

Transfusion-Associated Non-A, Non-B Hepatitis: The First Decade

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INTRODUCTION

Despite over a decade of intensive investigation, non-A, non-B hepatitis (NANBH) remains an etiologic, diagnostic, and therapeutic dilemma. At issue are the nature of the causative agent, the clinical significance of the disease and the methods for detection, intervention and prevention. This brief review will focus on only selected aspects of this dilemma and concentrate primarily on the known characteristics of the agent, the chronic sequelae of the disease, and emerging methods of viral inactivation.

THE AGENT

Is NANBV a Retrovirus?

Shortly after the 1984 International Hepatitis Symposium, two exciting reports [Seto et al., 1984; Prince et al., 1984] suggested that the NANB agent might be a retrovirus related to the human immunodeficiency virus (HIV). Seto et al. reported the finding of reverse transcriptase (RT) activity in 18 of 18 NANBH sera and only rarely in controls. The observed RT activity was sensitive to RNase and had characteristics that distinguished it from cellular DNA polymerases. The RT banded at a sucrose peak of 1.14 g/ml, and material from this peak caused apparent NANB infection in an experimentally inoculated chimpanzee. Seto and Gerety [1985] also described an approximately 77,000 dalton glycoprotein antigen in the serum of a patient with NANBH that they thought provided further evidence for a link between NANBV and HIV. Antibodies to this antigen reacted with 40% of NANBH sera compared to 3.4% of hepatitis A or hepatitis B sera, reacted with the aforementioned 1.14 g/ml sucrose fraction from NANBH infectious serum, and reacted by ELISA and immunoblot with disrupted HIV. Iwarson has extended these studies in Sweden, and, more recently, Alberti and coworkers in Italy (personal communication) found RT activity in 16 of 23 prospectively followed transfusion-associated NANBH cases and found that the RT activity resided in a band at 1.15 g/ml, was associated with a 70-90 nm retroviruslike particle, and could be biochemically distinguished from cellular and hepatitis B virus (HBV) DNA polymerase. Clinically, however, the RT was of low activity; fluctuated, with no direct relationship to the transaminase value; persisted even in patients who had recovered from their disease; and was also ob-

served in transfused patients who did not develop hepatitis.

Despite these provocative findings, Khan and Hollinger [1986] found no RT activity in serial samples from 11 pedigreed, transfusion-associated NANBH cases or from 11 dialysis patients with clinical NANBH. They also found no RT activity in three chimpanzees that had been infected with a Factor VIII (F VIII) concentrate known to contain the NANB agent. They further found that sera from eight NANBH cases did not react with HIV-infected cell lines and that there was no RT activity or retroviruslike particles in lots of F VIII, Factor IX, and gammaglobulin, all of which had been implicated in NANB transmission; furthermore, none of these implicated materials induced RT activity when inoculated into appropriate cell lines. Overall, Khan and Hollinger found no RT activity in 260 samples from NANBV-infected patients nor in components implicated in NANBH transmission. Itoh et al. [1986] failed to detect RT activity in sera from one NANBV-infected patient and two experimentally infected chimpanzees. Each serum had been demonstrated to transmit NANBH associated with cytoplasmic tubular structures, and each had an infectivity titer greater than 10^2 CID/ml.

In addition to these negative findings, I am aware through personal communications of seven other major laboratories that have been unable to confirm the finding of RT activity in very well pedigreed NANBH sera, including undiluted samples of the prototype H-strain and F-strain inocula. Furthermore, the retroviruslike particles observed by Prince et al. [1984] in liver cell cultures inoculated with H-strain NANBV are now recognized to represent foamy virus contaminants of the liver explant system rather than the NANB agent itself. Additionally, the size characteristics of the tubular forming agent are inconsistent with those of a retrovirus, as discussed below. Overall, it is my opinion that NANBV, at least the predominant tubular forming agent, is not a retrovirus.

Is the NANB Agent a Seronegative Variant of HBV?

