

Part I

Basic principles

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Background

An active female business executive, aged 39 years and a mother of three young children, visited her family doctor for an annual check-up. She had never been seriously ill. A few days after her visit, she received a phone call from the doctor's office, informing her that her liver enzymes were slightly elevated. Since this result may reflect an underlying problem in the liver, she was asked to come back in for more tests. She tested positive for antibodies against a recently discovered hepatitis virus that establishes a chronic, asymptomatic infection in most people, which is often accompanied by an underlying liver disease that is serious and often progresses toward cirrhosis. Cirrhosis, or end-stage liver disease, is accompanied by considerable morbidity and mortality. She left the doctor's office with the knowledge that within 10–15 years the virus infection could destroy her liver. She was treated with antiviral therapy, which was expensive, had considerable side effects, and was not effective beyond the period of treatment. She was then informed that her best chances for survival may eventually be a liver transplant. This is the clinical face that is often seen in chronic hepatitis C virus (HCV) infection. This book will present the basic features of the virus and associated diseases as well as discuss present and future treatment options, in the hope that it will stimulate further research and provide a brighter future for those infected with HCV.

1.1 The enigma of non-A, non-B hepatitis

The search for HCV is a great detective story that had to await the definition of hepatitis as an infectious disease entity. In addition, its discovery came about only after several other agents responsible for post-transfusion hepatitis (PTH) were identified and yet a considerable amount of PTH still existed without a known cause. During the 1940s, for example, it was realized that hepatitis could be transmitted by blood transfusion (Beeson, 1943) and that at least two forms of hepatitis existed (MacCallum, 1947). They were termed “infectious” or type A hepatitis and “serum” or type B hepatitis. Further characterization of the

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responsible agents showed that hepatitis A virus (HAV) (Feinstone, Kapilian & Purcell, 1973) was transmitted by ingestion of contaminated food and drink, while hepatitis B virus (HBV) (Blumberg, Alter & Visnich, 1965) was a major source of PTH. Moreover, HAV was associated with a single bout of acute hepatitis, while HBV was associated with both acute and chronic liver disease (CLD). There was great hope that the development of specific serological tests for HAV and HBV would eliminate most, if not all, PTH, since the rates of PTH in the United States, for example, had exceeded 20% during the 1960s (Alter et al., 1972). However, once screening of blood became widespread, it became apparent that up to 10% of individuals who received blood transfusions in the United States during the late 1970s still developed hepatitis (Feinstone et al., 1975; Knodell, Conrad & Dienstag, 1975; Seeff et al., 1975; Maugh, 1980). As a result, the term non-A, non-B hepatitis (NANBH) was created to account for the presumed existence of one or more infectious agents responsible for inflammatory liver disease that lacked serological markers of HAV and HBV (Prince et al., 1974; Alter et al., 1975; Feinstone et al., 1975). Hence, until the discovery of HCV (Choo et al., 1989), the diagnosis of NANBH was one of exclusion. In addition to the lack of HAV and HBV serology, the diagnosis of NANBH was also based upon differences in clinical presentation (Ch. 8) and epidemiological characteristics (Ch. 3) of NANBH compared with HAV and HBV (Krugman & Gocke, 1978) (Table 1.1).

It soon became apparent that a diagnosis of exclusion included a large number of possibilities that could account for NANBH. First of all, it was not known whether the etiology of endemic, sporadic, post-transfusion, or epidemic NANBH was linked to one or multiple agents (Alter, 1988). In 1984, two groups published provocative evidence that a NANBH agent might be a retrovirus (Prince et al., 1984; Seto et al., 1984). This was based on the findings that sera from patients with NANBH were consistently positive for reverse transcriptase (RT) activity, that retrovirus-like particles could be isolated from chimpanzee liver cell cultures innoculated with a putative NANBH agent, and that experimental inoculation of candidate sera into chimpanzees resulted in the development of NANBH. Retrovirus-like particles were also observed in the cytoplasm of hepatocytes from patients with acute or chronic NANB PTH (Iwarson et al., 1985). However, RT activity was low, had no direct relationship to the severity of liver disease, and was observed in patients receiving transfusions who did not develop PTH. Independent observations also failed to detect RT activity in sera obtained from multiple, pedigreed, NANB PTH cases, or in sera obtained from chimpanzees inoculated with a NANB agent (Itoh et al., 1986; Kahn and Hollinger, 1986).

