpanzee liver cells infected with the NANBH TFA. Dermott (1985), however, suggested that the above viruses might be similar or identical to the (latent) simian foamy viruses previously described by Hooks and Gibbs (1975). Iwarson et al. (1985) reported the appearance of tubules in cultured NANBH-infected chimpanzee liver cells that were identical in morphology to the tubules seen in NANBH-infected chimpanzee hepatoctyes. The liver cell tubules referred to above were noted by Bradley et al. (1985b) as being most similar to the test-tube and ring-shaped structures (TRF and CTS) previously identified by Shamoto et al. (1981) and Sidhu et al. (1983) in lymphocytes obtained from patients with adult T-cell leukemia and acquired immune deficiency syndrome (AIDS), respectively.

Delta virus, a "defective" agent that requires the presence of HBV for replication, was also shown to induce similar tubular structures in chimpanzee hepatocytes but not in human hepatocytes (see below for further discussion). At best, the above findings were interesting phenomena but confounded an area of viral research that was already plagued by numerous blind alleys and "red-herrings." The questionable presence of NANBH infectivity in chimpanzee PBLs, the lack of detectable RT activity in proven-infectious materials, and the puzzling (non-specific) nature of the tubular structures observed in NANBH-infected chimpanzee hepatocytes, however, all mitigated against the possibility that the major agent of human NANBH was a retrovirus.

Earlier findings by BRADLEY et al. (1979) and YOSHIZAWA et al. (1980) that 27nm diameter virus-like particles (VLPs) were associated with infection of chimpanzees with either FVIII concentrates or fibrinogen, respectively, were greeted with little enthusiasm by a highly skeptical scientific community. The inability of other laboratories to reproduce the reported findings did little to support the association of these VLPs with NANBH. In retrospect, however, the recovery from acute-phase chimpanzee liver tissues of extremely fragile, often ragged 27nm diameter VLPs coated with what appeared to be an IgM isotype antibody is consistent with the notion that these VLPs were NANBH TFA (HCV) capsids aggregated by anti-C22 (core) antibodies that are now known to be among the first to appear in circulation in response to infection.

8 Comparative Morphology of Ultrastructural Alterations in Chimpanzee Hepatocytes

The NANBH (virus)-induced ultrastructural alterations observed in chimpanzee hepatocytes were extensively reviewed by BRADLEY et al. (1985). However, for purposes of historical perspective, selected aspects of the above review and other published works are described below and demonstrate the intense interest many scientists had in this area of NANBH research during the late 1970s to mid-1980s. SHIMIZU et al. (1979) first described the finding of double-walled tubular structures in the hepatocyte cytoplasm of chimpanzees infected with the "H" strain of the NANBH TFA (HCV). Subsequently, numerous other workers, including BRADLEY et al. (1980), TSIQUAYE et al. (1980), PFEIFER et al. (1980), and YOSHIZAWA et al. (1982), reported finding identical structures in the hepatocyte cytoplasm of chimpanzees infected with a wide variety of NANBH inocula. In total, six different types of ultrastructural alterations were observed in chimpanzees infected with the (chloroform-sensitive; see below) TFA. All of the changes were confined to the cytoplasm of infected or affected liver cells and included: (1) dense reticular inclusion bodies; (2) convoluted membranes derived from proliferated smooth endoplasmic reticulum; (3) characteristic tubular structures (already referred to above); (4) bundles of tightly-packed granular microtubules; (5) crystalline arrays

of 25nm "particles" in endothelial cells; and (6) highly structured crystals of proteinaceous material resembling paracrystalline arrays of tubulin found in reovirus-infected cells. It is of interest to note, as described earlier, that many of these ultrastructural alterations were also found by CANESE et al. (1984) in the hepatocyte cytoplasm of chimpanzees infected by delta virus.