Space does not permit a review of the extensive literature suggesting an interrelationship between NANBH and HBV. There have been three primary lines of evidence to suggest such a relationship: 1) Antigen-antibody systems which correspond to the

surface, core, and e antigens of HBV and HBV-like virions with associated DNA polymerase have been described in sera from patients with NANBH [Trepo et al., 1983; Hantz et al., 1980]. 2) Monoclonal antibodies directed against HBsAg have been found to react with both the liver and the serum of some patients diagnosed as having NANBH [Wands et al., 1982]. These sera and tissues were negative for HBsAg when reacted against conventional polyclonal anti-HBs reagents. 3) HBV DNA has been identified in the serum and liver of patients with acute and chronic hepatitis who are negative for HBV markers by conventional assays [Shafritz et al., 1982; Brechot et al., 1985; Charney et al., 1982].

More recently, Wands et al. [1986] inoculated chimpanzees with sera that were negative for HBsAg by polyclonal assays but positive by monoclonal assay and also positive for HBV DNA. Two HBV-susceptible and two HBV-immune chimpanzees, after a long incubation period, developed reactivity with the monoclonal, but not with the polyclonal, anti-HBs and also demonstrated positive reactions for HBV DNA; some animals developed elevated serum transaminase. Since this occurred in HBV-immune as well as HBV-susceptible chimpanzees, it was postulated that the infectious agent was antigenically distinct from HBV but shared epitopes that could react with the monoclonal anti-HBs. The question relevant to this discussion is the extent to which these proposed HBV variants account for cases previously diagnosed as NANBH. This question has two elements. First, in what proportion of classic transfusion-associated NANB cases would one find serologic or genomic evidence for the HBV variant? and, second, if such evidence exists, does that imply that the variant was responsible for the observed hepatitis or might these markers represent a residuum of previous HBV infection? There has been considerable divergence of opinion regarding these issues [Feinstone et al., 1984]. The first aspect can be directly addressed by instituting a multicenter study that under code utilizes the best pedigreed NANBH sera, the optimal HBsAg monoclonal antibody, and the most specific hybridization technique. I cannot estimate the proportion of unequivocal NANBH cases that might then be classified as potentially related to an HBV variant, but I suspect that the number is low, since hepatitis B and NANBH are clinically, immunologically, and biologically distinct diseases. This is further evidenced by the finding of Fields et al. [1983] that nucleic acid derived from an NANB-infected chimp liver having an infectivity titer of 10^7 did not hybridize with HBV DNA.

Nonetheless, some cases of NANBH diagnosed by serologic exclusion may be due to this proposed HBV variant. It is essential to establish the contribution of HBV to the spectrum of diseases now classified as NANBH. This could best be accomplished in a controlled, blinded study in which the sera to be tested and the techniques to be utilized are predetermined in a collaborative protocol.

What Are the Physicochemical Properties of the NANB Agent?

Feinstone et al. [1983] and Bradley et al. [1983] have demonstrated the chloroform sensitivity of the cyto-

plasmic tubular-forming NANB agent, indicative of essential lipid in its structure. Conversely, the chloroform resistance of the nontubular-forming agent has been demonstrated. Bradley et al. [1985] have also shown that the tubular-forming agent is smaller than 80 nm in diameter.

More recently, He and coworkers [1987] performed comprehensive filtration studies in which every step of the filtration process contained internal controls to prove that the membranes were passing or retaining viruses of appropriate sizes. The Hutchinson NANB agent and control viruses of known dimension were subjected to filtration on 100, 80, 50, and 30 nm Nucleopore polycarbonate membranes. Filtrates were then inoculated into chimpanzees and/or tissue culture, and infectivity was assessed by standardized methods. The NANB agent passed through 100, 80, and 50 nm filters but was completely retained by the 30 nm filter. By comparison with the retention or passage of viruses of known sizes, the NANB agent is estimated to be between 30 and 60 nm in diameter.