There was also evidence presented that a NANB agent might be related to HBV. For example, there were antigen and corresponding antibody specificities in NANB sera that crossreacted with the nucleocapsid (core; anti-HBC) and “e”

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Table 1.1. Comparison of infections with hepatitis A or B virus and non-A non-B hepatitis

| Characteristic | Hepatitis A | Hepatitis B | Non-A, Non-B hepatitis |
|--|---------------------------|--------------------|------------------------|
| <i>Presentation</i> | | | |
| Average incubation period (days (range)) | ~25 (15–50) | 70 (50–180) | 40–60 (15–180) |
| Onset | Acute | Usually chronic | Usually chronic |
| Age affected | Children and young adults | All ages | All ages |
| <i>Symptoms</i> | | | |
| Arthralgias | Uncommon | Common | Uncommon |
| Nausea and vomiting | Common | Common | Common |
| Fever | Common | Uncommon | Uncommon |
| Jaundice | Uncommon | Common | Uncommon |
| <i>Laboratory values</i> | | | |
| Alanine/ aspartate aminotransferase elevation | Transient | Prolonged | Prolonged |
| Duration of alanine aminotransferase elevation | 1–3 weeks | 1–8 or more months | 1–8 or more months |
| Anti-hepatitis A antibodies | Positive | Negative | Negative |
| Hepatitis B surface antigen (HBsAg) | Negative | Positive | Negative |
| <i>Disease association</i> | | | |
| Acute hepatitis | Yes | Yes | Yes |
| Chronic hepatitis | No | Yes | Yes |
| Cirrhosis hepatocellular carcinoma | No | Yes | Yes |
| <i>Transmission</i> | | | |
| Percutaneous | Rare | Common | Common |
| Oral | Common | Rare | ? |
| Virus in feces | Positive | Negative | Negative |
| Sexual | Rare | Common | Rare |
| Perinatal | None | Common | Rare |

Modified from Krugman and Gocke (1978) with permission from W. B. Saunders, and from Seeff (1992) with permission from Williams & Wilkins.

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antigens of HBV (Shirachi et al., 1978; Vitvitski, Trepo & Hantz, 1980; Trepo et al., 1983). These sera were also positive for virus-associated DNA polymerase activity (Hantz, Vitvitski & Trepo, 1980), which is also characteristic of HBV. In addition, monoclonal antibodies raised against HBV surface antigen (HBsAg) detected reactive material in both serum and liver samples from patients with NANBH who were HBsAg negative by conventional assays (Wands et al., 1982). When such sera were inoculated into HBV-susceptible or HBV-immune chimpanzees, several animals developed antigen reactivity in serum for this atypical antibody and also developed NANBH (Wands et al., 1986). Although interesting, the contribution of such HBV-like NANB agent(s) to PTH remains to be demonstrated.

Independent studies have demonstrated virus-like particles with morphology and density characteristics of picornaviruses, such as HAV, in the blood and liver samples from patients (Yoshizawa et al., 1980) and chimpanzees (Bradley et al., 1979) with documented NANBH. However, whether such particles are responsible for blood-borne or water-borne NANBH remains to be seen. Since the discovery of HCV (Choo et al., 1989), other blood-borne viruses, such as hepatitis G virus (Simons et al., 1995a,b; Linnen et al., 1996) and transfusion-transmitted virus (Nishizawa et al., 1997), have been discovered, but their relationship to PTH also remains to be firmly established. Greater success was met with the identification of the hepatitis E virus as a major agent responsible for epidemic, water-borne NANBH, which is a major problem in southern Asia and northern Africa (Wong et al., 1980). There is also evidence that several herpes viruses, such as Epstein-Barr and human cytomegalovirus, are also associated with NANBH in some settings (Prince, 1983). Although the detection of anti-HCV has greatly reduced the incidence of PTH during the 1990s, the documentation of non-A, non-B, non-C hepatitis (Alter and Bradley, 1995) has stimulated the search for new viral candidates, which are now regularly described at hepatitis symposia throughout the world.

1.2 The chimpanzee as a model of non-A non-B infection

The transmission and propagation of NANB agent(s) in chimpanzees during the 1970s (Alter et al., 1978; Hollinger et al., 1978; Tabor et al., 1978, 1979; Tsiquaye & Zuckerman, 1979) represented a major breakthrough in the eventual identification and characterization of HCV. These studies demonstrated that NANBH was really caused by one or more infectious agents, that the agent(s) could be transmitted from chronically infected people, and that chronic infection and mild hepatitis could be shown to develop in an animal model. The agent(s) were then shown to be serially transmitted in chimpanzees using serum from animals obtained at the time of acute or chronic hepatitis (Tabor et al., 1979). Additional