Comparative morphologic studies by BRADLEY et al. (1985b) of the TFA-induced ultrastructural alterations revealed the occurrence of similar changes in cells infected by other, well characterized viruses. For example, cytoplasmic tubules observed by ROBERTS and HARRISON (1970) in plant cells infected with strawberry latent ringspot virus (SLRV, a picornavirus) were strikingly similar to the tubular structures found in TFA-infected chimpanzee hepatocytes. Both structures were derived from proliferated smooth endoplasmic reticulum (SER) and consisted of double-walled tubules surrounded by a membranous sheath. Tubules or cylindrical structures have also been visualized by HARRISON et al. (1982) and MONATH et al. (1983) in the cytoplasm of mosquito cells and visceral target organs of hamsters infected with St. Louis encephalitis virus (SLEV), a member of the Flaviviridae family. These cylinders were shown to be associated with virus replication and were also derived from proliferated SER. Tubules identical in morphology to those observed in NANBH TFA-infected chimpanzee hepatocytes were observed by SIDHU et al. (1983) in lymphocytes of patients with AIDS; similarly, SHAMOTO et al. (1981) reported identical tubules in the cytoplasm of lymphocytes from cases of adult T-cell leukemia. Paired, convoluted membranes enclosing an osmiophilic substance (Erc) were also observed by HAMPTON et al. (1973) in plant cells infected with pea seed-borne mosaic virus (PsbMV), a rod-shaped, non-enveloped RNA virus. ERc identical in morphology to that described above was also seen by HARRISON et al. (1982) in the lamina propria of hamster ileum infected with SLEV. Structures identical or very similar to the dense reticular inclusion bodies seen in infected chimpanzee hepatocyte cytoplasm were also observed in the cytoplasm of cell infected by a variety of RNA viruses, namely, reovirus (DALES 1973), influenza virus (COMPANS and CHOPIN 1973), coronavirus (mouse hepatitis virus; DAVID-FERREIRA and MANAKER 1965), poliovirus (DALES et al. 1965), SLEV (MONATH et al. 1983), and seadog (Tyuleniy) virus (SDV, a member of the Flaviviridae family; Zhdanov 1982, personal communication). Of great interest was the fact that the dense reticular inclusion bodies most closely resembled those found in cells infected with SDV or SLEV, both members of the Flaviviridae family. The above inclusion bodies appeared to contain masses of highly convoluted and densely-stained 25nm diameter filaments entangled in amorphic, proteinaceous materials that were absolutely identical in morphology to those found in SDV-infected cells and in the mid-gut epithelium of *Culex pipiens* (mosquitoes) infected with SLEV (MONATH et al. 1983). In the latter case, these structures were found to be spatially related to virus particle formation and were thought to represent viroplasmic foci (virus "factories"). Microtubular aggregates also have their counterpart in other virus infections. Nearly identical aggregates were previously found by VIDANO (1970) in the mid-gut tissue of an insect vector (*Laodelphax striatellus*) infected with maize rough dwarf virus (MRDV9), a small, RNA-containing virus. The para-

crystalline structures composed of proteinaceous materials, probably tubulin (the protein precursor of microtubules), were also observed in chimpanzee hepatocytes, as noted above. These same structures were described by DALES (1973) in cells infected with reovirus.

The constellation of disease-associated ultrastructural changes observed in chimpanzee hepatocytes was confined to the cytoplasm, and, as noted, bore a striking resemblance to ultrastructural alterations found in other kinds of cells infected with plant, insect vector, or animal viruses. The results of this early study of the comparative morphology of PT-NANBH-associated changes strongly supported the notion that the major agent (virus) had an RNA genome since all of the above viruses shared the common property of an RNA (and not DNA) genome.

9 Viral Interference

Early studies at the CDC and elsewhere showed that coinfection with HAV and HBV could occur in both humans (HINDMAN et al. 1977) and experimentally infected chimpanzees (DRUCKER et al. 1979) without obvious viral interference. In later studies, two patients with chronic HBV infections and intercurrent episodes of hepatitis A (in contrast to coinfection with HBV and HAV) were found to have depressed or even negative markers of HBV replication, including hepatitis B "e" antigen (HBeAg), HBV-DNA, and DNA polymerase activity with concomitant appearance of leukocyte (α)-interferon during the acute-phase of disease, as reported by DAVIS et al. (1984). Nevertheless, both patients exhibited significantly elevated ALT activity and early seroconversion to anti-HAV, indicating that chronic HBV infection did not interfere with superinfection by HAV.