The NANB agent is thus small and is lipid-encapsidated. There are only a limited number of candidate viruses that fulfill these criteria. First are the alpha and flavi RNA viruses previously known as the Toga viruses. These viruses cannot yet be excluded as the agent of NANBH, and their homology to NANBV is currently being investigated by Bradley at the CDC. The second group of small, lipid-encapsidated viruses is the hepadna viruses, and the possible association between NANBV and HBV has been previously discussed. Most intriguing is the possibility that NANBV is a delta-like agent. Both delta and NANB have been shown to have very similar hepatic histopathologic effects by light microscopy, and they induce identical cytoplasmic tubular structures and nuclear changes by EM. They also show identical reactivity with the Shimizu monoclonal antibody [Shimizu et al., 1986]. Both agents demonstrate interference with coexistent HBV infection, progress to chronicity in a high proportion of cases, show a high infectivity titer in the early acute phase and low titers in the chronic phase, and show poor or little immune response to surface or internal antigens. Despite these similarities, Bradley, in collaboration with Chiron Laboratories, failed to show nucleic acid homology between the tubular-forming NANB agent and the delta agent (Bradley, personal communication). Although the associations between delta and NANBV are intriguing, it is equally probable that NANB will turn out to be a new class of viral or subviral agents.

THE UNIVERSE OF NANB

NANBH has become a confusing conglomerate of terms and diagnostic entities. As is shown in Figure 1, we now have within the universe of NANB the primary percutaneous form, including transfusion-associated hepatitis; the sporadic form, the endemic form such as in Costa Rica, the epidemic form, as observed in the Asian subcontinent; the HBV variant form; and the likelihood that some cases of NANBH represent nonvirally induced elevations in serum transaminase. Within the percutaneous group are the primary tubule-forming, chloroform-sensitive agent designated type 1

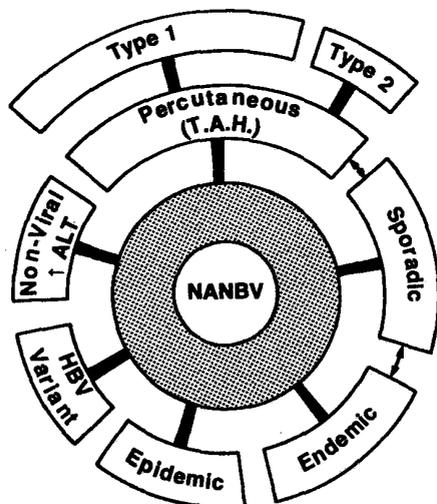


Fig. 1. The universe of non-A, non-B hepatitis (see text).

and the nontubule-forming, chloroform-resistant agent designated type 2. We know little of the type 2 agent beyond those physical characteristics. I propose that the epidemic form be immediately removed from this universe, since it has no clinical correlations with the NANB agent, since it is more suggestive of a hepatitis A variant, and since there is now a serologic system and an identified particle [Kane et al., 1984]. Second, as alluded to above, we require the studies to determine what proportion of NANBH represents HBV variant disease and then must rename that appropriately. Third, I urge that, before an entity is diagnosed as NANBH, there not only be extensive serological exclusion of other viral entities but also intensive historical and laboratory exclusion of ALT elevation. Without a serologic marker, I cannot think of a good way currently to distinguish the percutaneous, sporadic, and endemic forms of NANBH but would recommend studies in chimpanzees to determine whether well documented sporadic and endemic cases induce the type 1 ultrastructural alterations and whether these agents are chloroform-sensitive.

DETECTION OF THE NANB AGENT

In the absence of a specific NANBH serologic assay, Shimizu et al. [1986] have extracted lymphocytes from both chimpanzees and humans in the convalescent phase of NANBH, transformed these lymphocytes with Epstein-Barr virus, cloned them in microculture, and tested the monoclonal antibodies by immunofluorescence and immunoperoxidase against NANBV-infected and -uninfected liver tissue. The characteristics of the reaction patterns of the chimpanzee-derived and human-derived antibody are quite similar, but the effects on the liver are distinct between the species. In chimpanzee liver, the reaction with monoclonal antibody was originally thought to be specific for NANBV, but identical reactions were subsequently observed in delta-infected livers. In human liver, the reactivity is even

less specific, being seen also in some patients with acute hepatitis B infection and some with alcoholic liver disease. In the chimpanzee, the fluorescence pattern is granular and is located within hepatocyte microtubular structures. In the human, the fluorescence is homogeneous and primarily in Kupfer cells. In the chimpanzee, reactivity was seen in both acute- and chronic-phase infection, whereas, in humans, this reaction is rare in chronic NANBH. Disappointingly, from the standpoint of assay development, the antibody is not found free in either chimpanzee or human serum, nor does serum block the reaction of lymphocyte-derived antibody. Hence there is no immediate optimism that this approach will lead to a diagnostic assay independent of chimpanzee inoculation.