7 The chimpanzee as a model

studies in which outbreaks of NANBH from contaminated factor VIII (Bradley et al., 1979) or factor IX (Wyke et al., 1979) concentrates, used to treat hemophiliacs, also induced NANBH in chimpanzees, firmly establishing the chimpanzee as a reliable animal model of NANB infection. Hepatocytes isolated and cultured from infected chimpanzees during the acute phase of NANBH infection continued to replicate virus for several weeks, as indicated by the ability of tissue culture supernatants to infect susceptible animals (Jacob et al., 1990). These chimpanzees developed elevated alanine aminotransferase (ALT) levels in serum (reflecting the release of an intracellular enzyme into the blood from damaged hepatocytes), inflammatory liver disease, and the appearance of cyto-plasmic tubule structures commonly observed in the livers of those with NANBH (Jacob et al., 1990). Further characterization showed that these tubular structures derived from the proliferation of membranes associated with the smooth endo-plasmic reticulum (ER) (Shimizu et al., 1979). These ultrastructural changes, however, were not specific to the agent(s) responsible for NANBH, since they were also observed in different cell types infected with a variety of RNA viruses, including strawberry latent ringspot virus (a picornavirus) (Roberts & Harrison, 1970), St Louis encephalitis virus (a flavivirus) (Harrison, Murphy & Gardner, 1982), human immunodeficiency virus (HIV) (Sidhu et al., 1983), hepatitis delta virus (Canese et al., 1984), and poliovirus (Dales et al., 1965). These findings were consistent with the hypothesis that a NANB agent may have a RNA genome. Further work supported the existence of at least two agents associated with NANBH (Shimizu et al., 1979) and showed that one of these agents was sensitive to pretreatment with chloroform (Bradley et al., 1983; Feinstone et al., 1983), suggesting the presence of an envelope. Infection with the chloroform-sensitive virus was also associated with the development of cytoplasmic tubular structures. To determine the appropriate size of the NANB agent(s), infectious samples were passed through membranes with different pore sizes, and the eluants used for chimpanzee challenge studies. Using this approach, the size of this putative NANB agent was estimated to be 30–60 nm, with a 37 nm inner core (Bradley et al., 1985; He et al., 1987; Jacob et al., 1990), which was consistent with it being a toga- or flavivirus. The buoyant density of the infectious agent, 1.08–1.11 g/ml, in sucrose gradients was also consistent with a togavirus (Bradley et al., 1991; Miyamoto et al., 1992). Although a chloroform-resistant NANB agent was also reported (Bradley et al., 1983), the enveloped NANB agent had many of the characteristics that were later shown to be associated with HCV.

The presence of one or more NANB agents is further suggested by the finding of multiple episodes of NANBH in patients with biopsy-proven hepatitis (Mosley et al., 1977) and in hemophiliacs given multiple doses of factor VIII (Hruby and Schauf, 1978). At least two agents were also suggested by the existence of PTH of

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both short- and long-term incubation (Craske & Spooner, 1978; Norkrans et al., 1980). The appearance and transmission of tubular structures in the livers of chimpanzees that were serially infected with some but not other sources of infectious material also suggested the existence of at least two NANB agents (Shimizu et al., 1979). In addition, immunity appeared to develop against agent(s) from a single source, but cross-protection against putative NANB agents in different inocula was not consistently observed (Tsiquaye & Zuckerman, 1979; Bradley et al., 1980; Feinstone et al., 1981; Yoshizawa et al., 1981), since in some challenges, chimpanzees would experience a new bout of hepatitis. Following the discovery of HCV, it became evident that these results reflected the genetic heterogeneity of the virus (Chs. 3 and 14). Independent observations also showed two separate episodes of NANBH in chimpanzees inoculated with chloroform-resistant followed by chloroform-sensitive strains (Bradley et al., 1983). Consequently, it was not clear how many NANBH agents were present.