In sharp contrast to the above findings, BRADLEY et al. (1983), TSIQUAYE et al. (1983), BROTMAN et al. (1983), and LIAW et al. (1982) documented the phenomenon of viral interference in chimpanzees and humans simultaneously or sequentially infected with HBV and the NANBH TFA or HAV and the NANBH TFA (HCV). Studies at CDC by BRADLEY et al. (1983) revealed a profound effect of persistent NANBH infection on superinfection by HAV. Neither of two NANBH-infected chimpanzees (both with persistent viremia and persistently or intermittently elevated ALT activity) developed additional or increased elevations in ALT activity when challenged with proven infectious HAV. In addition, both challenged animals demonstrated a delayed anti-HAV antibody response (28 and 43 days after inoculation) when compared to two control chimpanzees (14 days after inoculation) who had received the identical inoculum. Furthermore, neither superinfected chimpanzee was shown to have detectable HAV antigen by an immunofluorescent antibody assay (FA) in acute-phase liver biopsy specimens; all daily stool specimens, with one possible exception in one animal, were also negative for HAV antigen when tested by a sensitive RIA. Bradley et al. (unpublished data) also found that chimpanzees with biochemically and electron microscopically resolved

PT-NANBH could also interfere with HAV infection. The latter finding suggested that even presumed low-level replication of the NANBH TFA (HCV) was sufficient to interfere with superinfection by HAV. With regard to hepatitis B, BRADLEY et al. (1983) and TSIQUAYE et al. (1983) also showed that acute PT-NANBH infection in HBsAg carrier chimpanzees depressed the replication of HBV as judged by the decrease in surface antigen titer and serum DNA polymerase activity during the acute-phase of NANBH. BROTMAN et al. (1983) and Dolana et al. (1983, unpublished studies) showed that coinfection of chimpanzees with HBV and the NANBH TFA also delayed, moderated, or obviated the appearance of serologic markers of HBV infection. These studies showed that the NANBH TFA was a "dominant" agent and further supported the growing (if not already established) consensus that PT-NANBH was, indeed, caused by a virus.

10 Physicochemical Properties of the Major PT-NANBH Agent

Although numerous and persistent attempts were made at CDC and in other laboratories to visualize the virus of PT-NANBH, it became readily apparent by 1980–1981 that the titer of virus in most sera, plasmas, and liver/liver preparations was far too low to permit detection by electron microscopy or immune electron microscopy(IEM). In fact, the titers of most proven-infectious materials were found to be less than 1×10^3CID/ml as summarized by BRADLEY (1984). Since direct electron microscopy detection of virus particles generally requires particle concentrations on the order of $1 \times 10^{8-10}$/ml, there was little realistic hope of visualizing the presumed viral agent of PT-NANBH. Furthermore, even the use of a more sensitive technique, IEM, still required virus particle concentrations of approximately $1 \times 10^{6-7}$/ml, far above the majority of titers reported for proven-infectious inocula. As a consequence, routine examination of known infectious plasmas and liver materials never revealed the presence of virus or VLPs that could be reproducibly detected. The absence of any information on the morphology or properties of the major agent of PT-NANBH prompted BRADLEY et al. at CDC to consider, as early as 1980–1981, alternative approaches to the characterization of the causative agent(s). As noted earlier, BRADLEY et al. (1983) defined the possible existence of two distinct agents of PT-NANBH by noting their differential sensitivity to treatment with chloroform, a lipid solvent. Since it was obvious from all previous reports that there was one major agent of PT-NANBH, as judged by the consistent appearance in chimpanzees of the ultrastructural alterations described above, a decision was made at CDC to further define the properties of the PT-NANBH TFA (HCV). Furthermore, FEINSTONE et al. (1983) also reported that the TFA (as well as HBV) could be inactivated by treatment with chloroform, a finding that was consistent with the notion that the agent was a lipid-containing or enveloped virus. The combined findings by BRADLEY et al. and FEINSTONE et al., that the TFA was chloroform-sensitive, mitigated against any notion that the agent