Schaff et al. (this volume) have recently expanded the observations of Seto and Gerety [1985] that antibody to a 77,000 mw glycoprotein reacted with NANBV-infected liver tissue of both human and chimpanzee origin. In addition, a DNA probe was generated that hybridized specifically with NANBV-infected liver. Reactions with the antiglycoprotein antibody and with the DNA probe are suggested as additional approaches to the detection of NANBV in liver tissue.

Several laboratories are now attempting a molecular approach to elucidation of the NANB agent. In a generic summation, messenger RNA is extracted from NANBV-infected liver, and a complimentary DNA library is generated. The complimentary DNA is hybridized with messenger RNA from normal liver, double-stranded material is removed, and residual single-stranded nucleic acid is then presumably specific for the NANB agent; this material can be cloned and used as a molecular probe. To my knowledge, at least four laboratories have used some variation of this technology and have not as yet been successful in generating a specific probe. One potential problem with this technique is that the NANB agent may not result in the production of an abundant class of polyadenylated messenger RNA. Linke et al. (this volume) suggests that NANBV messenger may exist as a lower-abundance species or in a structure that renders it refractory to these cloning procedures.

RELATIONSHIP OF NANBV TO CHRONIC HEPATITIS, CIRRHOSIS, AND HEPATOCELLULAR CARCINOMA

Although there is still considerable skepticism regarding the significance of chronic NANBH, evidence continues to accumulate that, in at least a proportion of cases, this is a significant and sometimes a fatal illness. In a composite of eight studies reviewed by Dienstag [1983], 102 patients with NANBV-induced chronic ALT elevation were biopsied, of whom 41% had chronic active hepatitis (CAH) and 20% had cirrhosis. Within this group, there have been five liver-related fatalities, representing 25% of the patients who developed cirrhosis. Included in the above analysis is an ongoing NIH study in which 32 patients have undergone initial liver biopsy; 69% demonstrated a generally mild form of CAH, and three patients (9%) had cirrhosis observed between 4 and 11 years after transfusion. Of primary interest are follow-up biopsies in 13 patients with CAH; 46% had improved histologic lesions,

15% had a stable form of CAH, and 39% went on to more severe disease, one developing a more severe CAH and four additional patients developing cirrhosis. In total, seven patients (22%) developed cirrhosis in our series, and, in two, the cirrhosis was directly or indirectly related to the patient's demise.

A considerable body of data on chronic hepatitis is now accumulating in the hemophilia population. In a collaborative study [Aledort et al., 1985], 155 liver biopsies were collected and examined under code by some of the world's leading hepatopathologists. Although most patients had mild lesions, 7% had severe CAH, and 15% had cirrhosis. It was thought that these chronic lesions were NANBV-related because the patients were HBsAg-negative, because there were no ground-glass hepatocytes, and because the light microscopic appearance was most consistent with that of NANBH. Assuming that the 7% severe CAH would progress to cirrhosis, an approximate 20% incidence of cirrhosis in chronic NANBH is again established. Chronic hepatitis is not a benign disease among hemophiliac patients, and, in five different series, 5-11% of hemophilia patients died of chronic end-stage liver disease. The most devastating data in hemophilia come from Hay et al. [1985], who studied 79 patients, of whom 71% had chronic ALT elevations; 34 were biopsied, among whom 26% had CAH and 12% had cirrhosis on initial biopsy. Follow-up biopsy was obtained in nine patients; two patients initially diagnosed as chronic persistent hepatitis (CPH) and two as CAH went on to cirrhosis, and one with CAH died of portal hypertension. Overall, five patients in this series had esophageal varices, and the incidence of cirrhosis and/or portal hypertension was 26%.