The difficulty in identifying agents associated with NANBH was further highlighted by the failure to isolate any infectious virus particle from human or chimpanzee sera. In addition, many laboratories reported “unique” antigen–antibody systems that appeared to be associated with NANBH (Kabiri, Tabor & Gerety, 1979; Shirachi et al., 1978; Spertini & Frei, 1982; Prince, 1983; Jacob et al., 1990). In one promising approach, lymphocytes obtained from chimpanzees with NANBH were transformed by Epstein–Barr virus, and tissue culture supernatants were used to stain for NANB antigens in infected liver (Shimizu et al., 1985). Although two antibodies stained infected but not uninfected liver, their cross-reactivity with delta antigen encoded by hepatitis delta virus (Shimizu et al., 1986) suggested that either the NANB agent was delta-like or that the reactions were nonspecific. This work, and others cited above, have not been independently confirmed in the literature, nor did any of them correctly identify a NANBH agent in samples of pedigreed human sera from infected patients, from normal individuals, or from chimpanzees known to be infected with a NANBH virus. Attempts to find antigens encoded by a NANB agent in the liver and serum were also disappointing, implying that their levels of expression may be too low for detection. Likewise, electron microscopic (EM) examination of serum and liver samples revealed a variety of virus-like particles (Bradley et al., 1979; Yoshizawa et al., 1980), but the relationship between any of these particles and the agents responsible for NANBH remained elusive. The reason for all of these difficulties, it turns out, was the very low titer of virus particles in most infectious sera from patients or experimentally infected chimpanzees, which was below the levels of detectability of most methodologies used at the time to detect markers of the NANB agent(s). This was supported by the fact that most sera had titers less than 1×10^3 chimpanzee infectious doses (Bradley et al., 1979). Consequently, it was realized that much

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more sensitive assays would have to be developed if reproducible detection of the NANB agent(s) was going to succeed. This early work in chimpanzees did show that a major agent for NANBH consisted of a chloroform-sensitive agent that was less than 80 nm in diameter and was associated with acute and chronic NANBH. Infections could also be shown to result in characteristic ultrastructural changes in the liver. Together, these results suggest that a major etiological agent of NANBH was a small, enveloped, RNA virus that probably replicated in the cytoplasm of infected hepatocytes.

1.3 Surrogate markers

The difficulties in establishing reproducible markers of HCV infection during the 1970s and 1980s (Section 1.1 and 1.2) prompted studies aimed at the evaluation of surrogate markers in the hope that such an effort would further reduce the incidence of NANB PTH. Exclusion of donor blood with ALT levels (ALT levels throughout refer to serum levels) greater than two standard deviations above the logarithmic mean prevented about 30% of NANB PTH (Alter et al., 1981; Aach et al., 1991). In attempts to reduce the incidence of NANB PTH further, antibodies against HBV core antigen (anti-HBc), which had been used to detect low levels of HBV in a PTH setting (Hoofnagle et al., 1973, 1978), were evaluated. For example, a large transfusion-related study of 1151 patients showed that 18.7% of those transfused with a single unit of anti-HBc-positive blood developed NANBH, while this was observed in only 7.2% of individuals receiving a single transfusion of anti-HBc-negative blood (Stevens et al., 1984). Similar results were obtained in independent studies (Koziol et al., 1986; Sugg, Schenzle & Hess, 1988), although still other studies showed no benefit of anti-HBc screening (Hanson & Polesky, 1987; Hoyos et al., 1989). Among the studies with positive results, anti-HBc screening prevented another 20–30% of NANB PTH. While the significance of isolated anti-HBc detection in PTH remains to be fully elucidated, it is clear that some anti-HBc-positive blood is infectious (Debure et al., 1988), and that isolated anti-HBc may reflect either very low levels of HBV or an HBV-like NANB agent that triggers a cross-reactive anti-HBc response (Lai et al., 1990a). While the combined use of ALT and anti-HBc as surrogate markers significantly reduced NANB PTH, the utility of these tests was limited by problems with standardization of “normal” ALT values and the nonspecificity of anti-HBc testing (Kroes, Quint & Heijtkink, 1991). Use of these tests resulted in the rejection of a significant proportion of the donor blood supply (Kline et al., 1987). Even so, surrogate markers provided a temporary solution to the problem of partially eliminating NANB PTH.