(virus) was either a viroid or a scrapie- or prion-like agent, since the latter agents were reported by PRUSINER et al. (1984) and MERZ et al. (1983) to be proteinaceous in nature. Since only certain families of viruses, both DNA and RNA, contain viruses with essential lipid, particle-sizing studies were initiated at CDC in 1983 in order to eliminate several possible families of viruses that contained lipid and exceeded a pre-determined diameter. Controlled pore (polycarbonate membrane) filters were used at CDC to sequentially filter pelleted plasma preparations through a final pore size of 80nm (BRADLEY et al. 1985a). Chronic-phase chimpanzee plasma was diluted 1:6 in TENB, pH 8.0 buffer (TENB: 0.05M Tris, 0.001M EDTA, 0.1M NaCl) and centrifuged at 120,000 × *g* for 5.0h at 20°C to pellet the TFA. The pelleting procedure assumed the NANBH TFA was a virus with a sedimentation coefficient of approximately 150S. The pellet was resuspended in TENB buffer and successively passed through 450-, 200-, and 80-nm filters. The final filtrate was used to inoculate a naive (never-before-used) chimpanzee. The latter animal developed elevated ALT activity 38 days after inoculation with a peak value at day 65; ultrastructural alterations indicative of infection with the TFA agent were also detected by EM in hepatocyte cytoplasm (Fig. 2). This disease profile was typical for many of the chimpanzees infected with the TFA at the CDC. In a later study, HE et al. (1987) also reported that the TFA agent would pass an 80-nm filter and partially pass a 50-nm (but not 30-nm) filter suggesting that the agent had a diameter of between 40 and 60nm, consistent with the earlier hypothesis by BRADLEY et al. (1985a), that the major etiologic agent of PT-NANBH was a small, enveloped virus. Additional studies conducted at CDC and elsewhere ruled out the possibility

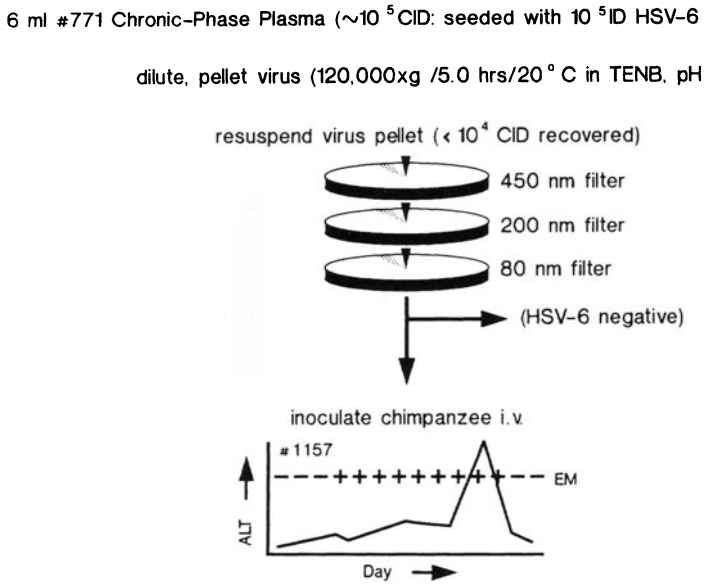

Fig. 2. Pelleting and microfiltration of the post-transfusion non-A, non-B hepatitis (PT-NANBH; HCV) agent

that the TFA was either a hepadnavirus or a delta-like virus (i.e., low or no significant nucleotide sequence homology, as revealed by carefully controlled hybridization assays). By a process of systematic virus-class "elimination" combined with the newly defined physicochemical properties of the TFA, BRADLEY further concluded that the agent was most like a small, enveloped RNA virus (in particular a flavi- or flavi-like virus) based in part on the results of an earlier comparative analysis of ultrastructural alterations induced in cells by well known flaviviruses. Figure 3 summarizes the process used at CDC to predict the most likely virus-candidate (i.e., family of virus) of the TFA (HCV) based on a combination of established physicochemical properties (not including its buoyant density in sucrose) and the findings of a comparative analysis of TFA-induced ultrastructural alterations observed in infected chimpanzee hepatocytes. The process was methodical and time-consuming; it also required the most expeditious, yet sparing, use of chimpanzees, a limited and highly expensive primate model for PT-NANBH research.