Some very important data on chronic NANBH come from Japan, where the posttransfusion hepatitis incidence is extraordinarily high and where Kiyosawa et al. [1982] retrospectively related almost half of chronic NANBH hepatitis and NANBV-related cirrhosis to prior blood transfusion. Kiyosawa et al. also showed that a long interval may be required for the development of these chronic sequela. In their series, it took a mean of 13.6 years from transfusion to the diagnosis of chronic hepatitis, 17.8 years to the diagnosis of cirrhosis, and 23.4 years to the development of hepatocellular carcinoma (HCC). AIDS look-back studies have recently indicated that greater than 50% of transfused patients die within 1 year of transfusion from the disease that necessitated the transfusion. Hence many transfused subjects will die of other illnesses before they progress to these NANBH-related chronic sequela; the observed incidence of severe chronic complications of NANBH may thus be less than that predicted from prospective studies. This does not, however, negate the seriousness of these events.

In the absence of a serologic marker, it is difficult to establish an association between NANBH and HCC. Nonetheless, there are several case reports suggesting this possibility, and a study by Okuda et al. [1984] suggests that at least 20% of HCC in nonalcoholic patients in Japan is NANBV-related. Recently, Muchmore et al. (this volume) demonstrated the development of HCC in an NANBV-infected chimpanzee.

In a very instructive case study, Kiyosawa et al.

[1984] performed serial liver biopsies in a patient progressing from acute to chronic NANBH to HCC. Two months following blood transfusion, this patient developed acute hepatitis, and a subsequent biopsy showed unresolved acute hepatitis. One year later, liver biopsy showed CPH, 4 years later progression to CAH, 3 years later bridging necrosis, and 2 years later cirrhosis. Three years subsequently, HCC was documented. The overall duration from transfusion to HCC was 18 years. I think time will show accumulating evidence for an association between NANBH and primary HCC.

PREVENTION OF NANBH AND ITS CHRONIC CONSEQUENCES

Surrogate Tests

Given the magnitude of these chronic consequences, the absence of a specific marker and the absence of a proven therapeutic intervention, attention must focus on means to prevent transfusion-associated NANBH. In the past 2 years, considerable controversy has centered on the adoption of surrogate tests to interdict the NANBV carrier blood donor. Figure 2 demonstrates the predicted efficacy of anticore as a surrogate marker for the NANBV carrier as demonstrated in three different prospective studies. In the large transfusion-transmitted virus (TTV) study [Stevens et al., 1984], in which 1,151 patients were followed, recipients of at least one unit of anticore-positive blood had a 18.7% incidence of NANBH as compared to 7.2% incidence in those receiving only anticore-negative blood. In the NIH study [Koziol et al., 1986], the comparable figures were 11.9% and 4.2%. More recently, Sugg et al. [1987], in the Federal Republic of Germany, demonstrated a 10.1% NANBH incidence in anticore-positive recipients and a 2.1 incidence in those receiving only anticore-negative blood. In this study, in which donors were already excluded for elevated ALT, anticore testing offered an additional 42% predicted efficacy. Overall, these three studies prospectively followed 2,049 patients, among whom hepatitis incidence was 14.5% when they received at least one unit of core-positive blood and 5.6% when they received only core-negative blood ($p < .001$). The predicted efficacy for core testing was 28%, diminished by the lower efficacy in the large TTV study. Based on these three studies, on prior data relating to ALT, and on the evidence for significant chronic liver disease following NANBH, the major blood organizations in the United States have elected to adopt both the ALT test and the anticore test as routine screening measures for all blood donations. Although I am in agreement with this decision, I wish to stress again that these are predicted efficacies, not proven efficacies, and that, in countries that can do so, an effort should continue to be made to perform a controlled, prospective study to demonstrate whether such costly measures are truly indicated.

Inactivation of Blood Products

The future of NANBH prevention may lie in viral inactivation rather than in serology. There are currently three major approaches to viral inactivation of blood products: heat, lipid solvents, and photochemical (nucleic acid) inactivation.