With the discovery of HCV, and the establishment of first-generation

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serological tests, a number of studies were conducted to examine whether it was still worthwhile to continue surrogate marker testing. An early study in the Netherlands showed that surrogate and first-generation anti-HCV tests were roughly comparable (van der Poel et al., 1990b), implying that specific anti-HCV testing may not be needed in countries that already did surrogate testing (van der Poel et al., 1990a). However, the finding that many anti-HCV-positive blood donors had normal ALT (Alter et al., 1989a; Katayama et al., 1990; van der Poel, 1999) suggested that surrogate testing missed many HCV-infected individuals. In addition, surrogate testing using ALT excluded 2–4% of donated blood units whereas only 0.2–0.4% of donated blood was excluded by anti-HCV testing, making it obvious that the specific antibody assay was a better test (Zuck, Sherwood & Bove, 1987; van der Poel et al., 1990b; Watanabe et al., 1990). Independent studies showed no difference in the incidence of NANB PTH in transfusion recipients receiving blood that had been screened for ALT and anti-HBc compared with those receiving unscreened blood (Esteban et al., 1990; Blajchman, Bull & Feinman, 1995). Further work showed that three cases of HCV “window” period donations (i.e., before the appearance of anti-HCV) out of a million transfused units could have been prevented by continuing ALT testing and demonstrated that continued ALT testing in the light of anti-HCV screening was of little value (Busch et al., 1995). Although it was possible that continued screening for anti-HBc would eliminate HBsAg-negative HBV mutants (Carman et al., 1990; Feitelson et al., 1995), it would also exclude 1–4% of donors in western countries (Sugg et al., 1988; Hetland et al., 1990; Koziol et al., 1986) and the great majority of donors in HBV endemic countries, such as Taiwan (Lin-Chu et al., 1990). A number of studies also showed no correlation between anti-HBc and anti-HCV positivity (Hetland et al., 1990; Lin-Chu et al., 1990; van der Poel et al., 1990a). Consequently, once anti-HCV testing was validated, surrogate testing was largely dropped as screening against NANB PTH.

1.4 The discovery of hepatitis C virus

The finding that most infectious sera had very low titers of virus (Section 1.2) meant that large volumes of high-titered serum or plasma samples would have to be created if attempts at cloning the virus genome were going to succeed. Accordingly, HCV was passaged from chimpanzee to chimpanzee so that the virus might “adapt” to its new host and grow to higher levels, as was done with HAV. Other chimpanzees were immunosuppressed in the hope that this would result in higher levels of virus (Bradley et al., 1984a,b). Eventually large pools of high-titered sera ($> 1 \times 10^5$ chimpanzee infectious doses/ml) were collected and used as starting material for the attempted cloning and further characterization of a major agent responsible for NANB PTH (reviewed in Bradley, 2000).

11 The detection of viral antibodies

In 1989, a group led by investigators at Chiron Corporation described a molecular clone obtained from a chimpanzee infected with the tubule-forming agent associated with NANBH that had been well characterized in animal transmission studies (Choo et al., 1989). Accordingly, plasma with a high concentration of the NANB agent(s) was centrifuged to pellet the putative virus(es) and the pellets extracted for total nucleic acid, since it was not known whether the genome of the agent(s) consisted of DNA or RNA. The extracted material was denatured, subjected to complementary DNA (cDNA) synthesis with random primers, and the products cloned into a lambda gt11 expression vector. In this vector, protein fragments encoded by the putative NANB agent were expressed as a fusion protein with β -galactosidase. The library of cloned inserts, which was then screened with a serum from a patient with NANBH, detected an epitope made by a 155 base pair (bp) clone (designated 5-1-1) that did not hybridize to human DNA or chimpanzee DNA from animals with NANBH. These results implied that the NANB agent(s) did not have a DNA genome. Further characterization showed that the 5-1-1-encoded epitope was recognized by sera collected from many patients with NANBH. This probe, as well as a larger overlapping clone (353 bp long), hybridized with RNA from the liver and sera of infected chimpanzees, but not with that from uninfected animals, suggesting that the NANB agent(s) had an RNA genome. Further characterization showed that the RNA detected was single stranded, was about 9.6 kilobases (kb) in length, had plus strand polarity, and had a genome organization similar to that of flaviviruses (Houghton et al., 1991; Weiner et al., 1991). This agent was referred to as HCV (Choo et al., 1989; Kuo et al., 1989), and its discovery was the culmination of many painstaking years of chimpanzee transmission studies (Section 1.2). The difficulty in identifying HCV earlier was a consequence of its usually low titers in the blood, the fact that HCV is genetically heterogeneous (Section 3.2), and its natural history, which often has a lengthy asymptomatic phase during the early years of chronic infection (Ch. 4). The low titer made it difficult to detect the genome and virus-encoded proteins by conventional methods. The sequence heterogeneity of HCV-encoded proteins made it difficult to detect them reproducibly in different infections. Finally, the asymptomatic course of early chronic infections provided few pathological landmarks to follow in the development of chronic hepatitis (Ch. 5).

1.5 The detection of viral antibodies

Following the discovery of clone 5-1-1, it was used to probe for other overlapping clones in the lambda gt11 expression library. Reconstruction of the clone 5-1-1 sequences within overlapping clones resulted in a larger recombinant, referred to as C 100, which was located mostly within the nonstructural (NS) protein 4 (NS4) region of HCV (Fig. 1.1). C 100 was then fused with the gene for human super-