Other studies were initiated at CDC in 1987 to determine the buoyant density of the PT-NANBH TFA (HCV) in sucrose using standard isopycnic banding methods. A total of five chimpanzees were used in two different study phases (conducted over a period of approximately 3 years) to determine, by back-titration of gradient fraction pools, the distribution and amount of infectious virus (in CID/gradient fraction pool) throughout the sucrose gradient. BRADLEY et al. (1991) reported that one ml of a chronic-phase chimpanzee plasma (from animal #910) that contained 1×10^6CID could be completely recovered in a gradient fraction pool that encompassed fractions with buoyant densities of 1.09–1.11g/cm^3 (Fig. 4). This buoyant density (combined with the previous findings described above and the concurrent knowledge that the TFA was itself HCV and that it shared genomic properties of both flavi- and pestiviruses) was consistent with the much earlier

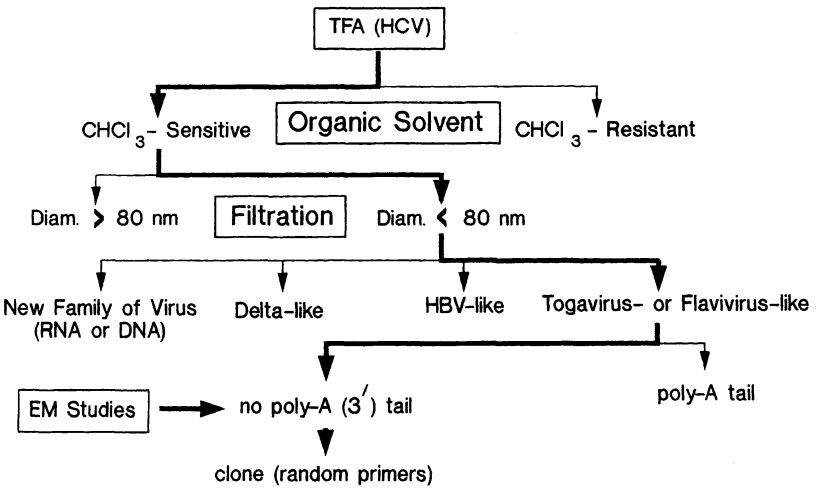

Fig. 3. Algorithm used at CDC to predict family of virus

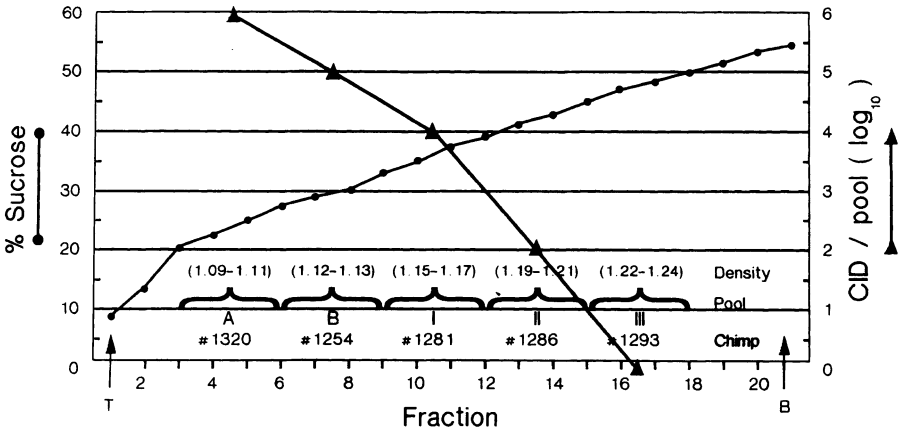

Fig. 4. Buoyant density determination of HCV (tubule forming agent, TFA); back-titration studies in chimpanzees

hypothesis that the TFA was a Togavirus (meaning either flavivirus or pestivirus). MIYAMOTO et al. (1992) confirmed the above findings when they found that HCV in human donor plasma also banded at a low density (i.e., 1.08g/cm^3). Both BRADLEY et al. (1991) and MIYAMOTO et al. (1992) acknowledged the fact that determination of this critical physicochemical property of HCV provided the means for the development of efficient purification procedures for HCV contained in large volumes of plasma (or tissue culture fluids/lysates).