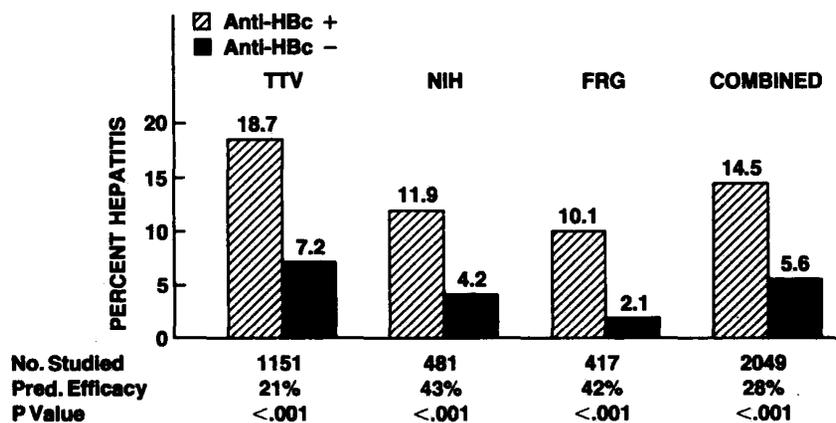


Fig. 2. Efficacy of anti-HBc testing as a surrogate marker for the detection of NANBV carriers. Depicted are the posttransfusion hepatitis incidence in recipients of anti-HBc⁺ vs anti-HBc⁻ blood in three prospective studies. TTV, transfusion-transmitted virus; NIH, National Institutes of Health; FRG = Federal Republic of Germany.

There have been several studies to investigate the safety of clotting factor concentrates heated in the dry state. Purcell and coworkers [1985] showed that heating to 60°C for 30 hr in the lyophilized state failed to prevent either hepatitis B or delta virus transmission but did completely prevent NANBH transmission. Hollinger et al. [1984] showed that none of four chimpanzees was infected by a dry-heated F VIII concentrate previously shown to transmit NANBH. However, when a dry-heated F VIII concentrate from the same manufacturer was administered to humans, Columbo and coworkers [1985] demonstrated that 11 of 13 developed NANBH. This study again emphasizes that testing in chimpanzees must remain only a prelude to clinical trials in humans.

More encouraging are results by Schimpf et al. [1987] demonstrating that material heated in solution in the presence of glycine and sucrose caused neither hepatitis B nor NANBH when administered to 26 hemophiliac patients that had been infrequently or never previously transfused. Heating in solution appears more efficacious than heating in the lyophilized state, presumably because lyophilization protects the virus as well as serum proteins.

It has been well documented that chloroform inactivates NANBV [Feinstone et al., 1983; Bradley et al., 1983], and this offers one approach to the inactivation of plasma products. More recent attention has focused on the use of tri-(n-butyl)-phosphate (TNBP) in combination with the detergent sodium cholate. As shown by Prince and coworkers [1986], this method inactivated 10⁴ CID 50 of both HBV and NANBV suspended in F VIII concentrate. In this study, six chimpanzees were protected, and all six were shown to be susceptible to infection after appropriate challenge.

The ideal inactivation procedure would be one that inactivates nucleic acid rather than destroys the membrane, since such a methodology might be applicable to cellular elements in which lipid was essential to cell integrity. Two such methods are currently under study and include β-propiolactone in combination with UV light [Prince et al., 1985] and psoralen in combination

with long-wave UV light (Agrion Corporation, personal communication). Both methods inactivate a broad spectrum of viruses, including single- and double-stranded RNA and DNA viruses. We have shown the latter method to inactivate 10⁴-10⁵ CID 50 of both HBV and NANBV in a variety of media, including F VIII concentrate. Preliminary studies indicate that this method is innocuous to red cells, and its effect on platelets is under study. Overall, several approaches to viral inactivation appear very promising, and I foresee a time when blood transfusion will no longer be a vehicle of infectious disease transmission.

In summary, NANB remains a frustrating and perplexing dilemma. Nonetheless, we know a little more about its physical properties, we know considerably more about its clinical outcome, and we know of multiple ways in which it can be inactivated. What we do not know exactly is where to go next, or what can be done to create the breakthrough that will allow progress with NANBH to parallel that with hepatitis B and hepatitis A. Some sophisticated approaches to this long-standing dilemma are under study, and, despite repeated past failure, I am optimistic that the resolution to this problem will be found in the not-too-distant future.

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