11 Development of High-Titered Liver and Large-Volume Plasma Pools

The development of high-titered plasma pools and liver tissue was essential to successful molecular cloning of the TFA (HCV). Chimpanzee studies were initiated at the CDC in 1979 specifically for the purpose of generating high-titered materials that would enable: (1) visualization of the virus by electron microscopy or IEM, (2) purification of the virus by accepted or revised procedures according to virus properties, (3) development of polyspecific or monoclonal antibodies, (4) attempts to propagate the virus in tissue culture, and (5) molecular cloning of the viral genome. The latter purpose became a more specific focus of our studies once it became apparent in the early 1980s that few, if any, sources available at the time had the requisite titer and volume for successful molecular cloning of the viral genome. Several different approaches were used to achieve the above goal and included: (1) serial passage of the virus in chimpanzees to "adapt" the virus to the host (with consequent increase of virus in liver, as previously reported for HAV by

BRADLEY et al. 1984; HCV passage studies were initiated in the early 1980s); (2) immunosuppression of the host chimpanzee to increase the severity of disease (and, hopefully, titer of the TFA; study reported by BRADLEY et al. 1984); (3) titration of selected acute-phase and chronic-phase livers from chimpanzees; and (4) titration of highly selected pools of plasma from persistently (chronically) infected chimpanzees. A detailed summary of the chimpanzee studies that primarily involved the use of liver can be found in BRADLEY (1990).

Cloning studies (of the PT-NANBH TFA) were conducted in close collaboration with Chiron Corporation. From the beginning it was apparent that the relative proportion of nonviral nucleic acid found in even high-titered liver (approximately 1×10^7CID/g of tissue) would make cloning difficult and underscored the need to develop other sources of virus that would be less complex. Although other investigators found that acute-phase (and even one chronic-phase) plasma generally had TFA (HCV) titers equal to or less than 1×10^3CID/ml, it was still apparent that, if achievable, a large-volume, high-titer plasma or plasma pool would be superior to liver as a source of virus for molecular cloning studies. The minimum acceptable infectivity titer of plasma (plasma pool) for cloning of the TFA genome was calculated to be 1×10^5CID/ml, assuming that there were ten defective particles for every infectious particle (1CID). Previous studies of the course of disease in chimpanzees housed at the CDC showed that chronic-phase plasma might be a richer source of virus than acute-phase plasma, since disease in many chimpanzees appeared to worsen with time after initial inoculation. As a result, several chimpanzees were intensively followed for periods of time up to 11 years in order to prospectively collect large volumes of plasma for characterization of the virus and (later) molecular cloning of the viral genome. Two chimpanzees, namely Don (#771) and Rodney (#910), were most carefully studied (Fig. 1), including determination of their ALT activity at relatively frequent intervals of time, and examination of serial liver biopsy specimens from each animal for histologic and EM evidence of disease severity that would be indicative of increased viral replication in liver. Based on the hypothesis (by this author) that the TFA was cytopathic and that the highest titers of virus would be found in plasma during periods of exacerbated, chronic-phase disease, units with the highest levels of ALT activity (relative to other reposited and catalogued units of plasma) were pooled and titered in chimpanzees. It should be noted that collection of chronic-phase plasma units was initiated at the CDC in 1979. A large pool of plasma was originally generated from units of plasma collected from chimpanzee #771 over a 4-year period after the acute-phase of disease. The original criteria for the initiation of a given plasmapheresis were: (1) a periodic episode of elevated ALT activity, and (2) EM or histologic evidence of increased viral replication in liver. Units of plasma collected at the peaks of recrudescent disease (including those collected well beyond 4years after inoculation) were selected, pooled, aliquoted, catalogued, and stored at –70°C until used. Inoculation of two chimpanzees with either of two different plasma pools showed that the titers of the TFA were 1×10^4CID/ml (first pool), and $1 \times$ 105CID/ml (second pool). Units of plasma were also prospectively collected from chimpanzee #910 using the above-described process. One pool con-

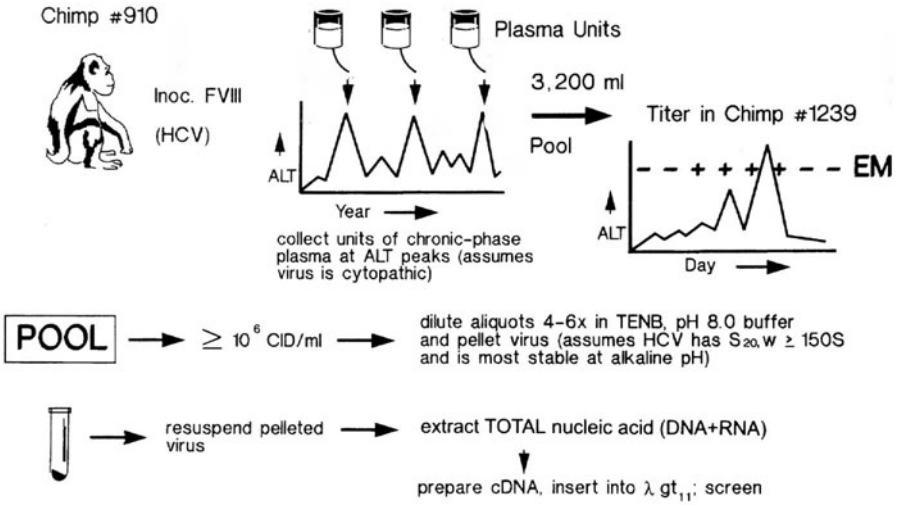

Fig. 5. Cloning of hepatitis C virus (HCV): production of large-volume, high-titer plasma pools

sisting of approximately 3,200ml was shown to have a titer of at least 1×10^6CID/ml; this pool contained all of the units of prospectively-collected plasma that were determined to have the highest levels of (chronic-phase) ALT activity (Fig. 5). A second pool of plasma consisting of units with a statistically lower (mean) ALT value was found to have a titer of 1×10^5CID/ml. The latter findings reaffirmed the hypothesis (and prediction) that large-volume, high-titered plasma pools could, in fact, be generated by a process that involved the collection of units of chronic-phase plasma from chimpanzees that had demonstrated the most severe disease, as indicated by relative values of ALT activity, EM evidence of worsening disease, and histologic evidence (in some instances) of more severe disease. The major criterion for the selection of any unit of plasma was its ALT value. It is of interest to note that liver tissue obtained from chimpanzee #910 during one period of exacerbated disease was shown to have a titer of 1×10^7CID/g.

12 Discussion

The isolation, characterization, and eventual molecular cloning of HCV have made it possible to: (1) develop blood donor or patient screening methods (for virus-specific antibodies and/or nucleic acid, i.e., RNA); (2) attempt propagation of HCV in tissue culture; (3) design and develop strategies for the production of recombinant vaccines; (4) devise rational approaches to the construction of inhibitors of HCV replication (such as synthetic organic molecules for virally encoded proteins, i.e., proteases, helicase, RNA-dependent RNA polymerase, or sequence-specific

oligonucleotides that could bind to the HCV genome). Although no tissue culture system has yet been developed that produces desirable quantities of HCV in vitro, the basic properties of HCV, including its buoyant density in sucrose, are now known and should facilitate the development of purification methods for HCV derived from tissue culture extracts. The latter methods, in all likelihood, will depend on some form of a quantitative test for viral RNA.

Nearly 14,000 articles have been published on one or more aspects of hepatitis C or HCV during the past decade. This explosive growth of information has provided the foundation for further exciting findings and developments in viral hepatitis C and reveals the level of interest in a field that was once considered to be moribund.

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